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Possible Inactivation of Ingested Ecdysteroids by Conjugation With Long-Chain Fatty Acids in the Female Tick *Ornithodoros moubata* (Acarina:Argasidae)

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Ornithodoros moubata females proved to be extremely sensitive to ingested 22,25-dideoxyecdysone; 15–20 ng provoked molting in all females and temporarily inhibited vitellogenesis. In contrast, this tick was very resistant to ingested ecdysteroids containing 22-OH groups, such as ecdysone, 20-hydroxyecdysone, ponasterone A, and makisterone A. Dosages about 500 times greater were necessary to produce supermolting and reduce fecundity [Connat et al: Z Ang Ent *96*, 520 (1983)]. Ingested tritiated ecdysone, 20-hydroxyecdysone, 2-deoxyecdysone, and ponasterone A were rapidly converted to apolar esterase-labile metabolites having approximately the same retention time as the AP2 identified as esters of ecdysteroids at C-22 with long-chain fatty acids (C16:0, C18:0, C18:1, C18:2) [Diehl et al: Int J Invert Reprod Dev *8*, 1 (1985)]. These products were then gradually transformed to the more polar apolar conjugates, AP1. A more detailed study with ingestion of large quantities of 20-hydroxyecdysone (10 μg/ml blood) demonstrated that only small amounts of free hormone were present in the hemolymph during the first day after the blood meal. The hormone was rapidly metabolized to AP2, then to AP1, in the intestinal cells and to a lesser extent in the peripheral tissues. Finally, AP1 accumulated in the intestinal cells and midgut content, probably because excretion outside the animal is impossible in this tick species.

In contrast, ingested 22,25-dideoxyecdysone was not metabolized to apolar products. This could account for its high biological activity. This compound was converted to unidentified more polar products. Two of them comigrated with ecdysone and 20-hydroxyecdysone on RP-18 HPLC column, but not on silica column, and therefore cannot correspond to these compounds.

We hypothesize that esterification of ecdysteroids at the C-22 position with fatty acids represents a detoxification mechanism for ingested ecdysteroids that might be present in blood from herbivorous or parasite-infected hosts.

Key words: ticks, *Ornithodoros moubata*, metabolism of ingested ecdysteroids, detoxification

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INTRODUCTION

It is surprising that a large number of insects are able to feed on plants containing phytoecdysteroids [for a review of these compounds see 3] and to develop successfully [4], especially since the compounds encountered most frequently are the molting hormone 20-hydroxyecdysone, ponasterone A, and ecdysone.

Several authors noted different sensitivities to ingested ecdysteroids in insects. Robbins and collaborators [5,6] reported that in five investigated species ingested ecdysone and 20-hydroxyecdysone were found to have little effect on larval growth. The fly *Musca domestica* was particularly resistant; ingestion of 150 μg 20-hydroxyecdysone/g of diet did not inhibit adult emergence. However, recently Singh and Russell [7] reported that the sensitivity depended on the quality of the diet in this species.

Other ecdysteroids could, however, disturb growth when ingested by the insects. That is the case, eg, for ponasterone A, which is the most active growth inhibitor in *Tribolium confusum* [6]. In the cecropia silkworm, 1 ppb of this compound in a synthetic diet blocked the development [8], and at lower doses it caused premature spinning [9]. Finally, it was found that synthetic ecdysteroid analogs such as Δ^7-5β-cholestene-2β, 3β, 14α-triol-6-one or 22,25-dideoxyecdysone could completely inhibit the development and the reproduction, as is the case in *Musca domestica* [5].

Metabolic studies of ingested tritiated ecdysteroids have been undertaken to determine the capability of certain insects to detoxify ecdysteroids. Hikino and collaborators [10] demonstrated that in *Bombyx mori* the high quantities of 20-hydroxyecdysone contained in the mulberry leaves [11] were only slowly absorbed by the body tissues but were very rapidly and easily excreted by the digestive tract; on the other hand, the steroidic nutrients of the insects like β-sitosterol were rapidly absorbed by the body tissues and excreted by the gut very slowly. For *Locusta migratoria*, Feyereisen and coworkers [12] also noted a high capability for detoxification of ingested ecdysteroids. A large amount of the ingested labeling was rapidly excreted in the feces. These ecdysteroids were excreted either unmetabolized or metabolized under free or conjugated form but at a lower rate when the endogenous hormone titer was high [12,13]. Recently, some of these metabolites were identified as acetyl or acetylphosphate derivates. Acetylation appeared especially active in the caeca [14].

In other species, the gut played a role in ecdysteroid inactivation by sulphation (in the southern armyworm *Prodenia eridania* [15]), or by epimerization of the hormones (in *Manduca sexta* [16], or *Pieris brassicae* [17]).

Several papers have dealt with the effects of ingested ecdysteroids in ticks. In female argasids, several micrograms of ingested ecdysteroids were necessary to induce supermolting, inhibition of oogenesis or, mortality [1,18–20]. In addition, we have demonstrated that the female *Ornithodoros moubata* was more sensitive to 22,25-dideoxyecdysone, which induced supermolting and reduction of fertility at doses about 500 times lower than ecdysone, 20-hydroxyecdysone, makisterone A, or ponasterone A [1]. We have thus undertaken metabolic studies with labeled ingested ecdysone, 20-hydroxyecdysone, 2-deoxyecdysone, ponasterone A, and 22,25-dideoxyecdysone for a better understanding of these differences in sensitivities.

MATERIALS AND METHODS

Animals

The argasid tick (soft tick) *Ornithodoros moubata* (Murray, 1877, *sensu* Walton, 1962) colony was kept at 27°C and 30–40% relative humidity in the dark. Adult females that had never fed previously were used in our experiment. After feeding, the females were kept in cotton-plugged glass tubes with a minimum of disturbance.

