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Chemistry, function and metabolism of tick ecdysteroids

P. A. Diehl, J.-L. Connat, and E. Dotson

7.1 INTRODUCTION

Ecdysteroids have been reported in many invertebrate groups including arthropods (Hetru & Horn 1980), molluscs (Romer 1979), nematodes (Koolman *et al.* 1984, Rees & Mendis, 1984), trematodes (Koolman *et al.* 1984, Nirdé *et al.* 1984), annelids (Porchet *et al.* 1984), and coelenterates (Sturaro *et al.* 1982). They have been detected in archaic arthropods such as xyphosurans (*Limulus*: Jegla & Costlow 1979) and pantopods (*Pycnogonum*: Behrens & Bückmann 1983), in myriapods (*Lithobius*: Joly *et al.* 1979, Leubert *et al.* 1982, *Hanseniella*: Juberthie-Jupeau *et al.* 1979), and in arachnid groups such as spiders (*Pisaura*: Bonaric & De Reggi 1977) and ticks (*Amblyomma*: Delbecque *et al.* 1978a, *Ornithodoros*: Germond *et al.* 1982). Except for arthropods, their physiological role has not yet been elucidated. Also, it is often not known if the ecdysteroids are synthesized *de novo* by the animals or whether they are derived from food.

Ecdysteroids have also been detected in many plants, sometimes even in surprisingly high concentrations (Bergamasco & Horn 1983). They could play a protective role directed against nonadapted phytophagous predators through interference with their hormonal system.

Much work has been devoted to the elucidation of the chemistry, biosynthesis, action, and metabolism of ecdysteroids in insects and crustaceans, as found and reviewed in excellent, recently published books edited by Downer & Laufer (1983), Hoffmann (1980), and Hoffmann & Porchet (1984). In contrast, detailed reports on ecdysteroids in organisms other than insects or crustaceans are still rare. Since the last review on tick hormones by Solomon *et al.* (1982), much new information about ecdysteroids

has become available. In this review we will first describe the effects of exogenous hormones on various processes such as diapause, molting, oogenesis, spermatogenesis, salivary gland degeneration, attachment and multiplication of cultured tick cells. Then we will present data on the chemical characterization and quantification of the hormones in various life stages and discuss their possible functions. The last part of the review will be devoted to the biosynthesis and the metabolism of ecdysteroids.

7.2 EFFECTS OF EXOGENOUS ECDYSTEROIDS

Owing to the availability of several ecdysteroids, many observations on the effects of exogenous hormones in ticks have been published. Part of this literature has already been discussed by Solomon *et al.* (1982).

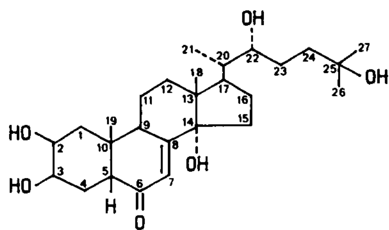
7.2.1 Termination of larval diapause

Wright (1969) was the first person to demonstrate an effect of exogenous ecdysteroids in ticks. He was able to terminate larval diapause in *Dermacentor albipictus* by external application of ecdysone (structure in Fig. 7.1(1)), 20-hydroxyecdysone (Fig. 7.1(2)), or 22,25-dideoxyecdysone (Fig. 7.1(3)). A similar effect of ecdysone or 20-hydroxyecdysone was noted in *Rhipicephalus sanquieneus* by Sannasi & Subramoniam (1972).

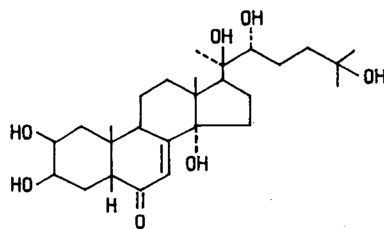
7.2.2 Influence on molting

In vitro feeding with porcine blood containing 20-hydroxyecdysone accelerated molting in nymphs of the argasid *Ornithodoros porcinus* (Mango, in Solomon *et al.* 1982). Similar treatment of *O. parkeri* nymphs had no effect on the molting period, but did induce a few second molts without an additional blood meal (Campbell & Oliver 1984). Also we observed no effect on the length of the molting period when fifth stage nymphs of *O. moubata* were fed on porcine blood containing 1 μg/ml of 22,25-dideoxyecdysone, a synthetic ecdysteroid which is highly active for the induction of supermolting of females (see below). As in *O. parkeri*, these nymphs underwent additional molts without taking another blood meal; 20% showed one, 50% two, and 30% three additional molts. The ticks were, however, unable to ecdyse successfully and eventually died imprisoned in their cuticles. In addition, these ticks frequently showed malformations or even an absence of appendages.

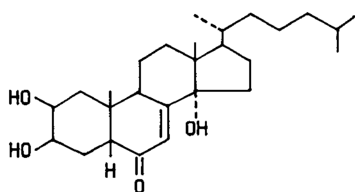
In ixodid *Hyalomma dromedarii* nymphs, topical application of 10 or 20 μg/tick of 20-hydroxyecdysone during or after feeding speeded up molting considerably. Applications of 1 or 5 μg had a similar effect, but only when applied to feeding nymphs 3 days after attachment to the host (Khalil *et al.* 1984). On the other hand, injection of 10 to 100 ng of 20-hydroxyecdysone into replete nymphs did not influence molting (Dees *et al.* 1984a). Furthermore, no effect was noted when *Dermacentor variabilis* nymphs were treated topically (1 or 10 μg/tick) or by injecting 10 or 100 ng of 20-hydroxyecdysone into each tick (Dees *et al.* 1984a; McDaniel & Oliver 1978).



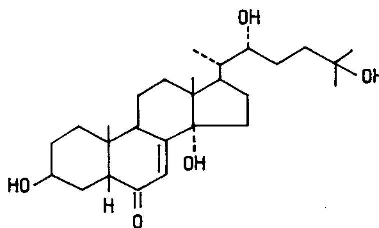
1) ECDYSONE



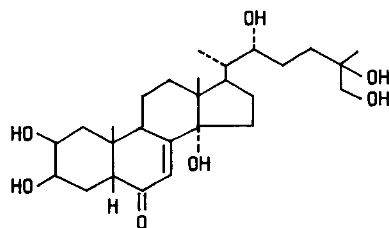
2) 20-HYDROXYECDYSONE



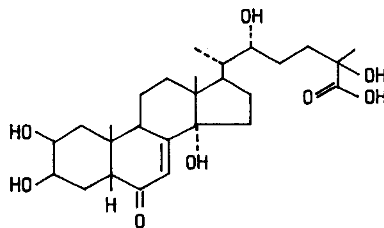
3) 22,25-DIDEOXYECDYSONE



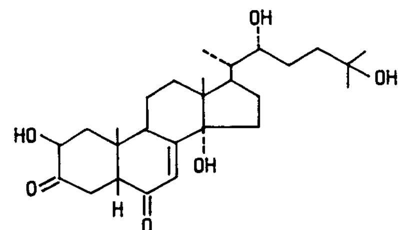
4) 2-DEOXYECDYSONE



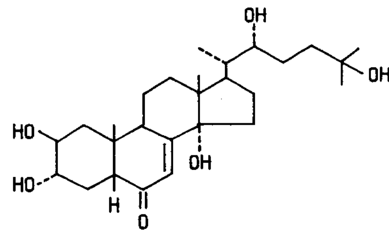
5) 26-HYDROXYECDYSONE



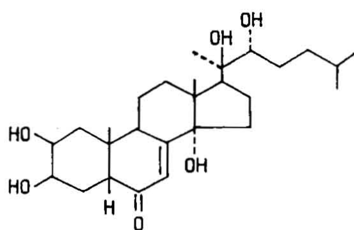
6) ECDYSONOIC ACID



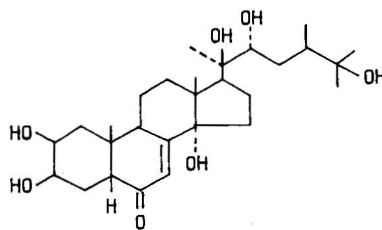
7) 3-DEHYDROECDYSONE



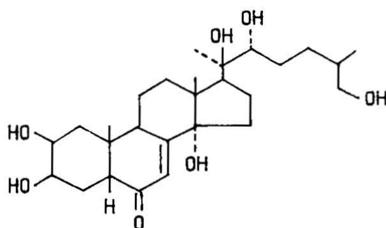
8) 3-EPIECDYSONE



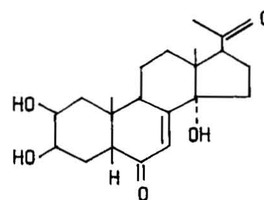
9) PONASTERONE A



10) MAKISTERONE A



11) INOKOSTERONE



12) POSTSTERONE

Fig. 7.1 — Structural formulae of several ecdysteroids with their trivial names. The systematic name of ecdysone (1) is 2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-ene-6-one.

