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Ecdysone metabolism in the tick *Ornithodoros moubata* (Argasidae, Ixodoidea)

by

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With 4 figures and 1 table

ABSTRACT

The metabolism of injected ^{3}H-ecdysone was studied in last instar nymphs of the tick *Ornithodoros moubata* at different stages of the molt cycle. After extraction from haemolymph or from whole animals, 5 radioactive fractions were detected by high-performance liquid chromatography (HPLC; reverse phase RP-18). Fraction 1 contains unknown metabolites less polar than ecdysone. Fraction 2 corresponds to the injected ecdysone. Fraction 3 represents ecdysterone (20-OH-ecdysone). The identification is based on the observations that the metabolite cochromatographs with authentic ecdysterone on RP-18, silica or diol columns. Furthermore, the TSIM, acetonide or acetate derivatives cochromatograph with the corresponding derivatives from authentic ecdysterone. Fraction 4 may represent 20, 26-diOH-ecdysone. Fraction 5 contains polar ionisable metabolites of yet unknown chemical nature.

Our results indicate that the metabolism of injected ^{3}H-ecdysone proceeds through 1) hydroxylation at C_{20}, and 2) probably hydroxylation at C_{26} to 20,26-diOH-ecdysone which yields then apolar and polar endproducts. In addition, ecdysone and ecdysterone may also be directly converted to apolar and polar metabolites. The formation of polar endproducts becomes very prominent during the 7^{th}-9^{th} day postfeeding when the endogenous ecdysteroid titer drops.

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INTRODUCTION

Recently the presence of ecdysone and ecdysterone, the principal molting hormones of insects and crustaceans, was also demonstrated in the ticks *Amblyomma hebraeum* and *Ornithodoros moubata* (DELBECQUE *et al.* 1978; GERMOND *et al.* 1982). Quite comparable to the situation in other arthropods the hormone titer fluctuated during the development of nymphs to adults. Apolysis was observed during rising hormone concentrations. Deposition of the adult epicuticle was temporarily correlated with the ecdysteroid peak whilst the following deposition of the adult exocuticle occured during decreasing hormone titers.

The ecdysteroid titer in insects is thought to be the result of a delicate balance between the rates of synthesis, metabolism and excretion of the hormones (see e.g. review by GILBERT *et al.* 1980). The prothoracicotropic hormone (PTTH) produced by neurosecretory cells controls the rate of synthesis of ecdysone by the prothoracic glands. Ecdysone is then hydroxylated by several peripheral tissues at C_{20} to ecdysterone (20-OH ecdysone) which is thought to be the active hormone. Inactivation of the ecdysteroids occurs by several pathways (see e.g. GILBERT *et al.* 1980; KOOLMAN 1980; LAFONT *et al.* 1980; OHTAKI 1981) leading to the production of e.g. 26-OH-ecdysone or -ecdysterone, C_{26}-acids, inokosterone, 3-dehydro-ecdysteroids, 3α-epimers, conjugates or poststerone (by side chain cleavage). Excretion of unchanged hormones may be another possibility.

In an effort to understand molting hormone metabolism and titer regulation in ticks we began several experiments with radiolabelled hormones. Here we report on the metabolism of ^{3}H-ecdysone in the argasid tick *Ornithodoros moubata*, injected at different physiological ages during nymphal- adult development. Special attention was paid to the question if hydroxylation of ecdysone to ecdysterone really occurs as one would expect from our earlier chemical study (GERMOND *et al.* 1982).

MATERIAL AND METHODS

Animals: Fifth stage nymphs of the tick *Ornithodoros moubata* (Murray, 1877; *sensu* Walton, 1962) were fed on defibrinated pig blood (at 37° C) through a Parafilm membrane. They were kept in glass tubes plugged loosly with cotton at 27° C and 30-40 % relative humidity in the dark. The nymphs molted to females 9-10 days after the bloodmeal.