Chemicals

Ecdysone and 20-hydroxyecdysone were purchased from Simes (Italy). Ecdysone-3-acetate and ecdysone-2-acetate were kindly provided by Dr. Rees (Liverpool, U.K.). Cold ponasterone A and tritiated ponasterone A (60 Ci/mmol) were a gift from Dr. Koolman (Marburg, Federal Republic of Germany). Unlabeled and [^{14}C]22,25-dideoxyecdysone (0.48 μCi/mmol) were synthetized by one of us (M.J.T.). Tritiated ecdysone (53.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Dr. René Lafont provided us with 2-deoxyecdysone and kindly prepared [^{3}H] 20-hydroxyecdysone by conversion of [^{3}H]ecdysone with *Locusta* Malpighian tubules in vitro. In addition, he generously supplied us with tritiated ecdysonoic and 20-hydroxy-ecdysonoic acid from *Pieris*. [^{3}H]2-Deoxyecdysone (80 Ci/mmol) was synthetized by Dr. C. Hetru and provided by Prof. J. Hoffmann (Strasbourg, France).

Before use in our experiments, all these hormones were purified by HPLC* on a reverse-phase column (RP-18), and 2-deoxyecdysone and ponasterone A were purified once more by HPLC on silica column.

Nutrition

A few microliters of methanolic solution of tritiated hormones together with the necessary quantity of cold hormone were added to fresh defibri-

*Abbreviations: AP1 and AP2 = apolar products (with reference to compounds that are appreciably less polar than ecdysone); E = ecdysone; 20E = 20-hydroxyecdysone; cpm = counts per minute; HPLC = high-performance liquid chromatography; IU = international units.

nated pig blood and vortexed. Females were fed at 37°C through a "Parafilm" membrane until repletion, approximately 30 min.

Dissections

Hemolymph was put in pure methanol, vortexed, sonicated, and then centrifuged. The whole body or dissected-out organs were homogenized in methanol. The pellets were reextracted twice after centrifugation. Supernatants were analyzed with HPLC.

Enzymatic Hydrolysis

Hydrolysis of apolar conjugates was accomplished with 50 μl (50 IU) or 200 μl (200 IU) pig liver esterase (E.C. 3.1.1.1; Boehringer, Mannheim, Federal Republic of Germany) in 950 or 1,800 μl, respectively, of 0.1 M borate buffer, pH 8. After an overnight incubation at 37°C, the ecdysteroids were extracted with methanol.

High-Performance Liquid Chromatography

HPLC analyses were done with Perkin Elmer Series 3 chromatograph, with an LC 55 variable-wavelength spectrophotometer at 242 nm. The samples were analyzed on a Lichrosorb RP-18 (7 μm; Merck, Darmstadt, F.R.G.) column with a solvent gradient Tris buffer, pH 7.5, 20 mmol, and methanol (0.8 ml/min). The conditions used were 30% methanol to 45% (10 min), isocratic 45% (15 min), 45–100% (20 min), purge methanol (20 min; gradient I). In complementary experiments, a shorter gradient (II) was used: isocratic 50% methanol (15 min), 50% to 100% (15 min), purge methanol (15 min). Cold standards ecdysone and 20-hydroxyecdysone were added to the samples to determine whether labeled hormones comigrated with the UV peaks of the internal standards.

Liquid Scintillation Counting

We used an automatic liquid scintillation counter KONTRON MR300 DPM and Riatron scintillation cocktail (Kontron, Zürich, Switzerland). In the case of HPLC fractions, results were expressed in uncorrected cpm, because only a small difference of quenching existed between the different HPLC fractions. However, to have a correct estimation of cpm in each organ after the dissection, a quench curve was established. A linear correlation existed between the standard channel ratio, and the counting yield estimated by addition of internal standards in the biological samples.

RESULTS

Metabolism of Ingested [^{3}H]Ecdysone and [^{3}H]20-hydroxyecdysone

We investigated the metabolism of ng and μg quantities (up to 10 μg/ml blood) of ingested ecdysone and 20-hydroxyecdysone in *Ornithodoros moubata* females. HPLC analysis of whole animal extracts, taken at different times after the blood meal, showed that the hormones were first converted into

very apolar metabolites AP2 (Fig. 1), which were eluted only during the methanolic purge on a RP-18 column. These products, appearing rapidly after ingestion, increased in quantity during the first 24 h (Fig. 1). They were composed of three major peaks (Fig. 2A), which were eluted, in the case of [^{3}H]E, at fractions 112, 115, and 119, followed by a peak of less importance at fraction 124. The AP2 obtained after ingestion of [^{3}H]20E presented an identical pattern of four peaks but with a slightly more polar behavior. They were eluted at fractions 111, 114, 117, and 120, respectively. These AP2 from both E and 20E ingestion comigrated with those obtained after injection of the respective hormones into the hemocoel of the females [21]. Those obtained with [^{3}H]20E also comigrated with the metabolites found in nymphs after ingestion of 20E, which were identified as esters at C-22 of the hormone with palmitic, stearic, oleic, and linoleic acids [2] and thus presumably corresponded to the same compounds. This is also strongly suggested by the fact that the AP2 were completely hydrolyzable with pig liver esterase liberating 20E. The esterase-labile AP2 obtained from [^{3}H]E corresponded presumably to homologous conjugates, slightly more apolar because of the lack of the 20-OH. In both cases of E and 20E ingestion, other less apolar products (AP1) appeared gradually (Fig. 1). At the same time, AP2 products progressively disappeared, suggesting a conversion of AP2 to AP1. AP1 represented a complex mixture of several peaks that were not completely resolved in our gradient system (Fig. 2B). These products became more and more polar with time. A part of them was ionizable. AP1 were totally esterase-labile and yielded principally the ingested hormone after hydrolysis. Thus, in the case of ingestion of E, only minute quantities of cpm were recovered at the retention time of 20E. AP1 were also obtained after injection of each hormone in the hemocoel of the females [21]; however, in this latter case, larger quantities of 20E were liberated after hydrolysis of AP1 coming from [^{3}H]E.