7.2.3 Supermolting of adults

Ordinarily, adults ticks do not molt. However, several argasids, but not ixodids, have been induced to supermolt experimentally either by topical application, ingestion, or injection of various ecdysteroids (Ahmed & Bassal 1982, Campbell & Oliver 1984, Connat *et al.* 1983, Kitaoka 1972, Mango *et al.* 1976, Pound *et al.* 1984, Solomon *et al.* 1982).

Great differences of sensitivities in inducing supermolting were noted, depending on the nature of the hormone, the type and timing of application, and the tick species. Some ticks are very sensitive, e.g. injection of only 1 ng of 20-hydroxyecdysone in olive oil into the hemocoel of *O. parkeri* produced supermolting (Pound *et al.* 1984). On the other hand, feeding on blood containing at least 6 μg/ml was needed to produce the same effect (Campbell & Oliver 1984). *O. moubata* proved to be extremely sensitive to ingested 22,25-dideoxyecdysone. About 15–20 ng provoked molting in every female. However, when applied topically, even 5 μg per tick showed no effect. In contrast to 22,25-dideoxyecdysone, this tick was very resistant to ingested ecdysteroids containing a 22-OH group like ecdysone, 20-hydroxyecdysone, ponasterone A (Fig. 7.1(9)), or makisterone A (Fig. 7.1(10)), presumably because of an efficient detoxification system in the midgut (Connat *et al.,* 1986). Doses of at least 10 μg/ml (makisterone A) or 20 μg/ml

(ecdysone, 20-hydroxyecdysone) were needed for supermolting (Connat *et al.* 1983). On the other hand, only 50 to 100 ng of ecdysone were necessary when injected into the hemolymph (see Chapter 8). Surprisingly, when compared with the high activity of 22,25-dideoxyecdysone, ingestion of the synthetic 2,22,25-trideoxyecdysone and of 2,22,25-trideoxy-5β-hydroxyecdysone at a dose of 5 μg/ml blood did not produce supermolting (unpublished results). The reason for their ineffectiveness is not yet clear.

In *O. porcinus*, supermolting apparently resulted in perfectly healthy specimens which could successfully feed, supermolt, and lay eggs. It has been speculated that supermolting may occur in nature when the host feeds on plants containing large amounts of phytoecdysteroids (Mango *et al.* 1979). In contrast, supermolting in *O. moubata* frequently resulted in malformations or absence of appendages and failure of ecdysis which rendered subsequent bloodmeals impossible (Connat *et al.* 1983). Ecdysis failures were also reported in *O. parkeri* (Campbell & Oliver 1984).

7.2.4 Effects on oogenesis

In addition to supermolt induction, exogenous ecdysteroids also influence argasid oogenesis (Connat *et al.* 1983, and Chapter 8, Kitaoka 1972, Solomon *et al.* 1982). It appears that molting and vitellogenesis are mutually exclusive processes. For example, in the presence of ingested 22,25-dideoxyecdysone, the ovary of *O. moubata* delayed vitellogenesis until after ecdysis, and fewer eggs reached maturity. After ingestion of high doses of ecdysone, 20-hydroxyecdysone, ponasterone A, or makisterone A, oogenesis was totally suppressed (Connat *et al.* 1983). Injection of small amounts (50 or 100 ng) of ecdysone into the hemocoel during full vitellogenesis on day 8 post-feeding induced egg resorption (see Chapter 8). In contrast to *O. moubata*, the ovary of *O. porcinus* resorbed the already developed eggs during the supermolt cycle (Solomon *et al.* 1982).

Surprisingly, ingested 2,22,25-trideoxyecdysone and 2,22,25-trideoxy-5β-hydroxyecdysone did not inhibit vitellogenesis in *O. moubata.* Doses of 1–5 μg/ml, however, induced egg desiccation, suggesting an influence on the process of egg waxing (see Chapter 8).

Exogenous ecdysteroids can also influence oogenesis in ixodids. For example, Mansingh & Rawlins (1977) observed high mortality and severe inhibition of egg production in *Boophilus microplus* after injection of 120 ng/tick of 20-hydroxyecdysone. Kitaoka (1972), however, did not see any effect when doses of 0.4–2 μg/tick of 20-hydroxyecdysone, ponasterone A, or inokosterone (Fig. 7.1(11)) were injected into the same species.

In *H. dromedarii*, topical application of 20 μg per tick of 20-hydroxyecdysone on day 1 post-attachment caused a drastic reduction of female fecundity and egg hatch (Khalil *et al.* 1984).

7.2.5 Effect on fovea dorsalis development and sex pheromone activity

Injection of 10 ng/tick of 20-hydroxyecdysone into replete *H. dromedarii* nymphs on the day of dropping increased the sex pheromone content in the resulting unfed females. The pheromone gland cells exhibited vesicular

disruption and release of secretory droplets in the cytoplasm characteristic of the actively secreting gland. Dosages of 100 ng did not show this effect (Dees *et al.* 1984a).

In contrast, similar experiments with *D. variabilis* did not increase the sex pheromone content, but induced comparable ultrastructural changes in the foveal gland cells. A correlation between ecdysteroids and foveal gland development may therefore exist (Dees *et al.* 1984b).

7.2.6 Effect on ixodid salivary glands

Perfusion or *in vitro* incubation experiments with ecdysone or 20-hydroxyecdysone strongly suggested that these hormones induce degeneration of the salivary glands in replete females of *Amblyomma hebraeum*. They might therefore be identical to the humoral 'tick salivary gland degeneration factor' (Harris & Kaufman 1981). For more details the reader is referred to Chapter 2 (see also section 7.3.4).

7.2.7 Effects on spermatogenesis and male fertility

In *D. variabilis*, injection of 20-hydroxyecdysone into unfed males increased the number of DNA synthesizing germ cells, suggesting an important role of ecdysteroids in spermatogenic induction (unpublished results, see discussion in Dumser & Oliver 1981). Topical application of 1 to 10 μg/tick of 20-hydroxyecdysone to fed nymphs did not influence spermatogenesis (McDaniel & Oliver 1978).

In contrast, topical treatment of *H. dromedarii* nymphs on day 10 post-engorgement with 1,5,10, or 20 μg of 20-hydroxyecdysone reduced male fertility. A similar detrimental effect was observed after treatment of feeding males with a dose of 20 μg per tick (Khalil *et al.* 1984).

7.2.8 Effects on tissue culture cells

Kurtii & Munderloh (1983) described dose-dependent effects of 20-hydroxyecdysone on two cell lines isolated from *Rhipicephalus appendiculatus* and *Anocentor nitens*. The rate of cell attachment to the culture substrate was reduced. Low concentrations (0.2 or 2 nM) stimulated, but higher concentrations (>200 nM), inhibited growth of the *Ripicephalus* cell line. Decrease of growth in the *Anocentor* cell line was less pronounced. Growth suppression was reversible. The hormone also induced cell detachment and vesicle formation.

7.2.9 Mortality

Several authors have observed mortality induced by the application of exogenous ecdysteroids. The lethal doses were generally high and variable, depending on the tick species, the nature of the hormone, the time of treatment, and the method of application.

Ticks appear to be more sensitive to the injection of hormones. For example, 50 ng of ecdysone injected into the hemocoel of *O. moubata* females on day 8 post-feeding induced 80% mortality (unpublished results). 100 ng of 20-hydroxyecdysone in olive oil injected into the hemolymph of *O. parkeri* females caused 40% mortality (Pound *et al.* 1984). 120 ng of 20-hydroxyecdysone induced high mortality in *B. microplus* females (Mansingh

& Rawlins 1977). Kitaoka (1972), however, did not see any lethal effect when doses of 0.4–2 μg/tick of 20-hydroxyecdysone, ponasterone A, or inokosterone were injected into the same species. Similarly, we observed that higher lethal doses were required for the Biarra strain of *B. microplus* (LD_{50} for 20-hydroxyecdysone: about 3–4 μg). 10 μg/tick provoked death in all females within 24 hours. Injection of 5 μg of 22,25-dideoxyecdysone, 2,22,25-trideoxyecdysone, or 2,22,25-trideoxy-5β-hydroxyecdysone were ineffective. In contrast to injection, topical application of 10 μg/tick of 20-hydroxyecdysone in 4 μl acetone was without effect. This clearly demonstrates that the route through the cuticle is generally much less efficient than injection.

A high sensitivity was noted for *Amblyomma variegatum* when the females were allowed to feed through capillaries on TC 199 containing ecdysone, 20-hydroxyecdysone, or 22,25-dideoxyecdysone before being placed on bovine hosts. For example, 1 μg/tick of ingested 20-hydroxyecdysone induced death in two out of three females. 1 or 10 μg of 22,25-dideoxyecdysone had no effect. Topical applications of 10 μg of 22,25-dideoxyecdysone or 50 μg of ecdysone per tick were ineffective. 50 θg of 20-hydroxyecdysone produced smaller replete females (unpublished results).

In conclusion, exogenous ecdysteroids exert several effects in ticks. In particular, the induction of supermolting and acceleration of molting, and the induction of salivary gland degeneration, demonstrate that ecdysteroids might be true endogenous tick hormones.