Chemicals: All solvents were of analytical or chromatographical grade (MERCK). Ecdysone, ecdysterone and Makisterone A were purchased from SIMES (Italy). 2-deoxyecdysone was a gift from Dr. Horn and Dr. Ohnishi. Ecdysone-23,24-^{3}H (N) (Sp. activity: 80 Ci/mmol) was purchased from NEW ENGLAND NUCLEAR (NEN). It was purified by high-performance liquid chromatography immediately before use. ^{3}H-ecdysterone and the labelled metabolites 20,26-diOH-ecdysone, 26-COOH-ecdysone and 26-COOH-ecdysterone from *Pieris* nymphs were kindly prepared by Dr. Lafont (Paris).

Injections: Labelled ecdysone was dried under a stream of N_2 and taken up in 0.9 % NaCl or medium TC 199. About 10^5 cpm in 2 μl were injected into a tick through an articulation membrane using a fine capillary tube.

Extractions: Haemolymph was taken with calibrated capillaries after puncturing the articulation membranes of several legs. It was vortexed in methanol. Whole animals

were homogenized in methanol-water (4:1). After centrifugation the pellets were extracted twice with methanol. The combined supernatants were kept overnight at −20° C for lipid precipitation.

Purification : In one experiment, the extract containing the various ecdysone metabolites was purified by passage through a SEP-PAK C_{18} cartridge (WATERS ASSOC.) according to the procedure described by LAFONT *et al.* (1982). Polar metabolites were eluted with methanol-water (1:4), ecdysteroids with methanol-water (3:2) and apolar metabolites with methanol.

Thin-layer chromatography (TLC) :

Aliquots from the various extracts were separated by TLC on precoated plates (MERCK, silica gel 60 F 254, 0.25 mm thickness with concentration zone) by chloroform-methanol (3:1) or chloroform-ethanol (3:1). Cold ecdysone and ecdysterone were added as internal standards and visualized under UV. Small bands (0.5 cm width) were scraped and the silica gel transferred into scintillation vials for determination of the radioactivity.

High-performance liquid chromatography (HPLC) :

Aliquots of the various extracts were suspended in 50-300 µl of methanol-water (3:7) and centrifuged at about 10,000 g. Cold ecdysone, Makisterone A, ecdysterone and 2-deoxy-ecdysone were added as internal standards. The samples were then separated by HPLC (PERKIN ELMER Series 3 chromatograph, with a LC 55 variable wavelength spectrophotometer at 242 nm) in the reverse phase mode (MERCK Hibar column RT; length: 25 cm; i.d. 4 mm; packed with Lichrosorb RP-18, 5 or 7 µm). The use of a precolumn considerably increased column life (packed with Perisorb RP-8; 30-40 µm). The ecdysteroid metabolites and internal standards were separated by a gradient of methanol-water (linear gradient 30 %-45 % methanol in 10′, 45 % for 15′, 45-100 % in 20′, followed by 100 % for 10′. Column temperature 27° C; flow 0.8 ml/min). Fractions of 0.4 ml or 0.8 ml were collected in 3 ml polypropylen vials and the radioactivity measured by scintillation counting.

Ecdysteroids were also separated by HPLC on a silica column (PERKIN ELMER Silica A; 0.26 × 25 cm) under isocratic conditions (1,3 ml/min, chloroform-isopropanol-water (15:3:0.2). Separation was also performed on a diol column (MERCK Diol, Lichrosorb 10 µm). Conditions: 1 ml/min of 88 % chloroform −12 % (isopropanol: water (100:4)).

Liquid scintillation counting (LSC) :

The HPLC fractions were mixed with a scintillation cocktail (Atomlight, NEN; 0.8 or 1.6 ml of cocktail per vial). Radioactivity was measured with a KONTRON MR 300 automatic liquid scintillation system, and corrected for quenching, if necessary.