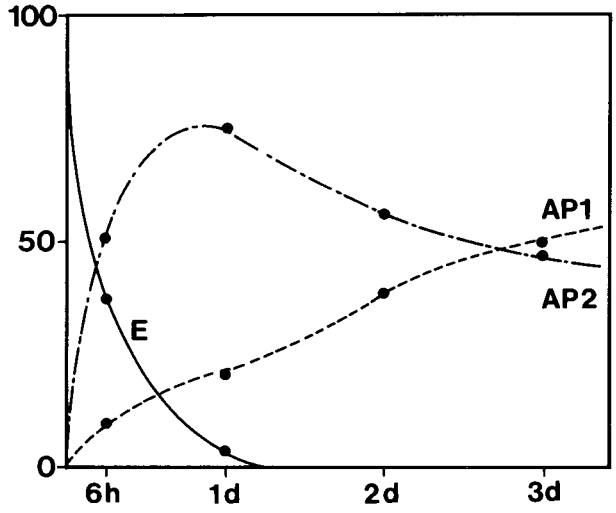

Fig. 1. Fate of ingested [^{3}H]ecdysone (5 μg/ml blood) in *O. moubata* females 6 h and 1, 2, and 3 days after the blood meal. Radioactivity of the compounds is expressed as a percentage of the total radiolabel recovered in the animal.

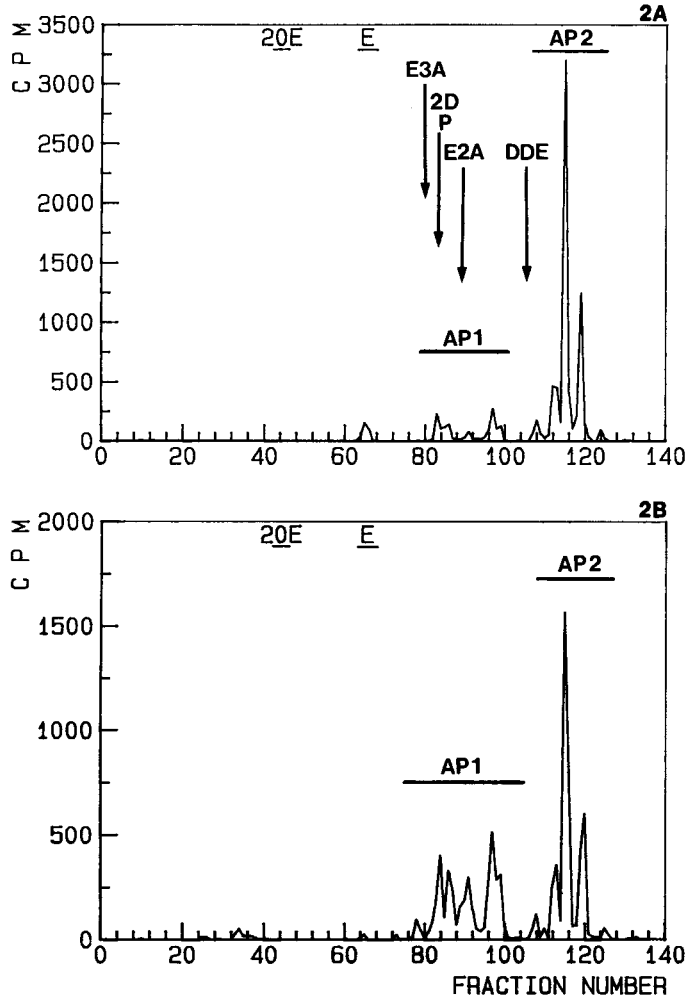

Fig. 2. HPLC reverse-phase radiochromatograms of metabolites in whole body extracts from *O. moubata* females after ingestion of [^{3}H]ecdysone (400,000 cpm/ml and 5 μg cold hormone). A, pattern after 24 h of metabolism; B, pattern after 3 days of metabolism. Solvent gradient I, see Materials and Methods, fractions of 30 s each. E and 20E indicate the retention time of ecdysone and 20-hydroxyecdysone standards, respectively, coinjected with the sample. The arrows indicate retention times of E3A, ecdysone-3-acetate; P, ponasterone A; 2D, 2-deoxyecdysone; E2A, ecdysone-2-acetate; DDE, 22,25-dideoxyecdysone.

At the end of the oviposition, the radiolabel contained in the females consisted only of this class of apolar compounds AP1. The oviposited eggs contained only AP2. The quantities of cpm recovered in the eggs corresponded to 10.2% and 2.3% of the total radiolabel after [^{3}H]E and [^{3}H]20E ingestion, respectively.

In a second phase, we investigated the fate of the radiolabel in coxal fluid, hemolymph, ovaries, intestinal cells, midgut content, and the remainder of the carcass after ingestion of high quantities of 20E (10 μg/ml blood).

In argasid ticks, much of the water and salts of the ingested vertebrate blood are discharged from the body as a fluid excreted from a pair of slitlike apertures located between coxae I and II. In our experiment, we have collected the combined coxal fluid of all females produced during feeding. They contained only negligible amounts of radioactive material (0.04% of the total radiolabel ingested by the females) in the form of very polar products (37%), 20E (47%), and AP1 (13%). It is possible, however, that part or all of the 20-hydroxyecdysone came from a minor contamination through the pierced parafilm membrane during nutrition of the ticks.