7.3 CHEMISTRY AND FUNCTIONS OF TICK ECDYSTEROIDS

Rapid progress in our knowledge of arthropod ecdysteroid chemistry, biosynthesis, metabolism, and mode of action has recently become possible through the introduction of several powerful analytical methods such as radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry or fragmentography, and NMR spectroscopy. For detailed reviews see Hetru & Hoffman (1983), Lafont & Koolman (1984), and Lafont *et al.* (1980). The application of these methods has allowed substantial progress to be made in our knowledge of tick ecdysteroids. In this section we will discuss the chemistry and the possible functions of these hormones for each of the various life stages.

7.3.1 Ecdysteroids in larvae

At present, very little is known about ecdysteroids in larvae. One of us (E.D.) studied the temporal correlation between ecdysteroid titer and the integument structure during the larval development of *O. moubata* at 28°C. Preliminary results indicated that only small amounts (about 13 pg of 20-hydroxyecdysone equivalents per larva) were present during the first 40 hours of development as measured by the RIA of De Reggi *et al.* (1975). Rising titers were observed during apolysis. High titers of about 70 pg/larva were present between 48–72 hours during epicuticle synthesis. The concen-

tration decreased during procuticle synthesis to basal values (about 13 pg) after 80–88 hours, shortly before ecdysis (which occurred after about 128 hours). The fluctuation in hormone titers in relation to cuticular events are very similar to the ones observed in fifth stage nymphs (see section 7.3.2). The chemical nature of the RIA-positive material has not yet been determined.

Dees *et al.* (1984b) found that RIA titer values in crude extracts rose from about 1.1 ± 0.3 pg in unfed *D. variabilis* larvae to a maximum of 23.5 ± 8.9 pg 20-hydroxyecdysone equivalents per tick on day 2 post-feeding. Three-week old unfed nymphs again had low values of 1.7 ± 1.2 pg/tick (Horn I-1 ecdysone antiserum from J. D. O'Connor). HPLC analysis in reverse phase mode demonstrated a RIA-positive fraction migrating like 20-hydroxyecdysone. Another very polar immunoreactive fraction was also present. Suprisingly, the ecdysteroid concentrations were considerably higher after fractionating extracts with HPLC.

7.3.2 Ecdysteroids in nymphs

Germond *et al.* (1982) performed a detailed study on the temporal correlation between integument structure and ecdysteroid titers in fifth stage nymphs of *O. moubata*. The titers, as measured by the RIA of De Reggi *et al.* (1975), in hemolymph or in whole animals were low during the first 3 days post-feeding. We were not able to confirm the small peak reported in an earlier preliminary note (Germond *et al.* 1980). During this period the hypodermal cells increased considerably in size, deposited a few nymphal procuticle lamellae (day 2–3), and initiated the mitotic period on day 2. The hormone titer began to increase between days 3 and 4, with the mitotic period ending on day 4. Apolysis and formation of the exuvial space took place during day 4–5 concomitant with sharply increasing hormone titers. Highest concentrations of about 500 pg 20-hydroxyecdysone equivalents per μl hemolymph and about 11 ng per tick were measured during deposition of the adult epicuticle between day 5–6. The titer began to drop with the beginning of procuticle deposition and digestion of the nymphal cuticle. It reached low values again shortly before ecdysis on day 9–10 post-feeding. Monitoring of the different HPLC fractions by RIA showed that the majority of the immunoreactive material migrated like ecdysone, and predominantly 20-hydroxyecdysone. This was confirmed by GC of the sylilated compounds. In addition, 20-hydroxyecdysone was also identified by the powerful method of GC coupled to mass fragmentography.

Molting hormone activity was also found in the hemolymph of fifth instar nymphs of *O. porcinus* by the *Musca* test and by RIA (Solomon *et al.* 1982). These authors reported an early low peak during day 3–5 post-feeding and a second higher peak at the time of general apolysis (day 9–11) preceeding ecdysis by several days. However, the correlation of the hormone peaks with epicuticle or procuticle deposition was not determined.

Ecdysteroids were also detected in nymphs of the ixodid *A. hebraeum* (Delbecque *et al.* 1978a; Diehl *et al.* 1982). As in the argasid nymph *O. moubata*, rising hormone titers paralleled apolysis and high concentrations

correlated with the beginning of adult cuticle deposition. Procuticle synthesis occurred during falling titers of ecdysteroids. A comparable situation has also been seen to occur in *A. variegatum* (Ellis & Obenchain 1984) and *D. variabilis* (Dees *et al.* 1984b).

Most of the immunoreactive material in *A. hebraeum* nymphs migrated like ecdysone and mainly 20-hydroxyecdysone on HPLC reverse phase columns (Diehl *et al.* 1982). This is in accordance with previous observations which identified these two hormones by the use of GC combined with mass spectroscopy or mass fragmentography (Delbecque *et al.* 1978a).

HPLC studies in *D. variabilis* nymphs demonstrated the presence of 3 RIA-positive fractions. One fraction presumably corresponds to 20-hydroxyecdysone because on reverse phase columns it showed the same retention time as the authentic reference compound; the two other fractions were more polar (Dees *et al.* 1984b).

In conclusion, the molting hormones ecdysone and predominantly 20-hydroxyecdysone are present in immature stages of argasid and ixodid ticks, thus resembling most other arthropods where 20-hydroxyecdysone is thought to be the active molt-controlling hormone. Some bugs like *Dysdercus* or *Oncopeltus*, however, are an exception to this general rule because they use makisterone A (Aldrich *et al.* 1982, Gibson *et al.* 1983). In addition, the tick ecdysteroids shows fluctuating titers which temporally correlate with molting events in a manner resembling insects or crustaceans. These observations and the ability of exogenous hormones to induce shortening of the molting period or provoke supermolting in argasids demonstrate that ecdysteroids probably play an important role in the humoral control of tick molting. However, evidence is still based mainly on the analogy to the insect or crustacean model. Thus, to substantiate the hypothesis further, more conclusive evidence such as the existence of specific hormone receptors or an induction of molt-specific genes is clearly needed.

In addition to their role in molting, the ecdysteroids might also be important in the control of spermatogenesis in developing nymphs (unpublished observations in *D. variabilis*, and see discussion in Dumser & Oliver 1981).

7.3.3 Ecdysteroids in males

Very little work has been done on ecdysteroids in males. In *D. variabilis*, the titer of RIA-positive material in crude extracts decreased from 386 ± 122 pg 20-hydroxyecdysone equivalents per tick at emergence to 38 ± 3 pg 2 weeks later. Males on day 8 post-feeding showed slightly higher values of about 131.6 ± 2.3 pg per male. On the other hand, considerably higher values were obtained after HPLC purification (Dees *et al.* 1984b). Similarly, in unfed *H. dromedarii*, titers in crude extracts decreased from 0.87 ± 0.39 ng 20-hydroxyecdysone equivalents in emerging males to 0.48 ± 0.14 ng per male at day 15 post-emergence. Slightly higher values of 2.03 ± 0.51 ng per tick were recorded from 15-day old males which when ecdysing from nymphs had been injected with 10 ng of 20-hydroxyecdysone (Dees *et al.* 1984a).

The only available information on the nature of male ecdysteroids comes from a preliminary study employing HPLC chromatography on reverse phase columns. It revealed three RIA-positive fractions in *D. variabilis*. One was migrating like 20-hydroxyecdysone; the two others were more polar (Dees *et al.* 1984b).

Dumser & Oliver (1981, see discussion) hypothesized that ecdysteroids might be important for the control of spermatogenesis in males. This was based on unpublished observations where injected 20-hydroxyecdysone provoked a very large increase in DNA synthesizing germ cells in unfed *D. variabilis* males. Furthermore, the presence of ecdysteroids in the hemolymph was detected, although the nature and quantity of the hormones and the detection method were not stated. More work is thus needed to explore the interesting possibility that molting hormones might be involved in the control of tick spermatogenesis.

7.3.4 Ecdysteroids in females

A detailed study on the ecdysteroids present during the single gonotrophic cycle of an ixodid tick was performed with *A. hebraeum* (Connat *et al.* 1985). During the first three days of the bloodmeal, the body weight increased slightly, and the cuticular dry weight remained stable. During this time hypodermal cells enlarged considerably, but did not yet secrete new endocuticle. This first phase was characterized by very low titers of about 12 or less pg 20-hydroxyecdysone equivalents per µl hemolymph as in unfed females. The ecdysteroid content in whole ticks increased slightly between days 2 and 3 from about 0.5 ng to 1 ng per female as determined by RIA (De Reggi *et al.* 1975).

The following 4 days showed a moderate increase of body weight. On the other hand, an important synthesis of endocuticular material took place, thus increasing the cuticle dry weight 4.3-fold. During this period hemolymphatic ecdysteroid levels remained low, but titers in whole animals began to rise quickly after day 5. At present it is impossible to decide if the slightly increased hormone level in whole ticks after day 2 might be responsible for the resumption of endocuticle synthesis. This seems rather unlikely, however, because in most immatures of ticks or other arthropods, procuticle synthesis proceeds generally in the presence of low ecdysteroid concentrations.