Formation of derivatives :

After purification by HPLC, the metabolite presumed to be ecdysterone was derivatised with N-trimethylsilylimidazole (TSIM) as described by GERMOND *et al.*, 1982. Cold ecdysterone (240 µg) was added as internal standard. Authentic ^{3}H-ecdysterone (NEN) was also derivatised. Silylation was performed during 3 hrs, 6 hrs and 20 hrs,

respectively. Ecdysteroid derivatives were separated by TLC on precoated silica gel 60 F 254 plates (0.5 mm; solvent system: toluene-ethyl-acetate (9:1)) and localized by UV absorption. The plate was then scraped (band width: 5 mm) and the radioactivity of the silicagel measured by LSC.

Similarly, formation of acetate and acetonide derivatives was performed according to KOOLMAN *et al.* (1979), except for the acetonide formation where the concentration of p-toluene sulfonic acid in acetone was adjusted to 1 mM. For kinetic analysis, acetylation was performed for 30′, 50′, 4 hours and 24 hours. Acetonide formation proceeded for 1 hour or 4 hours, respectively. Both types of derivatives were separated on precoated silica plates (MERCK; 0.5 mm; silica gel 60 F 254; solvent system: chloroform-methanol 8:2).

RESULTS

Ecdysone metabolism during the period of high endogenous ecdysteroid titer.

In a first experiment about 0.6 ng/tick of ^{3}H-ecdysone (about 10^{5} cpm) was injected into the haemolymph of last (5th) instar nymphs of *Ornithodoros moubata* which had fed 6 days ago. At this stage the endogenous ecdysteroid concentration in haemolymph and in whole ticks are highest (about 500 pg/µl haemolymph and 11 ng ecdysterone equivalents/tick; GERMOND *et al.*, 1982). After 24 hours the ecdysone metabolites from haemolymph and whole animals were extracted and separated by HPLC (Fig. 1). In both extracts 5 major radioactive fractions could be detected.

Fraction 1 contains several unidentified radioactive products which are less polar than ecdysone or 2-deoxy-ecdysone (apolar products: AP). In TLC, these metabolites are also less polar than ecdysone (see Table).

TABLE

TLC of apolar metabolites purified by HPLC (Fraction 1). Mobile phase: chloroform-ethanol (9 : 3).

Compound	Rf-value
cholesterol	0.86
2-deoxyecdysone	0.56
AP (fraction 1)	0.5
ecdysone	0.36
ecdysterone	0.27

Fraction 2 represents the injected ecdysone which has not yet been metabolized. It cochromatographs in HPLC and TLC with cold authentic ecdysone.

Fraction 3 corresponds to ecdysterone (20-OH-ecdysone). The identification is based on the observations that the metabolite cochromatographs with authentic ecdysterone not only in TLC but also in HPLC on silicic acid, diol or RP-18 columns. Furthermore, the metabolite together with authentic ecdysterone was derivatised to yield trimethylsilyl, acetate or acetonide derivatives, respectively. The various radio-