Figure 3 expresses the kinetics of metabolite formation in the different compartments. The radiolabel in the midgut lumen slowly decreased during the first 2 days (32.5% of the total radiolabel after 48 h) but remained, through the 11 days investigated, the quantitatively major compartment. In addition, we could see in this compartment the progressive replacement of the 20E by AP2 and then AP1. The half-life of 20E in the midgut content was approximately 10 h. After 11 days, 20E was no longer present, and AP1 represented

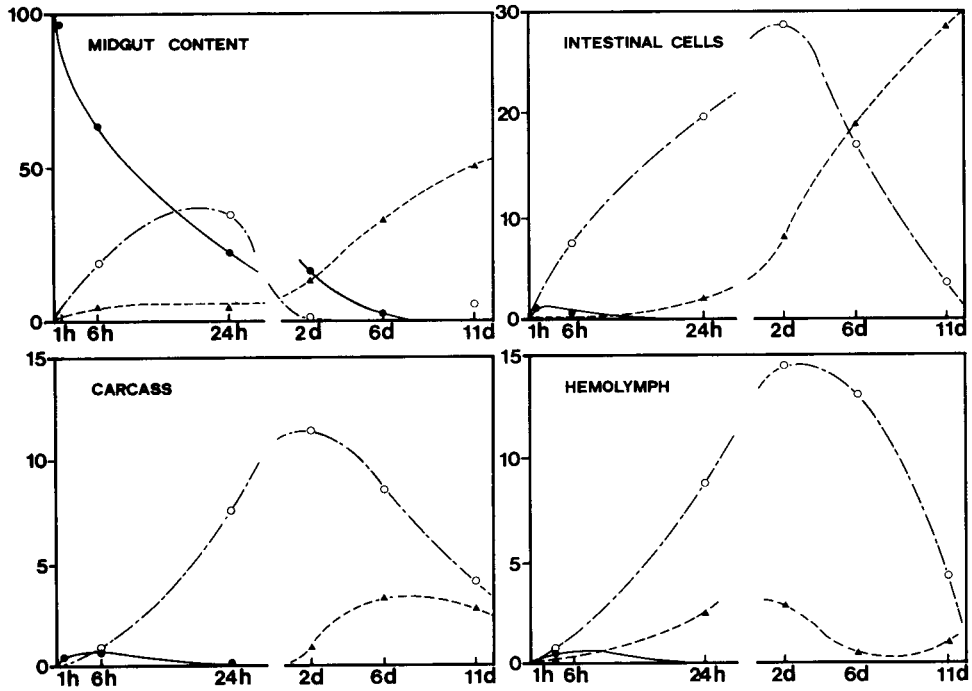

Fig. 3. Fate of the radiolabel in the different compartments of *O. moubata* females after the ingestion of blood containing 10 μg cold 20-hydroxyecdysone + 1,000,000 cmp [^{3}H]20-hydroxyecdysone per ml. Results are expressed in percent of the total radiolabel per female after 1, 6, 24 hours (h) and 2, 6, 11 days (d). In the case of the hemolymph, the total amount was based on the approximation that the average hemolymph volume is 50 μl [36]. In the case of midgut content, a large amount of nonwithdrawn hemolymph contaminated the dissection medium. We thus substracted the calculated radiolabel corresponding to this unwithdrawn hemolymph (total estimated amount of cpm corresponding to hemolymph minus cmp withdrawn). ●, 20-hydroxyecdysone; ▲, AP1; ○, AP2.

89.5% of the labeling in the midgut content and 49.9% of the total radiolabel in the animal.

In the intestinal cells, the radiolabel reached 37% of the total radiolabel found in the females 2 days after the blood meal. This quantity seemed to remain constant until the sixth day and then slightly decreased to 32% after 11 days. The kinetics of the different metabolites in the intestine cells (Fig. 3) showed that free 20E was found only in minute quantities during the first few hours (eg, 1.2% of the total radiolabel recovered in the animals 1 h after feeding). AP2 were produced (Fig. 4A) and reached their highest value (29% of the total radiolabel) after 2 days. In contrast, AP1 were produced only slowly (Fig. 4B) but increased continuously and by day 11 reached 89% of the metabolites of the intestinal cells (Fig. 4C) and 28.5% of the total radiolabel in the females. Thus, 11 days after feeding, 78.5% of the radiolabel in the female was AP1 and 9.4% AP2 associated with the digestive tract (intestinal cells and intestinal content).