The expansion phase during the last 12–24 hours is characterized by the ingestion of a huge amount of blood. This was accompanied by a further increase of the hormone level in whole animals to about 10 ng per tick, while hemolymphatic levels remained low. It is not yet known if the increasing hormone concentration could act as a signal for the induction of rapid feeding.

Within about 4 days after dropping from the host, the salivary glands had degenerated in parallel with vitellogenesis which was initiated after day 3. During this period, ecdysteroid concentrations increased considerably in the hemolymph (Fig. 7.2) and in whole body extracts. This could thus indeed be the humoral signal for salivary gland degeneration as suggested strongly

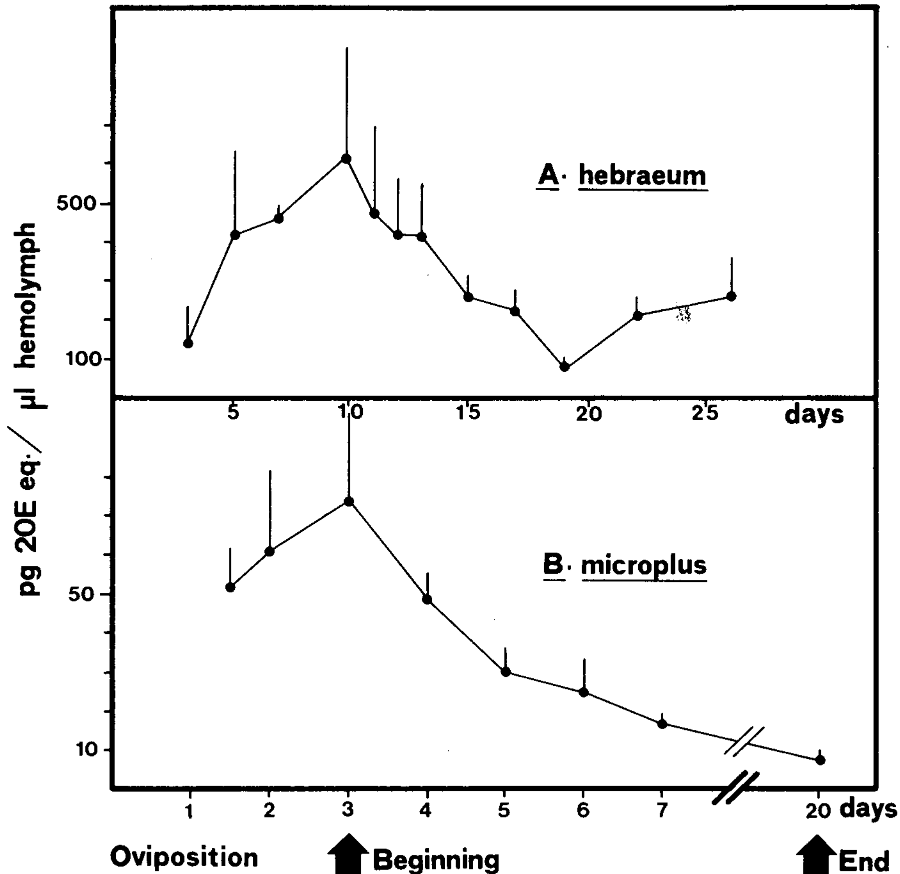


Fig. 7.2 — Hemolymphatic ecdysteroid levels during vitellogenesis and oviposition in females of *Amblyomma hebraeum* and *Boophilus microplus* (Biarra strain). The titers were measured with the RIA of De Reggi *et al.* (1975) at different days post-detachment and are expressed in pg 20-hydroxyecdysone equivalents per µl hemolymph. Each point corresponds to the mean ± S.D. of measurements from 3 females.

by the results of Kaufman's group (see Chapter 2). In addition, rising hormone levels could also play a role in oogenesis as, for example, the induction of vitellogenin synthesis in several mosquitoes or flies (see review by Hagedorn 1983).

During the following period of intense vitellogenesis and oviposition, the ecdysteroid titers in the hemolymph rose to a peak of about 600 pg per µl one day prior to oviposition and decreased thereafter (Fig. 7.2). Hormone levels in whole females continued to increase. About 700 ng of RIA-positive material was found in a single female and its eggs several days after the beginning of oviposition.

The ovary accumulated most of the RIA-positive material. Freshly laid eggs contained about 2744 ± 676 (SD) pg 20-hydroxyecdysone equivalents per mg. It was estimated that a female produced about 3.6 µg of 20-hydroxyecdysone equivalents during the gonotrophic cycle.

HPLC-RIA showed that the majority of the free ecdysteroids in hemolymph, ovary, and eggs were composed of ecdysone and mainly 20-hydroxyecdysone. This was confirmed by capillary GC combined with mass fragmentography. In addition, injection of tritiated ecdysone or 20-hydroxyecdysone at the beginning of oviposition led to an accumulation of free hormones in the freshly laid eggs (see section 7.5, Fig. 7.5c). Furthermore, some RIA-positive material less polar than ecdysone was also detected by HPLC. It may correspond to as yet unidentified precursors or perhaps to lipids interfering in an unspecific manner with the RIA.

Several insects such as *Locusta* (Lagueux *et al.* 1984) or *Schistocerca* (Rees & Isaac 1984) contain large amounts of conjugated ecdysteroids in their eggs. However, in *A. hebraeum* such conjugates, hydrolyzable by *Helix* juice or esterase do not seem to be present. This may indicate that the hormones of *A. hebraeum* females are either mostly in free form as in termite queens (Delbeque *et al.* 1978b) or partly conjugated as molecules not hydrolyzable by these enzymes.

Apart from induction of salivary gland degeneration, the possible significance of these large quantities of free ecdysteroids in *A. hebraeum* females is not yet clear. It may be necessary for vitellogenin synthesis or uptake, egg shell deposition, or maturation of Géné's organ. In addition, the hemolymphatic ecdysteroid peak before oviposition could represent a signal for initiating egg laying as reported in *Rhodnius* (Ruegg *et al.* 1981). Furthermore, the ecdysteroids transferred in large quantities into the eggs might represent a hormone source for the embryo (see section 7.3.5).

Very interestingly, a different situation has been observed in *B. microplus* females (Wigglesworth *et al.* 1985). In whole ticks, a major peak of RIA-positive free ecdysteroids (150–300 ng ecdysone equivalents/tick) was found just before complete engorgement, with very few polar conjugated ecdysteroids being detected. At peak titer, ecdysone was the major hormone, together with small amounts of 20-hydroxyecdysone. This decrease in titer of immunoreactive ecdysteroids before repletion may be accounted for by intense esterification of the hormones with fatty acids. These esters accumulate in the eggs, together with much smaller quantities of free hormones. Similarly, we found, in a preliminary comparative study, an accumulation of apolar hormone conjugates in eggs after injection of tritiated ecdysteroids into females of the Biarra strain of *B. microplus* (see section 7.5, Fig. 7.5e). In addition, we also noticed low titers of RIA-positive material in the hemolymph as compared with the titers found in *A. hebraeum* (Fig. 7.2). As in the latter tick, a peak of about 29.3 ± 7.5 pg ecdysone equivalents per µl (or about 70 pg 20-hydroxyecdysone equivalents per µl) was observed near the beginning of oviposition. However, as reported for whole females or eggs, much of this material was apparently esterified, because treatment with pig liver esterase increased the RIA response 10–15 times. Eggs from the Biarra strain contained lower amounts than *A. hebraeum* when measured in crude methanolic extracts (26 ± 2 pg ecdysone equivalents per mg eggs; or 65 ± 4 pg 20-hydroxyecdysone equivalents per mg). Hydrolysis with esterase increased the hormone

concentration to 2–5 ng ecdysone equivalents per mg eggs (or 5–10 ng 20-hydroxyecdysone equivalents per mg) thus confirming the results of Wigglesworth *et al.* (1985).

Therefore, considerable differences between *A. hebraeum* and *B. microplus* females apparently exist. In the former, large quantities of free hormones, mainly 20-hydroxyecdysone, accumulate after repletion in females and eggs with few hydrolyzable hormone conjugates present. In sharp contrast, considerable amounts of free hormones, mainly ecdysone, exist in the latter species prior to complete engorgement which are converted thereafter to esters and transferred to the eggs.

These interesting differences raise many questions. For example, how do ecdysteroids influence salivary gland degeneration in *Boophilus*? It may be that ecdysone is inactive in this respect or that the glands show a delayed response.

Preliminary results by Dees *et al.* (1984b) suggest that *D. variabilis* females resemble *A. hebraeum* with respect to the hormone titer changes. RIA of crude extracts of HPLC–RIA showed a large increase in immunoreactive material near the end of feeding that continues to rise during vitellogenesis. A transient decrease was noticed on day 8 post-attachment. Part of the RIA-positive material appeared to be 20-hydroxyecdysone. In contrast to the other two species, considerable amounts of more polar material were present in two fractions. Very low amounts (68.6 ± 32.9 pg of 20-hydroxyecdysone equivalents per mg) were detected in freshly laid eggs. However, hydrolysis experiments were not performed, and the isocratic HPLC conditions did not elute apolar hormone esters. Therefore, we do not yet know to what extent apolar or polar ecdysteroid conjugates are present in *D. variabilis* females and eggs.