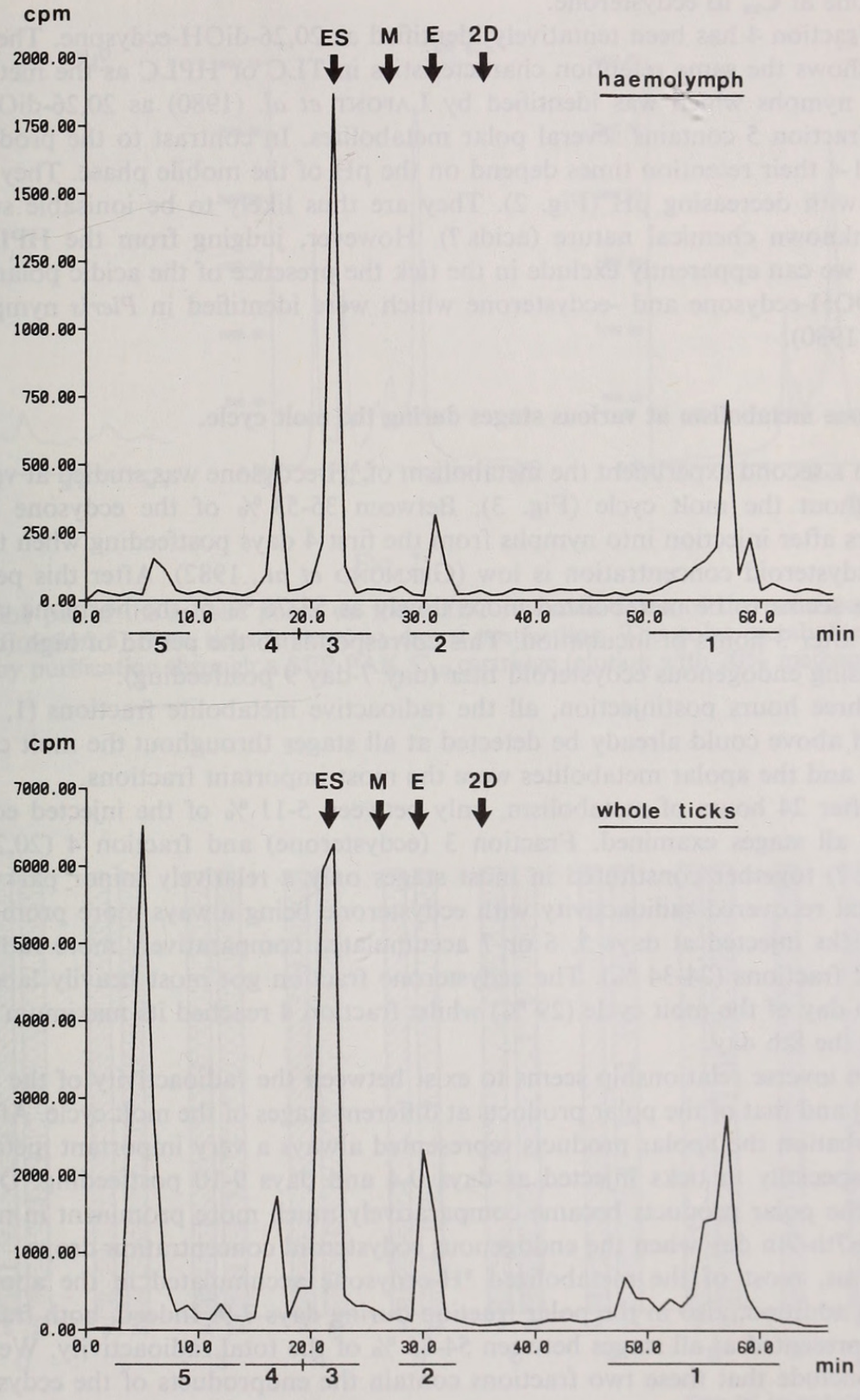

FIG. 1.

HPLC of the ecdysone metabolites in haemolymph and in whole ticks, 24 hours after injection into ticks from day 6 postfeeding. 5 major radioactive fractions (1–5) were detected. The positions of several ecdysteroid standards are indicated by arrows: ES (Ecdysterone), M (Makisterone A), E (Ecdysone), and 2D (2-deoxy-ecdysone).

active derivatives comigrated in TLC with the derivatives from the unlabelled standard. From these results we can conclude that the tick is indeed capable of hydroxylating ecdysone at C_{20} to ecdysterone.

Fraction 4 has been tentatively identified as 20,26-diOH-ecdysone. The tick metabolite shows the same retention characteristics in TLC or HPLC as the metabolite from *Pieris* nymphs which was identified by LAFONT *et al.* (1980) as 20,26-diOH-ecdysone.

Fraction 5 contains several polar metabolites. In contrast to the products in fractions 1-4 their retention times depend on the pH of the mobile phase. They become less polar with decreasing pH (Fig. 2). They are thus likely to be ionisable substances of yet unknown chemical nature (acids ?). However, judging from the HPLC retention times, we can apparently exclude in the tick the presence of the acidic polar metabolites 26-COOH-ecdysone and -ecdysterone which were identified in *Pieris* nymphs (LAFONT *et al.*, 1980).