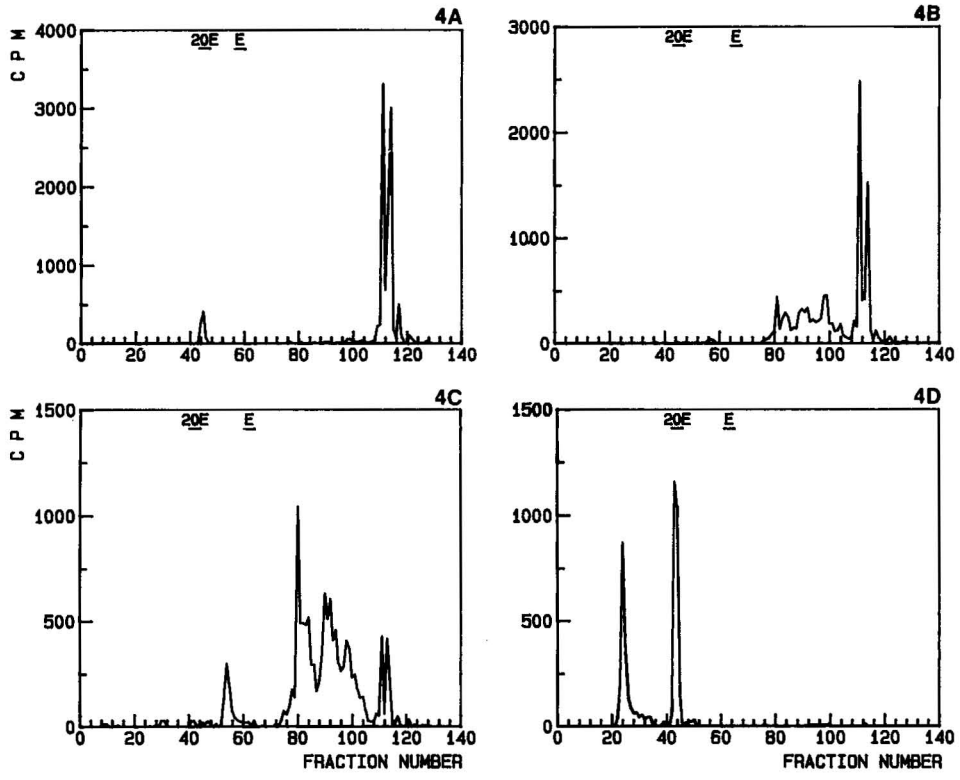

Fig. 4. HPLC reverse-phase radiochromatograms of metabolites found in the intestine cells of the *O. moubata* females after ingestion of blood containing 10 μg cold 20-hydroxyecdysone + 1,000,000 cpm [^{3}H]20-hydroxyecdysone per ml. Retention times of internal authentic ecdysone (E) and 20-hydroxyecdysone (20E) are indicated. Solvent gradient I, see Materials and Methods, fractions of 30 s each. A, metabolites 6 h after ingestion; B, metabolites 6 days after the blood meal; C, metabolites 11 days after the blood meal; D, same extracts as C submitted to hydrolysis by pig liver esterase.

In the hemolymph, the quantity of radiolabel increased gradually to reach its maximum value corresponding to 17.7% of the total radiolabel in the female 2 days after feeding (Table 1). HPLC analysis of combined hemolymph from 1 and 6 h showed the presence of 31.3% 20E, the remaining cpm corresponding to AP2. In that in our experiment 1 cpm corresponded to about 10 pg 20E, 6 h after the ingestion about 250 pg free 20E/μl was found in the hemolymph. Later, all the radiolabel in the hemolymph corresponded to apolar products (Fig. 3), which could have been released from the carcass and possibly also from the midgut cells. Maximum amounts of these apolar products circulated on day 2 postfeeding (Fig. 3).

In carcasses, free 20E could also be found during the first few hours. The hormone was rapidly converted into AP2, which accumulated during the first 2 days. Only small quantities of AP1 were present after 24 h. As was previously noted in hemolymph, a maximum quantity of radiolabel was present on day 2 in the carcasses. Thus 48 h after the blood meal a maximum of 30.3% of the total radiolabel in the females was found outside the digestive tract, principally in the form of AP2. We can suppose that after 2 days most of the metabolites produced either by the intestinal cells or by the peripheral organs (via the hemolymph) were finally transported into the midgut lumen.

Investigation of the radiolabel contained in the ovary showed that the percentage of cpm recovered in the ovary was always very low. It slowly increased and a maximum was found after 11 days, corresponding to 5.4% of the total radiolabel in the animal. HPLC analysis revealed that it corresponded to AP2 exclusively. Hydrolysis of these products gave only 20E.

Hydrolysis with esterase and subsequent HPLC analysis showed that, in the different extracts of carcasses, only 20E was liberated except in the 11-day extract in which a small peak having a retention time of 14 min (fraction 28) was also produced. It represented 9.5% of the total radiolabel in the extract. The same pattern was observed in the midgut content. However, in the intestinal cells, this polar unidentified product was present as of day 2, and its quantity increased with time. In the 11-day extracts, 42% of the radiolabel from midgut cells after hydrolysis corresponded to this product (and the remaining cpm to 20E; Fig. 4D). HPLC of this hydrolyzed extract at a lower

TABLE 1. Estimation of Radiolabel in the Hemolymph of Female *O. moubata* After Ingestion of Blood Containing 10 μg Cold 20-Hydroxyecdysone and 1,000,000 cpm [^{3}H]-20-Hydroxyecdysone/ml*

	Time					
Measure	1 h	6 h	24 h	2 days	6 days	11 days
Volume hemolymph withdrawn	25	25	5	10	10	36
cpm/μl	11.6	77	275	405	297	177.2
Percent of radiolabel in the female	0.3	1.6	11.5	17.7	14.1	5.2

*The hemolymph was withdrawn at different times after the blood meal, and its label content per μl was estimated by scintillation counting. Based on the approximation that the average hemolymph volume of female *O. moubata* is 50 μl [36], the total radiolabel contained in the hemolymph could be estimated and calculated as a percent of the total radioactivity found in the female.

pH showed that this product became more apolar and thus was presumably ionizable. It did not, however, comigrate with 20-hydroxyecdysonoic acid from *Pieris*.

In the case of the metabolism of ingested [^{3}H]E, the apolar products also accumulated in the intestinal cells and lumen. Hydrolysis with esterase of the AP1 contained in the intestinal cells yielded principally E but also a small ionizable polar peak with a retention time of 19 min (fraction 38) at pH 7.5. This product did not comigrate with ecdysonoic acid from *Pieris* and thus could be the homolog of those obtained with 20E.

Metabolism of [^{3}H]Ponasterone A

As in the case of [^{3}H]E and [^{3}H]20E metabolism, ingested [^{3}H]ponasterone A (25-deoxy-20-hydroxyecdysone; 1 μg/ml blood) was rapidly metabolized. The half-life of the ingested hormone was 3 h in our experimental conditions (Fig. 5). Four peaks of apolar products slightly less polar than those previously described were produced (Fig. 6). Their retention times corresponded to fractions 113, 116, 121, and 127. In addition, they were esterase-labile. Thus, by analogy, we also decided to call them *AP2*. This AP2 class (the more apolar) appeared very rapidly and represented 61.3% of the total radiolabel in the females 6 h after the blood meal (Fig. 5). The less apolar class of products AP1 appeared only slowly and represented 23.4% after 48 h. We always noticed a few cpm at the retention time of 20E, suggesting a minor hydroxylation at the C-25 position. Hydrolysis by esterase of both AP1 and AP2 was complete and liberated only one product having the same retention time as ponasterone A.

Six hours after the blood meal, the radiolabel circulating in the hemolymph corresponded to 9.5% ponasterone A, 21% AP1, and 70% AP2 and 24 h after the blood meal to apolar products exclusively (AP1, 15.5%; AP2, 84%).

Approximately 2% of the radiolabel in the females was incorporated in the freshly laid eggs, principally in the form of apolar products AP1 and AP2.