Preliminary experiments using thin layer chromatography combined with RIA, indicate that ecdysone and 20-hydroxyecdysone might be present in *Rhipicephalus appendiculatus* eggs, apparently coupled to a large egg protein (Whitehead *et al.* 1986).

In sharp contrast to immatures or ixodid females, we experienced many unexpected and frustrating difficulties with ecdysteroid analysis in the argasid female *O. moubata* (for a more detailed discussion see Chapter 8). Clearly, RIA-positive material was found in extracts of hemolymph, whole bodies, or eggs. The RIA responses were, however, variable, depending on the treatment before analysis. After simple methanolic extraction, only relatively low values were measured in the hemolymph of vitellogenic females or in eggs (3–7 pg 20-hydroxyecdysone equivalents per mg egg). Part of this material migrated like ecdysone or 20-hydroxyecdysone on reverse phase HPLC columns. However, we have not been able to confirm this by GC combined with mass fragmentography.

In contrast to the confusing situation in normally fed and mated *O. moubata* females, we encountered no difficulties in measuring RIA titer changes in supermolting females. Changes were comparable to the ones in fifth stage nymphs. Very high hemolymph peak titers of about 1800 pg 20-hydroxyecdysone equivalents/μl were temporally correlated with apolysis and deposition of the new epicuticle. Two peaks of RIA-positive material

migrated like ecdysone and 20-hydroxyecdysone (see Chapter 8).

In normally fed mated *O. porcinus* females, the hormone titers 'fluctuated rhythmically around mean background levels throughout the period of oogenesis' when assayed by the *Musca* test. Although this mean value was not quantified, it suggests that only a small amount of ecdysteroid material was present. But like *O. moubata*, hemolymph ecdysteroid content increased suddenly on day 10–12 post-feeding in supermolting females, concomitant with termination of vitellogenesis and induction of egg resorption (Solomon *et al.* 1982).

Thus, in contrast to some ixodid females, it is not known if ecdysteroids are present in argasid females and eggs. If they exist, they must be structurally different from classical hormones or masked in such a manner that they avoid induction of a molting cycle and egg resorption. It will be worth investigating whether molting hormones are used as humoral signals in fed, virgin females where oocyte resorption occurs naturally without any signs of supermolting.

7.3.5 Ecdysteroids in embryos

Clearly, freshly laid eggs of two well studied ixodid species contain maternal ecdysteroids, either mostly in free form (*A. hebraeum*) or in bound form as fatty acid conjugates (*B. microplus*). Very small amounts (68.6 ± 32.9 pg 20-hydroxyecdysone equivalents /mg of immunoreactive material) have been found in *D. variabilis* eggs (Dees *et al.* 1984b). They may, however, contain higher levels than suggested by RIA if the hormones are present as conjugates. This has not been tested. TLC–RIA studies revealed unspecified quantities of possibly free ecdysone and 20-hydroxyecdysone in *R. appendiculatus* eggs (Whitehead *et al.* 1986).

The fate of these maternal ecdysteroids during ixodid embryogenesis has not been elucidated in detail. Dees *et al.* (1984b) noted an increase of RIA-positive material in crude extracts from 3 different embryonic stages in *D. variabilis*. Such an increase, however, was not noted after HPLC–RIA. HPLC revealed the presence of 2 RIA-positive fractions, one migrating like 20-hydroxyecdysone and the other like more polar compounds. Wigglesworth *et al.* (1985), noted the occurrence of distinct hormone peaks during *B. microplus* embryogenesis. The free hormones might be enzymatically hydrolyzed from the conjugates which could therefore serve as a hormone storage form. This would be comparable to *Locusta* embryos where the free hormones are liberated from the maternal conjugates and are used for the control of serosal and embryonic cuticle deposition (Lagueux *et al.* 1979).

Freshly laid eggs of *O. moubata* apparently contain very low amounts of RIA-positive material; it is not clear if this response is due to endogenous ecdysteroids (see section 7.3.4). Preliminary investigations suggest titer variations during embryogenesis. However, owing to the presence of very little immunoreactive material and to a rather poor synchronization of embryos, the studies are not yet complete. In contrast to *B. microplus*, *O. moubata* embryos apparently do not use fatty acid hormone esters as a

potential hormone source, as was speculated (Connat *et al.* 1984); at least not when the developing oocytes were experimentally loaded with such conjugates after injection of tritiated ecdysone or 20-hydroxyecdysone into the hemocoel of vitellogenic females (see section 7.5.2).

Therefore it appears that much more work is required to elucidate the nature, origin, titer changes, and the probable functions of ecdysteroids during tick embryogenesis. One of the possible functions of these hormones might be to control the deposition of extra-embryonic layers or embryonic cuticles as in *Locusta* (Lagueux *et al.* 1979); formation of such structures has been observed in *O. moubata* embryos (Vogel 1975).

7.4 BIOSYNTHESIS OF ECDYSTEROIDS

It is now generally agreed that in most immature insects prothoracic glands or homologous structures synthesize ecdysone from cholesterol or other phytosterols under the influence of a stimulatory prothoracicotropic hormone (for a review see Hoffmann & Hetru 1983). However, in several cases e.g. *Tenebrio* pupae (Delachambre *et al.* 1984), ecdysteroids can also be produced outside the prothoracic glands, possibly in a generalized tissue such as the hypodermis itself, or in oenocytes. In addition, the follicular cells in some females are capable of ecdysone biosynthesis.

In contrast to insects, ecdysone synthesis in crustaceans occurs in Y-organs under the control of an inhibitory hormone from the X-organ/sinus gland complex. Other sources are not precluded (see Spindler *et al.* 1980). In opilionids belonging to the class Arachnid, oenocytes in the walking legs produce ecdysone and 20-hydroxyecdysone (Romer & Gnatzy 1981).

In ticks, nothing is known about the biosynthesis of ecdysteroids, its possible site(s), or its control. Cytological studies suggested several possible endocrine tissues such as the lateral organs, the retrocerebral complex or the peritracheal glands in addition to the 'brain' with its numerous neurosecretory cells (see Chapter 6). However, experiments such as gland ablation, and demonstration beyond doubt of the endocrine nature of the tissue, are still lacking.

Cox (1960) was the first to attempt to demonstrate the humoral control of tick molting. In 1 out of 3 series, 2 fed females and 1 male of the argasid *Ornithodoros turicata* supermolted after implantation of 3–4 'brains' from donor fed 3rd instar nymphs. In addition, by the use of ligatures, molting in some nymphs could be restricted to the anterior part containing the 'brain'. Cox thus concluded that the presumptive hormone was produced in the anterior part by the 'brain'. However, this remains merely as a suggestion because many nymphs molted in both parts even when ligated very early. In addition, it was not determined if the non-molting posterior parts were still viable and responsive to molting hormones. Thus, although the work by Cox suggests that the 'brain' is important for molting control, it does not yield information on the structure of the supposed 'brain' hormone(s) which

might be of neurosecretory nature.

Ellis & Obenchain (1984) tried to study the origin of ecdysteroids in *A. variegatum* nymphs by measuring the amounts of RIA-positive material released into the culture medium by several tissues. Low levels were observed in media from midgut, heart, pericardial tissue, and central nervous system cultures. Titers were higher and variable in medium from fat body associated with tracheal trunk cultures. This evidence suggested that some cell type associated with this explant (e.g. the nephrocytes) could be responsible for ecdysteroid production. This remains speculative, however, because the amounts of RIA-positive material present in the different tissues before or after incubation have not been measured. The results might therefore simply reflect a release, but not biosynthesis, by these tissues. In addition, other tissues, e.g. the hypodermis, were not tested, although in the light of results with *Tenebrio* (Delachambre *et al.* 1984) and *Locusta* nymphs (Porcheron *et al.* 1984) this tissue appears now as a possible site of ecdysone biosynthesis.

In *A. hebraeum* females, the RIA-positive material appeared much later in the ovary than in the rest of the animal (Connat *et al.* 1985). In addition, the vitellogenic ovary avidly took up injected tritiated hormones (see section 7.5). Thus, in contrast to several insects, it appears that the source of maternal ecdysteroids might reside outside the ovary. However, as only total immunoreactivity was measured, we cannot exclude a secretion of low- or non-immunoreactive precursors by the ovary and their subsequent transformation to ecdysone and 20-hydroxyecdysone outside the ovary.

The biosynthetic pathway, presumably from dietary cholesterol up to ecdysone, remains to be elucidated. We noticed, however, in several studies in *O. moubata* nymphs or females, that the possible precursors 22,25-dideoxyecdysone, 2-deoxyecdysone (Fig. 7.1(4)) or ponasterone A did not yield ecdysone or 20-hydroxyecdysone to any appreciable extent, although this has been reported in some insects. Thus, it is possible that these ticks do not utilize these precursors. Alternatively, it may be that these compounds when injected or ingested do not gain access to the ecdysteroid producing tissues.