Ecdysone metabolism at various stages during the molt cycle.

In a second experiment the metabolism of ^{3}H-ecdysone was studied at various stages throughout the molt cycle (Fig. 3). Between 35-53 % of the ecdysone was present 3 hours after injection into nymphs from the first 4 days postfeeding when the endogenous ecdysteroid concentration is low (GERMOND *et al.*, 1982). After this period the ecdysone seems to be metabolized more slowly as 53-78 % of the hormone could still be found after 3 hours of incubation. This corresponds to the period of high (day 5-6) and decreasing endogenous ecdysteroid titer (day 7-day 9 postfeeding).

Three hours postinjection, all the radioactive metabolite fractions (1, 3, 4, 5) described above could already be detected at all stages throughout the molt cycle. Ecdysterone and the apolar metabolites were the most important fractions.

After 24 hours of metabolism, only between 5-11 % of the injected ecdysone was left in all stages examined. Fraction 3 (ecdysterone) and fraction 4 (20,26 diOH-ecdysone ?) together constituted in most stages only a relatively minor part (8-20 %) of the total recovered radioactivity with ecdysterone being always more prominent. However, ticks injected at days 5, 6 or 7 accumulated comparatively more radioactivity in these 2 fractions (24-34 %). The ecdysterone fraction got most heavily labelled during the 7th day of the molt cycle (29 %) whilst fraction 4 reached its maximum value (9 %) during the 8th day.

An inverse relationship seems to exist between the radioactivity of the apolar fraction (1) and that of the polar products at different stages of the molt cycle. After 24 hours of incubation the apolar products represented always a very important metabolite fraction, especially in ticks injected at days 0-4 and days 9-10 postfeeding. On the other hand, the polar products became comparatively much more prominent in nymphs during the 7th-9th day when the endogenous ecdysteroid concentration drops.

Thus, most of the metabolized ^{3}H-ecdysone accumulated in the apolar fraction and, in addition, also in the polar fraction during days 7-9. Indeed, both fractions together represented at all stages between 54-86 % of the total radioactivity. We may therefore conclude that these two fractions contain the endproducts of the ecdysone metabolism.

The metabolic pathway of ecdysone.

We propose that the ecdysterone produced in the first metabolic step is further hydroxylated to compound 4 which is tentatively identified as 20,26-diOH-ecdysone