Metabolism of [^{3}H]2-Deoxyecdysone

Ingested [^{3}H]2-deoxyecdysone (2 ng/ml blood) was also metabolized to apolar products with a short half-life of 3 h (Fig. 7). As with E, 20E, and ponasterone A metabolism, AP2 products (fractions 117-118, 123, 129-130; Fig. 8) were produced first and AP1 (the less apolar) afterwards. After 4 h, apolar products (AP1 + AP2) represented 94.3% of the total radiolabel recovered in the females. Analysis after 48 h showed that AP1 were the metabolites that accumulated. Hydrolysis of the extracts by esterase was complete and liberated only one peak with a retention time corresponding to 2-deoxyecdysone. Surprisingly, 2-deoxyecdysone was not found to be converted to E. Six hours after ingestion, radiolabel was present in the hemolymph mostly in the form of apolar products (94%). Only 4% corresponded to free 2-deoxyecdysone.

Metabolism of [^{14}C]22,25-Dideoxyecdysone

Finally, we investigated the metabolism of ingested [^{14}C]22,25-dideoxyecdysone. In contrast to the previous ecdysteroids studied, this compound

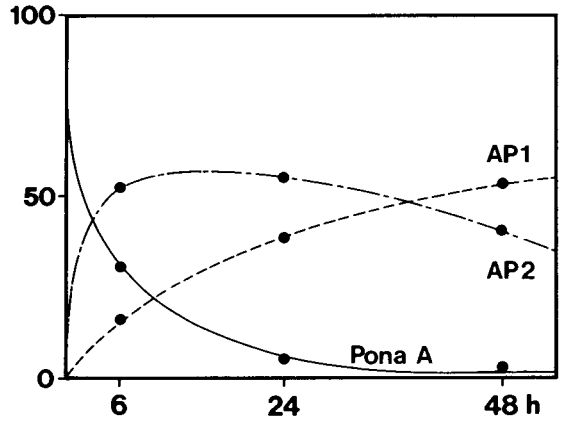

Fig. 5. Fate of ingested [^{3}H]ponasterone A (1 μg/ml blood) in females *O. moubata* 6, 24, and 48 h after the blood meal. Radioactivity of the compounds is expressed as a percentage of the total labeling recovered in the animal.

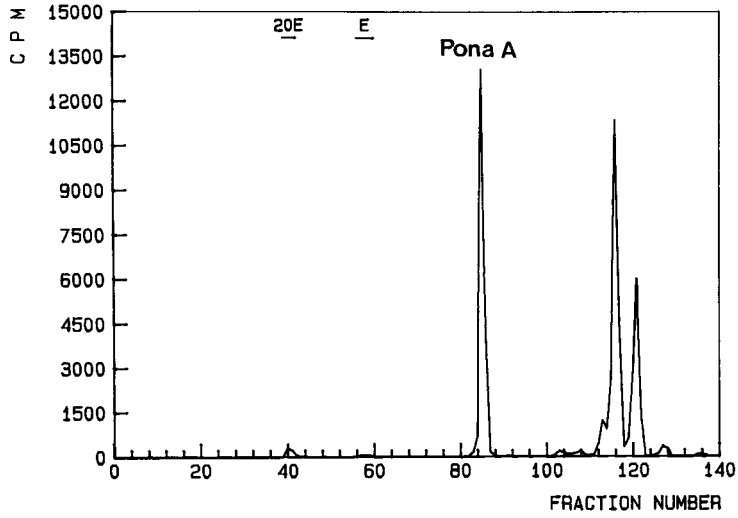

Fig. 6. HPLC reverse-phase radiochromatogram of metabolites in whole body extracts from *O. moubata* females 6 h after the ingestion of [^{3}H]ponasterone A (Pona A). E and 20E indicate the retention times of ecdysone and 20-hydroxyecdysone standards, respectively, coinjected with the sample. Solvent gradient I, see Materials and Methods, fractions of 30 s each.

proved to be very effective in inducing a supermolting cycle when ingested by the females [1]. Because of the low specific activity of our substrate, a group of *O. moubata* females was fed with blood containing 4 μg/ml, a concentration very near the lethal dose but which still induces supermolting, delays vitellogenesis, and reduces fecundity.

After 24 h, only one peak of radioactive material with the retention time of 22,25-dideoxyecdysone was recovered, showing that this product had not yet been metabolized. However, after 2 days, no more radioactivity was found in this fraction. In contrast to the other ecdysteroids, no metabolites

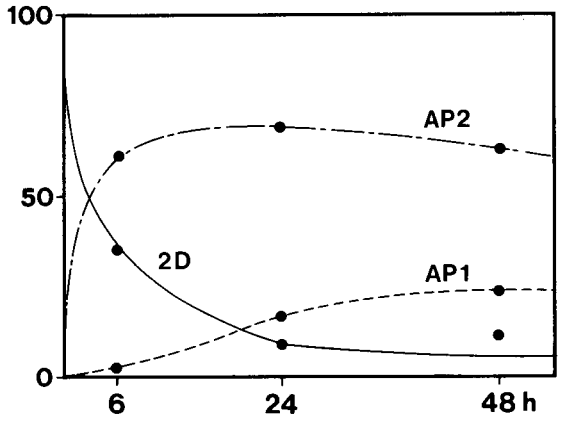

Fig. 7. Fate of ingested [^{3}H]2-deoxyecdysone (2 ng/ml blood) in *O. moubata* females 6, 24, and 48 h after the blood meal. Radioactivity of the compounds is expressed as a percentage of the total labeling recovered in the animal.