In short, the site(s), the pathway, and the control of ecdysteroid biosynthesis in ticks are unknown.

7.5 METABOLISM OF ECDYSTEROIDS

After the introduction of powerful new analytical techniques, our knowledge of ecdysteroid metabolism in insects and to a lesser extent in crustaceans increased greatly (for a recent review see Lafont & Koolman 1984). The conversion of ecdysone into 20-hydroxyecdysone by an ecdysone 20-monoxygenase is a reaction occurring in many tissues, but especially in the fat body and the digestive tract. Ecdysone and 20-hydroxyecdysone are further metabolized via several pathways. (1) They are converted into the

corresponding 26-carboxyecdysteroids (ecdysonoic acid (Fig. 7.1(6) and 20-hydroxyecdysonoic acid) via the 26-hydroxylated metabolites (26-hydroxyecdysone (Fig. 7.1(5)) and 20,26-dihydroxyecdysone). (2) Many ecdysteroids become conjugated and form either more polar compounds (e.g. phosphates, acetophosphates, nucleotides, sulfates) or less polar compounds (acetates). (3) Injected ecdysone or 20-hydroxyecdysone can lead in some insects to the formation of 3-dehydroecdysone (Fig. 7.1(7)) or 3-dehydro-20-hydroxyecdysone. (4) Several ecdysteroids can undergo epimerization at the C3 position, leading to formation of 3-epiecdysone (Fig. 7.1(8)) or 3-epi-20-hydroxyecdysone. (5) Side chain cleavage between C_{20} and C_{22} might lead to poststerone (Fig. 7.1(12)), although this appears to be a minor reaction. Most of these metabolites are inactivation products which are excreted by the animal. Others, such as ecdysteroid-22-phosphates and/ or nucleotides, are stored in eggs where they represent a hormone source for the embryo.

Compared with insects or crustaceans, our research on ecdysteroid metabolism in ticks is only beginning. Nevertheless, studies have already led to the identification of ecdysonoic acids and to the discovery of a class of apolar conjugates which is new for arthropods. We shall now review our current knowledge of hormone metabolism for each of the life stages.

7.5.1 Metabolism in nymphs

In fifth stage nymphs of *O. moubata*, injection of tritiated ecdysone into the hemocoel at various times during the post-feeding stage demonstrated the tick's ability of efficiently transforming this hormone to 20-hydroxyecdysone (Bouvier *et al.* 1982, and see Fig. 7.3a,b). The ticks metabolized 20-hydroxyecdysone further presumably to 20,26-dihydroxyecdysone and then to polar compounds of yet unknown chemical nature (Fig. 7.3a–c). These are ionizable, but do not correspond to any of the known polar compounds from insects such as ecdysonoic acids or conjugates hydrolyzable by *Helix* enzymes (e.g. phosphates). This polar pathway was especially active when endogenous ecdysteroid titers were decreasing, leading to an accumulation of polar products in the digestive tract. It is not operative with ingested ecdysteroids and also not in females (see section 5.2). The pathway can thus be hypothesized to act as an important inactivation mechanism for endogenous nymphal hormones.

In a second, but apolar, pathway, injected ecdysone, 20-hydroxyecdysone, or the probable 20,26-dihydroxyecdysone (after HPLC purification) were rapidly converted into apolar metabolites (Bouvier *et al.* 1982; AP2 in Fig. 7.3a) These metabolites were always prominent, constituting from about 30 to 75% of the injected label after 24 hours of metabolism.

Such apolar metabolites were even more rapidly formed after ingestion of ecdysone or 20-hydroxyecdysone (Diehl *et al.* 1985; and see Fig. 7.3d,e). In some experiments, up to 95% of the ingested hormone was already converted to apolar compounds after 24 hours, even with quantities of up to 35 μg/ml blood. Recent studies with ingested 20-hydroxyecdysone

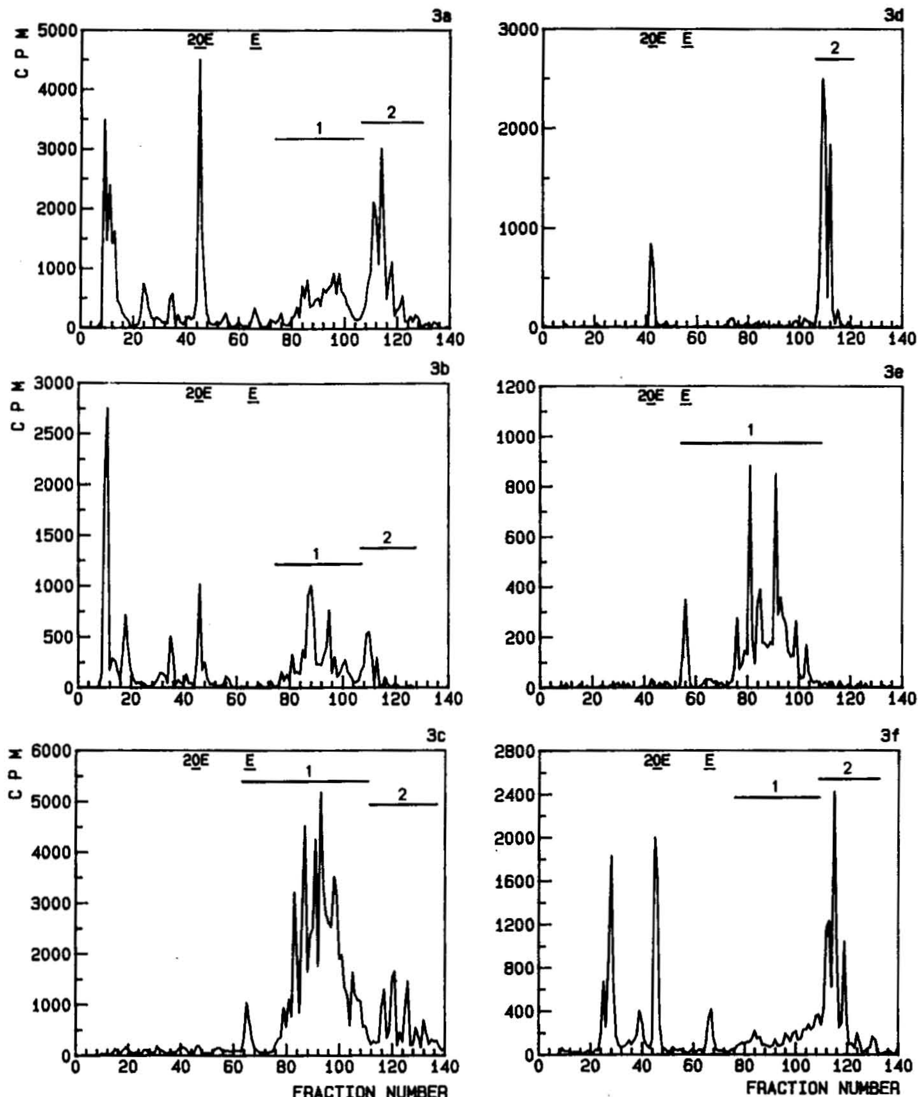


Fig. 7.3 — Metabolism of ecdysteroids in nymphs of *Ornithodoros moubata* and *Amblyomma hebraeum*. Tritiated ecdysteroids and their metabolites were extracted with methanol and subjected to HPLC on a Perkin–Elmer Series 3 chromatograph fitted with a reverse phase column RP-18 (Hibar Lichrosorb RP-18, 7 µm; column dimensions : 4×250 mm). Solvent system: linear gradient of methanol — tris/perchloric acid buffer (20 mM, pH 7.5) at 0.8 ml/min from 30–45% in 10 min, 45% for 15 min, 45–100% for 20 min, followed by 100% methanol for 20 min. Fractions were collected every 30 seconds and their radioactivity (counts per min: CPM) monitored by liquid scintillation counting in a Kontron MR 300 counter. Samples were dissolved in Riatron scintillation cocktail (Kontron; 1:1.75 v/v). The retention times of the internal cold standards ecdysone (labeled E) and 20-hydroxyecdysone (labeled 20E) were monitored with a spectrophotometer operated at 242 nm. For more technical details see Connat *et al.* (1984), or Diehl *et al.* (1985). 3a–c: metabolite pattern in whole animals after injection of ^{3}H-ecdysone (100 000 cpm/animal; sp. activity 50 Ci/mM) into the hemocoel of fifth stage nymphs of *O. moubata*. 3a: injection on day 8 post-feeding, extraction 24 hours later. 3b: ditto, but extraction after 48 hours. 3c: injection 24 hours before feeding, extraction on day 7 post-feeding. Ecdysone was metabolized to 20-hydroxyecdysone and to several polar metabolites (3a,b) and to apolar conjugates AP2 (labeled 2) which are slowly transformed into apolar conjugates AP1 (labeled 1). 3d–e: HPLC radiochromatograms of extracts of fifth stage nymphs of *O. moubata*, 6 hours (3d) and 13 days (3e) after ingestion of 20-hydroxyecdysone (10 µg cold hormone + 100 000 cpm of tritrated hormone/ml pig blood). The ingested 20-hydroxyecdysone was rapidly converted to apolar conjugates AP2 (3d) and later slowly to slightly more polar conjugates AP1 (3e).
3f: metabolite pattern, 6 hours after injection of 100 000 cpm of ^{3}H-ecdysone into *A. hebraeum* nymphs on day 2 post-dropping. The hormone was converted to 20-hydroxyecdysone and to polar compounds (acids ?). Furthermore, apolar conjugates AP2 were rapidly formed, together with a small amount of AP1.