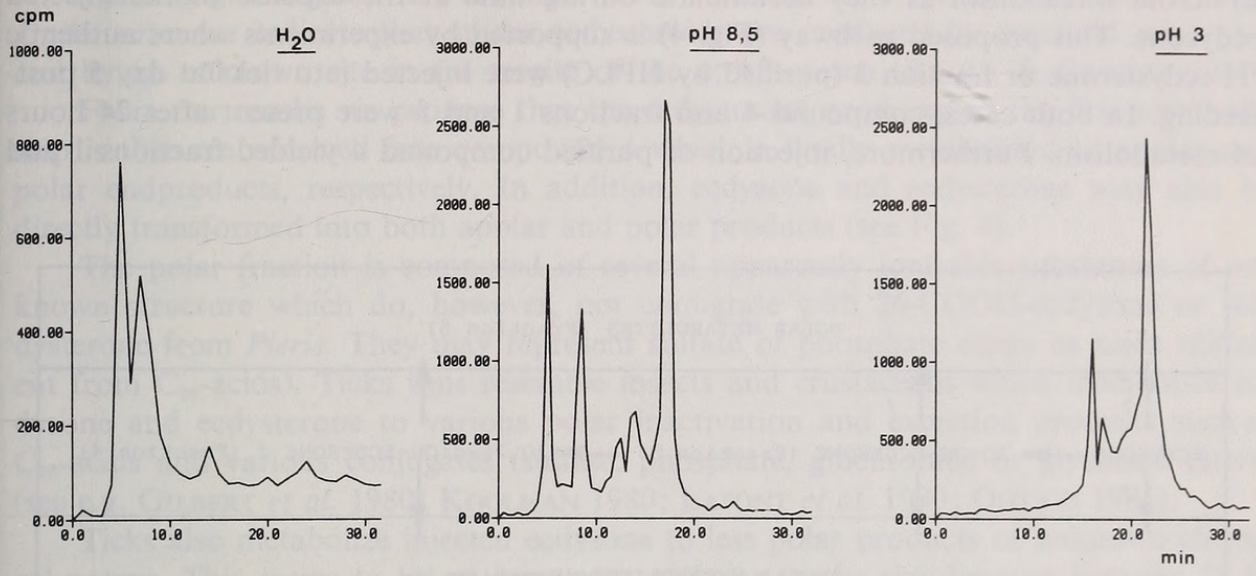

FIG. 2.

Influence of the pH of the mobile phase on the HPLC retention times of the polar ecdysone metabolites (Fraction 5) from ticks injected at day 6 postfeeding. The polar metabolites were obtained by purification through a SEP PAK C_{18} cartridge (elution with 20% methanol).

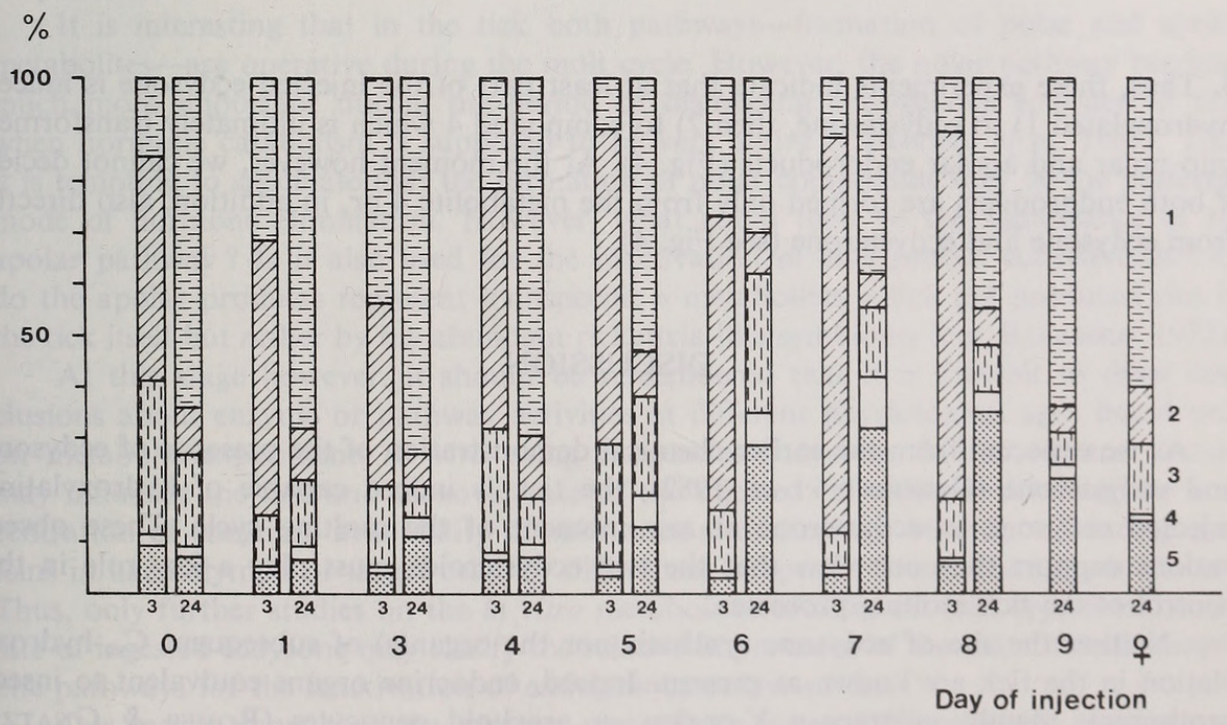

FIG. 3.