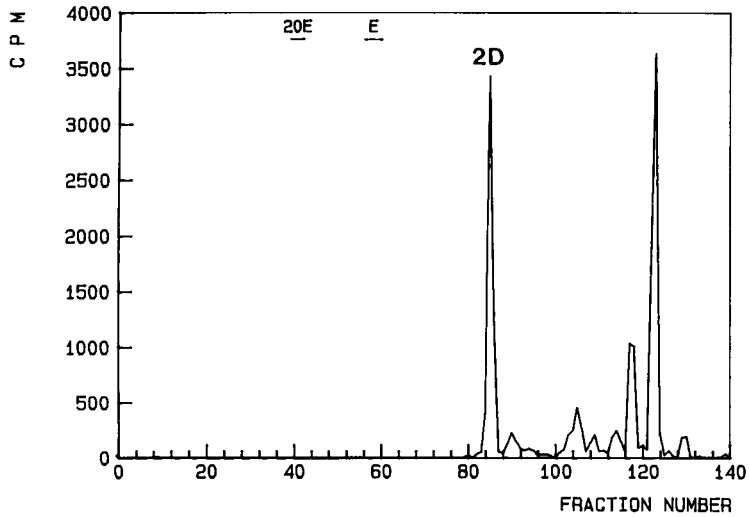

Fig. 8. HPLC reverse-phase radiochromatogram of metabolites in whole body extracts from *O. moubata* females 6 h after the ingestion of [^{3}H]2-deoxyecdysone (2D). E and 20E indicate the retention times of ecdysone and 20-hydroxyecdysone standards, respectively, coinjected with the sample. Solvent gradient I, see Materials and Methods, fractions of 30 s each.

less polar than this hormone were formed. The radiolabel corresponded to five unidentified peaks more polar than 22,25-dideoxyecdysone (Fig. 9). These five peaks were recovered each day in varying amounts during the induced molting cycle. Analysis of the hemolymph collected 3 days after feeding demonstrated the presence of only peaks 2 and 4, which comigrated with 20E and E, respectively, on different reverse-phase column and different solvent gradients. However, injection of the corresponding fractions on a silica column showed that these metabolites were not 20E and E. They did not comigrate with the standards contained in the collected fraction. They

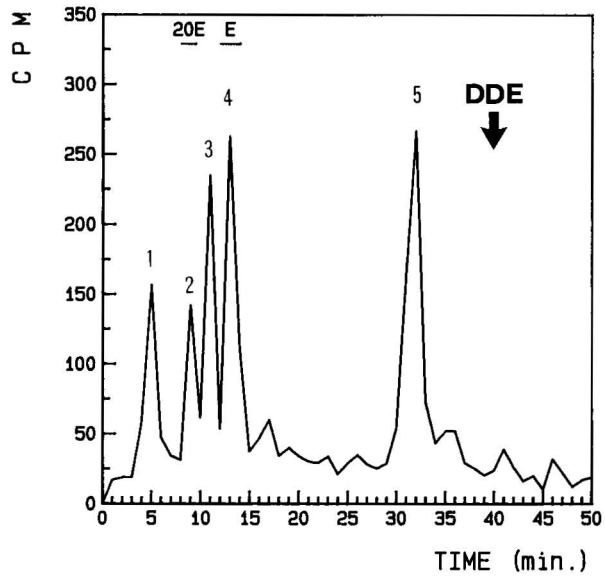

Fig. 9. Reverse-phase HPLC analysis of an extract of whole *O. moubata* females 3 days after ingestion of [^{14}C]22,25-dideoxyecdysone. Retention times of some cold standards are indicated. E and 20E indicate the retention times of ecdysone and 20-hydroxyecdysone standards, respectively, coinjected with the sample. Retention time of 22,25-dideoxyecdysone is indicated (DDE). Solvent gradient II, see Materials and Methods, fractions of 1 min each.

were not recovered in the other fractions of the HPLC run and were thus possibly irreversibly bound to the column. Their nature has not yet been elucidated.

DISCUSSION

Metabolism of Ecdysteroids With 22-OH

To complete our knowledge about the metabolism of ecdysteroids in the tick *Ornithodoros moubata*, and to understand the capacity of the female to ingest high quantities of them [1], we investigated the metabolism of ingested ecdysone, 20-hydroxyecdysone, 2-deoxyecdysone, and ponasterone A. Both ecdysone and 20-hydroxyecdysone were rapidly converted into very apolar metabolites, called *AP2*. This metabolic pathway was previously observed when the hormones were injected into the hemocoel of nymphs [22] or of females [21]. AP2 have been identified as esters of 20-hydroxyecdysone at the C-22 position with long-chain fatty acid (C16:0, C18:0, C18:1, C18:2) in stage 5 nymphs after ingestion of high quantities of cold 20-hydroxyecdysone [2]. However, the conjugates of the free hormone with fatty acids, as is the case with injected hormone, were not the final products of this metabolic pathway. Gradually, their abundance decreased, and concomitantly the slightly more polar apolar metabolites, AP1, appeared. AP1 were produced continuously and corresponded to a mixture of several products that were in part ionizable. Their nature has not yet been elucidated, but they remained esters; as AP2, they are labile to hydrolysis with esterase. They yielded either

the intact free hormone, suggesting modifications on the fatty acid moiety, or polar metabolites of ecdysteroids, indicating that modification on the steroidic nucleus could occur, eg, in the conjugate form. Ingested ponasterone A and 2-deoxyecdysone were also rapidly metabolized to apolar compounds with approximately the same retention times as AP2 and AP1 obtained by metabolism of [^{3}H]ecdysone or [^{3}H]20-hydroxyecdysone. In addition, these compounds liberated the original hormones after esterase hydrolysis. These two facts suggest that they also correspond to esters with long-chain fatty acids. This pathway was also observed after injection of these two hormones in the hemocoel (J.-L. Connat, unpublished results). Surprisingly, in both cases of ingestion and injection these hormones were not used as precursors for ecdysone or 20-hydroxyecdysone synthesis.

Our study followed the metabolites in the different compartments of females that ingested blood containing a large amount of the molting hormone 20-hydroxyecdysone. Only a little free hormone circulated in the hemolymph during the first day after feeding compared to the large quantity present in the midgut. However the ecdysteroids levels reached 250 pg 20-hydroxyecdysone/μl at this time, which is about half the maximum level found in a nymph during apolysis [23]. It was thus surprising that the integument of the female did not show any response to this hormonal stimulation. We can hypothesize either that at this moment the hypodermal cells were not yet able to respond because of an absence of receptors or that the exposure to the stimulus was not long enough to induce a response.