revealed that these apolar compounds (AP2) are conjugates of 20-hydroxy-ecdysone esterified at C_{22} with the common long-chain fatty acids $C_{16:0}$, $C_{18:0}$, $C_{18:1}$ or $C_{18:2}$ (Fig. 7.4; Diehl *et al.* 1985). This represents a new class

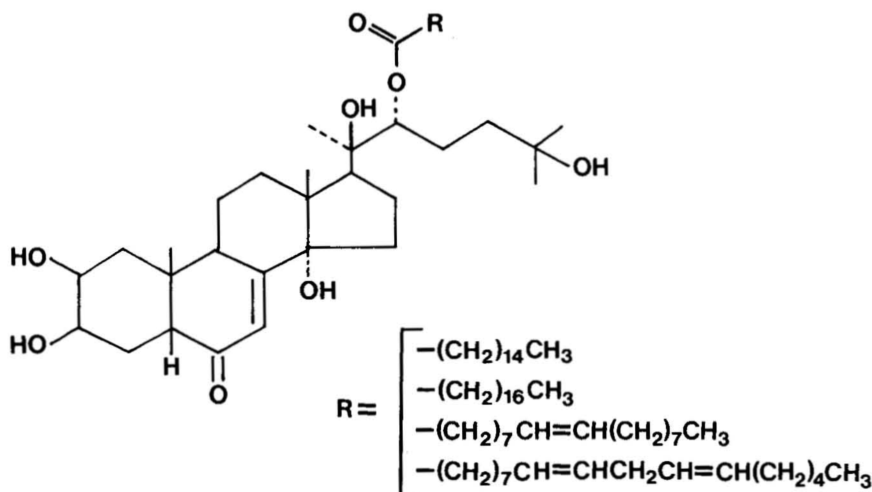


Fig. 7.4 — Structure of the 4 major apolar conjugates AP2. 20-hydroxyecdysone is esterified at C_{22} with palmitic, stearic, oleic, or linoleic acid.

of hormone conjugates amongst zoo- or phytoecdysteroids. They behave as very apolar compounds only on reverse phase columns, owing to their fatty acid tail. On silica, in contrast, they are much more polar than suspected from their behaviour in the reverse phase mode.

Conjugates AP2 were not the final products of the apolar pathway. As they gradually disappeared, other apolar conjugates (AP1) were formed which are slightly more polar (Fig. 7.3a–c,e). We do not know the chemical nature of AP1. These ionizable compounds retain their ester nature because they can be readily hydrolyzed by pig liver esterase to yield the original hormones. AP2 may be transformed into AP1 by a modification of the fatty acid tail through, for example, oxidation. Other possibilities include transesterification or an additional esterification.

A preliminary analysis of the metabolite distribution in various organs demonstrated that most of the ingested hormone was retained in the digestive tract where it was transformed by the midgut cells to AP1 via AP2. AP1 accumulated in the midgut lumen. However, during the first few hours after feeding, a small quantity of free hormone crossed the midgut epithelium and circulated in the hemolymph. Later, only AP2 and some AP1 were found in the hemolymph. The free hormone was apparently quickly removed from circulation by peripheral tissues which tranformed it into AP2 and finally into AP1.

The physiological significance of the apolar pathway is not clear. We postulate that conjugation might inactivate exogenous ecdysteroids which could be present in the blood of herbivorous hosts on which the ticks had fed (see also 7.2). In addition, it could also inactivate endogenous ecdysteroids. Several studies showed that these conjugates apparently do not serve as a hormone storage form from which nymphal ecdysteroids might be derived when hormone titers rise (unpublished work).

In vitro cultures demonstrated that the transformation of ecdysone to 20-hydroxyecdysone and the metabolism to polar or apolar compounds was especially active in the midgut, but Malpighian tubules and the rest of the animal were also capable of the same reactions (Vuillième, personal communication).

Interestingly, injected 2-deoxyecdysone and ponasterone A were not converted to ecdysone or 20-hydroxyecdysone, but were rapidly metabolized to esterase-labile apolar conjugates and to a slight extent to polar compounds.

Fed nymphs of the ixodid *A. hebraeum* were capable of transforming ecdysone into 20-hydroxyecdysone during several periods of the molting cycle (Fig. 7.3f). They produce apolar hydrolyzable conjugates of the same type produced by *O. moubata.* In addition, they accumulated polar compounds of yet unknown nature.

7.5.2 Metabolism in females

Connat *et al.* (1984) studied the fate of ecdysone or 20-hydroxyecdysone in females of *O. moubata.* After injection into the hemocoel of mated vitellogenic females, ecdysone was converted into 20-hydroxyecdysone and the 2 classes of apolar conjugates AP1 and AP2 (which are presumably the same as in nymphs). 20-hydroxyecdysone was directly transformed into AP1 and AP2. Polar products were not produced. AP2 conjugates accumulated principally in the ovaries and eggs, and accounted for about 25% of the total injected hormone by the end of vitellogenesis (Fig. 7.5a). Metabolism in fed virgin females was comparable, but ovaries incorporated less AP2. Cultured ovaries were also capable of hydroxylating ecdysone to 20-hydroxyecdysone and of producing AP2. It was hypothesized that the apolar conjugates accumulating in the eggs could represent a storage form of hormone for the embryo. New studies showed, however, that this might not be the case (see section 7.5.3).

Similarly, injected 2-deoxyecdysone and ponasterone A were converted to apolar products and AP2 accumulated in the eggs. As in nymphs, no ecdysone or 20-hydroxyecdysone were formed from these potential precursors.

Because of the great sensitivity of *O. moubata* females to supermolting after ingesting 22,25-dideoxyecdysone, we investigated the metabolism of several ingested ecdysteroids (Connat *et al.* 1986). As in the nymphs, ecdysone, 20-hydroxyecdysone, 2-deoxyecdysone, or ponasterone A were all rapidly converted to apolar conjugates. Most of the hormone was stored in the midgut mainly in the form of AP1 and some as AP2. In contrast

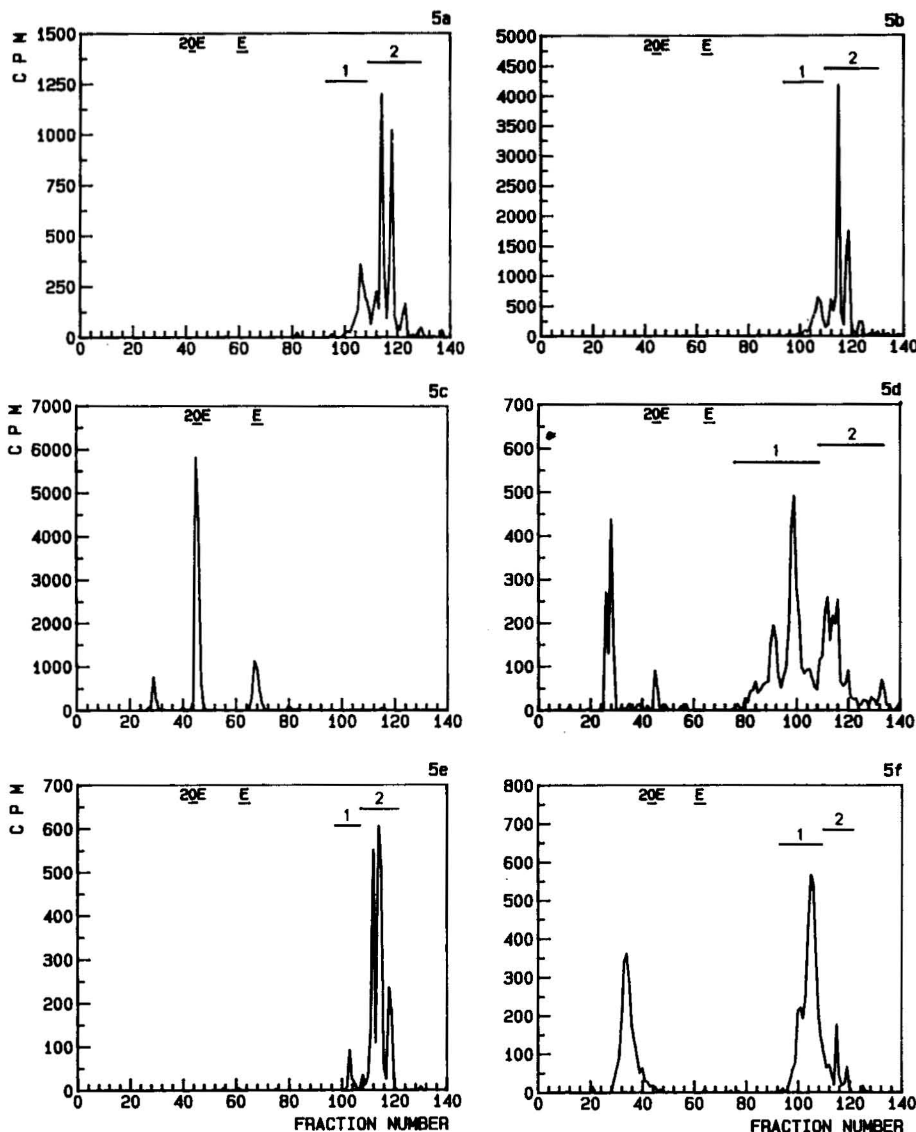


Fig. 7.5 — Ecdysteroid metabolites in freshly laid eggs after injection of ^{3}H-ecdysone into vitellogenic females and their subsequent fate during embryogenesis. For techniques and symbols see Fig. 7.3.