The radioactivity of the 5 different ecdysone metabolite fractions 1–5 expressed in % of the total recovered radioactivity after HPLC. Ticks were injected at several stages after the blood meal. ♀: freshly molted females. The animals were extracted 3 or 24 hours after the injection (3–4 ticks per assay).

(see Fig. 4). The metabolism then proceeds further to yield 1) polar ionisable products, and 2) also apolar substances. These are likely to represent the endproducts of the ecdysteroid metabolism as they accumulate during time at the expense of the injected ecdysone. This proposed pathway (Fig. 4) is supported by experiments where authentic ^{3}H-ecdysterone or fraction 3 (purified by HPLC) were injected into ticks at day 5 post-feeding. In both cases, compound 4 and fractions 1 and 5 were present after 24 hours of metabolism. Furthermore, injection of purified compound 4 yielded fractions 1 and

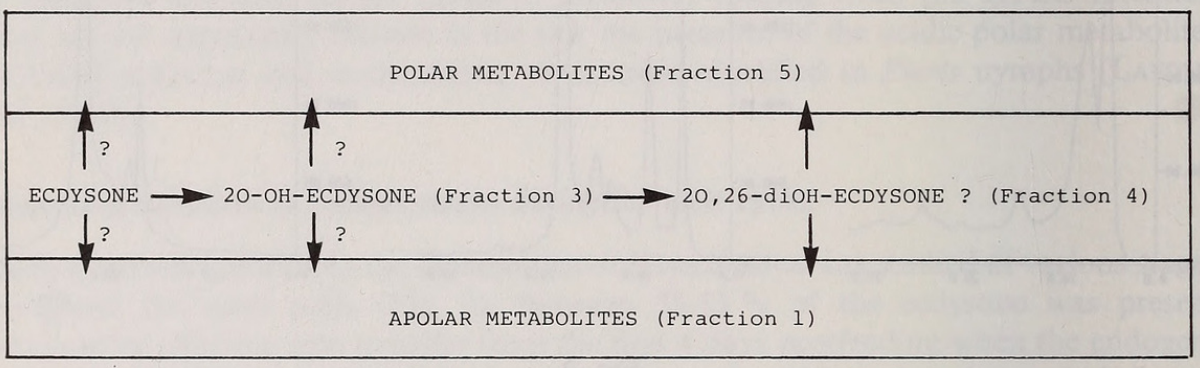

FIG. 4.

The proposed pathway of ecdysone metabolism in the tick. Ecdysone is hydroxylated 1) to ecdysterone, and then probably 2) to metabolite 4 (possibly 20, 26-diOH-ecdysone) which gives then rise to apolar (1) and polar metabolites (5). In addition, ecdysone and ecdysterone may perhaps yield directly apolar and polar products (?).

5. Thus, these experiments indicate that at least part of the injected ecdysone is indeed hydroxylated 1) to ecdysterone, then 2) to compound 4 which is ultimately transformed into polar and apolar endproducts (Fig. 4). At the moment however, we cannot decide if both endproducts are formed only from the metabolite 4 or, in addition, also directly from ecdysone and ecdysterone (see Fig. 4).

DISCUSSION

As we expected from the earlier chemical demonstration of the presence of ecdysone and ecdysterone (GERMOND *et al.* 1982), the tick is indeed capable of hydroxylating injected ecdysone to ecdysterone at any moment of the molting cycle. These observations support thus our view that the two ecdysteroids must play a key role in the control of the tick molting processes.

Neither the site of ecdysone synthesis nor the organ(s) of subsequent C_{20}-hydroxylation in the tick are known at present. Indeed, endocrine organs equivalent to insect prothoracic glands, crustacean Y-organs or arachnid oenocytes (ROMER & GNATZY 1981) remain to be discovered in argasid ticks. This obvious lack of information contrasts with the situation in insects or crustaceans where it appears now well established that synthesis of ecdysone occurs in prothoracic glands and in as yet unidentified extra-prothoracic tissues. The released ecdysone is then hydroxylated to ecdysterone in several peripheral tissues such as fat body or Malpighian tubules (for review see e.g.