Two days after feeding, the hemolymph contained large amounts of radiolabel (17.7%), but it corresponded to apolar products, which thus seemed to be inactive for the induction of a supermolt cycle. We must also note that the tissues of the body did not accumulate large amounts of metabolites. In addition, the proportion of radiolabel contained in the hemolymph and in the carcasses decreased after 48 h and increased in the midgut. Thus the gut seemed to play a very important role (1) limiting the passage of free ecdysteroids into the hemolymphatic compartment and (2) accumulating the AP1 constituting the final products of the inactivation metabolism. We must note here that this latter property could be due to the fact that in *O. moubata* the hindgut does not communicate with the midgut, and excretion from the animal is not possible. Preliminary studies with the closely related *O. parkeri*, which possesses a functional gut, showed that ingested tritiated ecdysteroids were also rapidly metabolized to AP2 then to AP1, but within one week following the blood meal already 42% of the ingested hormone were excreted as AP1 in the feces. This latter case could represent the classical method of detoxification in ticks and explain the accumulation in the midgut of *O. moubata*.

In crustaceans, the gut seemed to also play a great role in elimination of ecdysteroids. Metabolites of injected tritiated ecdysone accumulated in midgut gland and hindgut of the crab *Gecarcinus lateralis* [24].

In insects, Hikino and colleagues [10] reported a similar situation in *Bombyx mori* in detoxifying phytoecdysteroids contained in the diet. Ingested 20-hydroxyecdysone was absorbed very slowly by the tissues and metabolized. Part of the unidentified metabolites was excreted into the digestive tract. On

the other hand, steroidic nutrients were rapidly absorbed into the body tissues and excreted into the digestive tract very slowly. More recent work on *Locusta migratoria* [14] showed that ingested ecdysteroids were rapidly converted to 3-acetates mainly in the gastric caeca and then conjugated with phosphate and rapidly excreted. Most of the hormones and their metabolites did not enter the hemolymph. Similar results were observed in *Schistocerca gregaria* [25]. Thus locusts, similar to *Ornithodoros spp.*, are also capable of esterification with fatty acids. However, the short-chain acetic acid is used in the C-3 position instead of long-chain fatty acids in the C-22 position [2].

Metabolism of 22,25-Dideoxyecdysone

In contrast to ecdysone, 20-hydroxyecdysone-, ponasterone A-, and 2-deoxyecdysone-, all of which possess a 22-OH group, ingested 22,25-dideoxyecdysone was not metabolized in the apolar pathway. Only unidentified polar metabolites were produced, suggesting that the animal was not able to produce long-chain fatty acid conjugates at the C-2, C-3, or C-14 position. This variation in metabolism could account for the differences in sensitivity of the females to these ecdysteroids. Only small amounts of ingested hormone (20–30 ng) were necessary to disrupt vitellogenesis and provoke supermolting, whereas doses about 500 times greater of ecdysone, 20-hydroxyecdysone, and ponasterone A were necessary to produce the same effects [1]. 22,25-Dideoxyecdysone has been reported to be a precursor for biosynthesis of ecdysone and 20-hydroxyecdysone in several species. This, however, does not explain the activity of this compound in our tick; it was not converted to the molting hormones. Thus we can hypothesize that 22,25-dideoxyecdysone itself and/or its metabolites is hormonally active in *O. moubata*.

The Role of the Apolar Pathway

For females previous studies indicated that large amounts of AP2 accumulated in the eggs after the injection of tritiated ecdysone or 20-hydroxyecdysone. It was hypothesized that these products represent a storage form for embryonic development [21]. Recent studies showing that these products remained very stable during embryogenesis [Dotson, unpublished results, 26] indicate that this might not be the case and argue for the inactivation nature of these products. In fact, accumulation of AP2 in the ovaries could be artifactual. The lipophilic nature of these metabolites might allow them to be bound to vitellogenins. In the case of ingestion, a lower percentage of radiolabel is presumably circulating in the hemolymph, and this probably accounts for the very low incorporation into the ovary, in contrast to when the hormone was injected during vitellogenesis. On the contrary, in the ixodid tick *Boophilus microplus* the apolar products seem to function as a storage form for the embryo [27].

The apolar pathway in the female *O. moubata* is thus interpreted to be a detoxification mechanism against ingested ecdysteroids that inhibit vitellogenesis. Injection of 100 ng ecdysone in the hemolymph was sufficient to induce supermolting and egg resorption [28, Connat and Diehl, in prepara-

tion]. On the other hand, ingestion of about 10–20 μg of the same hormone was needed to obtain the same effects. This fact unambiguously demonstrates the very important role of the midgut as a barrier against the release of the hormone into the hemocoel. In addition, our data demonstrate the capacity of accumulation of inactivation products by the intestine cells and their liberation in the intestine lumen. This apolar pathway has also been observed in other life stages after either injection into the hemocoel or ingestion of different ecdy-steroids [2,22,26].

In conclusion, in ticks, esterification of ecdysteroids with fatty acids in position C-22 might fulfill three different functions: (1) In nymphs, it is particularly active at the moment of low ecdysteroids titers [22] and thus could inactivate the endogenous hormone; (2) in female *B. microplus*, in contrast to *Amblyomma hebraeum*, which accumulate free ecdysteroids in the eggs [29,30], or *O. moubata*, which seem not to use the conjugates, it could serve to produce an hormonal storage form for embryos [27]; (3) in all the different life stages of ticks, it might inactivate exogenous ecdysteroids that could be ingested with blood from hosts that feed on plants containing phytoecdysteroids [3] or hosts infested with worms [31–33]. The choice of this esterification position could be an adaptive mechanism, because most active phytoecdysteroids contain a 22-OH, which is very important for molting activity [34,35].

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