5a: mainly AP2 and small amounts of AP1 accumulated in freshly laid eggs after injection of ^{3}H-ecdysone into vitellogenic females of *O. moubata*. At the end of embryogenesis (day 9 at 28° C), the pattern had hardly changed (5b).

5c: mostly 20-hydroxyecdysone together with small amounts of ecdysone and of polar metabolites (acids ?) were present in freshly laid eggs of *A. hebraeum* which were oviposited 3 days after injection of ^{3}H-ecdysone into females at the beginning of oviposition. The free hormones were converted during embryogenesis to apolar conjugates AP1 and AP2 (5d, embryos shortly before hatching).

5e: only apolar conjugates, mainly AP2, accumulated in eggs of *B. microplus* (Biarra strain), oviposited 3 days after injection of ^{3}H-ecdysone into females at the beginning of egg laying. At the end of embryogenesis, the conjugates AP2 were nearly completely transformed into conjugates AP1 and to polar compounds (presumably ecdysonoic acid and 20-hydroxyecdysonoic acid, 5f).

22,25-dideoxyecdysone was not transformed into apolar conjugates, because of the absence of a 22-OH group, but was metabolized to more polar compounds of unknown structure. Two of the metabolites co-migrated on reverse phase columns, but not on silica, with ecdysone or 20-hydroxyecdysone. It was concluded that 22,25-dideoxyecdysone (or its metabolites) might be very active because of the lack of conjugation. The apolar pathway, especially active in the midgut, might thus serve to inactivate those phytoecdysteroids with a 22-OH group present in the blood of herbivorous hosts.

In contrast to injection experiments, only small amounts of labelled AP2 accumulate in eggs after ingestion of the hormone.

When injected either 7 days before or at the beginning of oviposition, *A. hebraeum* females slowly converted ecdysone to 20-hydroxyecdysone, to some polar compounds, and to apolar conjugates with similar retention times as AP1 and AP2 in *O. moubata*. 20-hydroxyecdysone was metabolized only to apolar esters. Polar and apolar metabolites accumulated mainly in the midgut. In contrast to *O. moubata*, freshly laid eggs contained mainly free hormones and to a small extent polar compounds after ecdysone was injected into the tick (Fig. 7.5c). The radio label in eggs amounted, in some experiments, to 67% of the injected hormone. This capacity of incorporating free ecdysone or 20-hydroxyecdysone agrees well with our chemical studies.

Wigglesworth *et al.* (1985) investigated the fate of labelled ecdysone injected into newly detached *B. microplus* females at day 22–23. It was metabolized primarily (80%) to apolar compounds, corresponding to hydrolyzable ecdysone fatty acid esters. Acetonide formation indicated that the 2- and 3-hydroxyls of ecdysone remained unsubstituted in these compounds. These labelled apolar conjugates were transferred to the eggs and accounted for the majority of the ecdysteroids (the level of free hormone being low). Esterification occurred, at least *in vitro*, in the Malpighian tubules, ovaries, fat body, and gut.

For comparison, we also investigated the fate of ecdysone in *B. microplus* females (Biarra strain). However, the hormone was injected at a later stage (1 day after the beginning of egg-laying). Freshly laid eggs contained only apolar products. The major radioactivity had a retention time similar to AP2 in *O. moubata* nymphs and females. Some small amounts corresponded to AP1 (Fig. 7.5e), and hydrolysis with pig liver esterase liberated ecdysone. About 90% of the injected hormone passed into the eggs, with the remainder being present mainly as AP1 (75%) in the midgut. Our data support the results of Wigglesworth *et al.* (1985), showing a strong tendency of *B. microplus* eggs to accumulate apolar hormone esters.

7.5.3 Metabolism in embryos

Preliminary studies in *A. hebraeum* embryos indicated that labelled free hormones initially present, were converted to apolar products (Fig. 7.5d). This conjugation began in embryos older than 16 days. Production of polar metabolites during embryogenesis apparently did not occur. From our data,

however, we can not as yet state unequivocally if the maternal free ecdysteroids are used by the embryo.

Maternal ecdysteroid esters in *B. microplus* embryos are suspected as being inactive hormone storage forms from which free hormones can be enzymically hydrolyzed at certain times during embryogenesis. Other sources are not, however, precluded (Wigglesworth *et al.* 1985). Recent studies showed that the developing embryo metabolized hormones to 26-carboxyecdysteroids (Rees *et al.* 1984). This indicates that the pathway leading to acids is not restricted to insects or crustaceans (see Lafont & Koolman 1984), but is also operative in *B. microplus* embryos.

Our comparative study with the Biarra strain of *B. microplus* appears to support the conclusions of Rees and his co-workers. During embryogenesis, the labelled products corresponding to AP2 initially present were partly converted to more polar metabolites similar to AP1 and to an appreciable extent to other polar metabolites which may be ecdysonoic acids (Fig. 7.5f). Hydrolysis of the apolar fraction produced mostly polar products indicating that, after formation, these metabolites were partly re-esterified.

In contrast to *B. microplus*, studies of *O. moubata* embryogenesis indicated that labelled apolar AP2 conjugates, which accumulated after injection of hormones into vitellogenic females, were not metabolized to any appreciable extent, not even during larval development (Fig. 7.5b). No free labelled ecdysone, 20-hydroxyecdysone, or polar products were detected. This is contrary to the previous hypothesis that apolar conjugates, at least those of experimental origin, may be used as a source of hormone for the developing embryo of this species (Connat *et al.* 1984).

In vitro experiments with *O. moubata* demonstrated that a freshly laid egg and an embryo at any stage during its development could efficiently conjugate hormones to form AP2. On the other hand, the capacity to hydroxylate ecdysone to 20-hydroxyecdysone was manifested only after 24–48 hours of development (at 28°C). As in the *in vivo* condition no formation of polar metabolites was observed.

7.6 CONCLUSIONS

In the preceding sections we reviewed our current knowledge of tick ecdysteroids based on published and unpublished observations. Clearly, ticks possess ecdysteroids (ecdysone, 20-hydroxyecdysone) which are important in controlling processes such as molting or ixodid salivary gland degeneration. In addition, these hormones are strongly suspected of being involved in the hormonal control of other events such as diapause, spermatogenesis, oogenesis, and embryogenesis, but additional work is needed to substantiate these observations. We have also progressed in our understanding of ecdysteroid metabolism. Results indicate that polar metabolites (in part ecdysonoic acid and 20-hydroxyecdysonoic acid) and a new class of apolar conjugates, i.e. ecdysteroid esters with long-chain fatty acids, can be formed.

On the other hand, several important research areas remain totally

unexplored. We do not know the biosynthetic pathway, its localization, or its probable control by other hormones such as neurosecretions. In addition, we have no information on the hormone action at the gene level or on possible interactions with other postulated hormones such as juvenile hormone-like substances which appear to be important for the control of oogenesis.

We expect that the results presented here will be of interest not only for tick endocrinology, but also for comparative arthropod endocrinology. In addition, we think that the studies on basic tick endocrinology might also be useful for applied biology. Indeed, resistance to new classical acaricides can develop rapidly. Thus, interference with hormone biosynthesis, action, or metabolism could provide a new approach to tick control. For example, the synthesis of hormone analogues inducing precocious ixodid salivary gland degeneration or the disturbance of molting and oogenesis would be highly desirable. In view of the effects of exogenous ecdysteroids, this does not appear too unrealistic.

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NOTE ADDED IN PROOF

Ovaries from *Amblomma hebraeum* females massively converted *in vitro* ecdysone to 3-epiecdysone presumably through the intermediate formation of 3-dehydroecdysone. Only small amounts of 20-hydroxyecdysone were produced. Epimerization occurred also to some extent in carcasses, but not in gut (Connat, J. L., Lafont, R., & Diehl, P. A., *Mol. Cell Endocrinol.*, in press).