GILBERT *et al.* 1980). In crustaceans the ecdysone synthesized by the Y-organs is also hydroxylated in peripheral organs such as the hypodermis, hepatopancreas, gut, or genital organs (e.g. LACHAISE & FEYEREISEN 1976). Furthermore, preliminary results from *Opilio ravennae* indicate that spider ecdysteroids are produced by arachnid oenocytes and by an unknown site in the tergite of the opisthosoma (ROMER & GNATZY 1981).

From our results we propose that the ecdysterone produced in the first metabolic step is then transformed into compound 4 which is finally metabolized to apolar and polar endproducts, respectively. In addition, ecdysone and ecdysterone may also be directly transformed into both apolar and polar products (see Fig. 4).

The polar fraction is composed of several apparently ionisable substances of unknown structure which do, however, not comigrate with 26-COOH-ecdysone or -ecdysterone from *Pieris*. They may represent sulfate of phosphate esters or acids (different from C_{26}-acids). Ticks thus resemble insects and crustaceans which metabolize ecdysone and ecdysterone to various polar inactivation and excretion products such as C_{26}-acids and various conjugates (sulfate, phosphate, glucuronide or glycoside esters) (see e.g. GILBERT *et al.* 1980; KOOLMAN 1980; LAFONT *et al.* 1980; OHTAKI 1981).

Ticks also metabolize injected ecdysone to less polar products of unknown chemical nature. This seems to be an important pathway in the tick because between 28 % and 73 % of the total radioactivity may be found in this fraction. This is in contrast to the situation in other arthropods where the pathway leading to less polar metabolites appears to be generally of minor importance. In several insects (*Locusta*, *Drosophila*, *Calliphora*, *Daphnis*, *Periplaneta)* ecdysone and ecdysterone can be converted to 3-dehydro-ecdysone and -ecdysterone (FEYEREISEN *et al.* 1976; KOOLMAN 1978) or even to less polar products (unidentified product R in *Locusta :* FEYEREISEN *et al.* 1976). The authors suggest that this reaction leads to the inactivation of ingested exogenous ecdysteroids.

It is interesting that in the tick both pathways—formation of polar and apolar metabolites—are operative during the molt cycle. However, the polar pathway becomes much more important during the period of decreasing endogenous ecdysteroid titer when hormone catabolism is supposed to be very active (GERMOND *et al.* 1982). Thus it is tempting to speculate that the formation of polar compounds may be the preferred mode of hormone elimination. However, what could then be the significance of the apolar pathway ? Is it also used for the inactivation of endogenous ecdysteroids ? Or do the apolar products represent « unspecific » metabolites which are produced not by the tick itself but rather by the abundant rickettsia-like symbionts (e.g. BALASHOV 1972) ?

At this stage however, it should be remembered that it is difficult to draw conclusions about enzyme or pathway activities at different physiological ages based only on metabolic experiments *in vivo* using exogenous ecdysone. Indeed, several factors may influence the fate and turnover rate of the injected ^{3}H-ecdysone like e.g. the concentration of enzymes, availability of substrates and enzymes, binding to binding proteins in haemolymph or target cells, or differences in speed of haemolymph circulation. Thus, only further studies on the *in vitro* metabolism of different tick organs or on the fate of ingested ecdysone may clarify the relative importance of the two different metabolic pathways for the inactivation of endogenous ecdysteroids.

Our results show that the greatest part of the injected ^{3}H-ecdysone is converted into polar or apolar metabolites during an incubation period of 24 hours as most of the radioactivity (80-90 %) is recovered during extraction and chromatography. Thus it is likely that the two other possibilities of hormone inactivation present in insects —namely 1) excretion of unchanged hormone and 2) side chain cleavage (see e.g. GILBERT *et al.* 1980; OHTAKI 1981)—do not play a significant role in the tick.

Acknowledgements

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