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Presence and Metabolism of Ecdysteroids during Embryonic Development of the Argasid Tick *Ornithodoros moubata* and of the Ixodid Tick *Amblyomma hebraeum*: A Comparative Study

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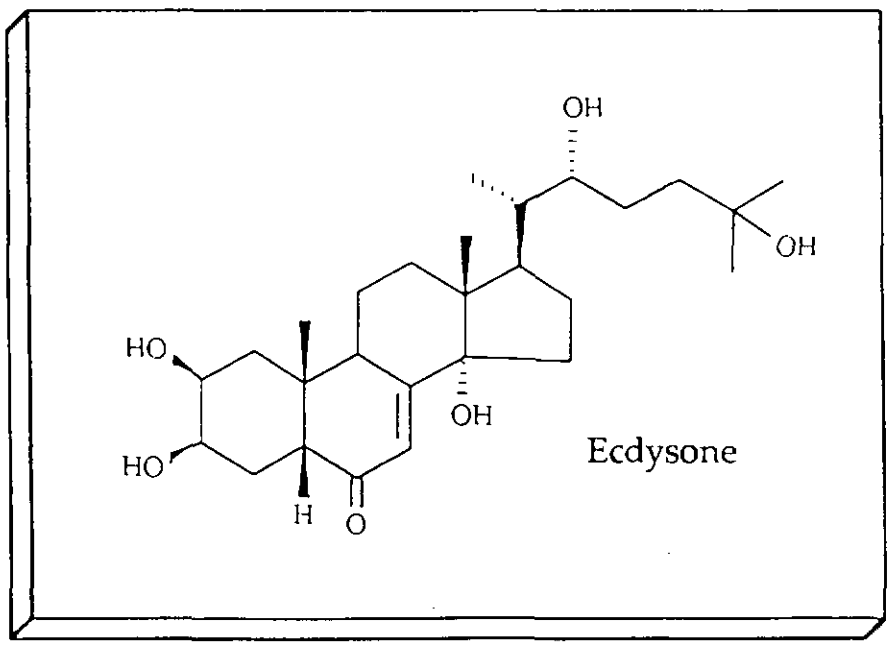

Ecdysone

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SUMMARY

The ultrastructure and timing of embryonic and larval cuticles and their relationship with ecdysteroid titers was investigated during embryonic and larval development of the argasid tick *Ornithodoros moubata* and during embryonic development of the ixodid tick *Amblyomma hebraeum*. In addition, *in vivo* and *in vitro* metabolism of radiolabelled ecdysone ([^{3}H]-E) and 20-hydroxyecdysone ([^{3}H]-20E) was studied in the embryos of these tick species along with those of the ixodid tick *Boophilus microplus*.

Embryonic cuticles A, B and C were found in both *O. moubata* and *A. hebraeum* and were reduced in structure when compared with larval and nymphal cuticles. Because they are present in ticks from the two principal tick families, they may be a general feature in tick embryonic development. Larval cuticle development (and first instar nymphal cuticle production in *O. moubata*) progressed similarly to that described in immatures of other tick species and consisted of epicuticle and exo- and endocuticle.

In *O. moubata* embryos, no peak of ecdysteroid-RIA-positive material was detected during production of cuticles A, B and C. However, two peaks (one corresponding to the shortening of the germ band and one to the deposition of larval epicuticle) and one peak (which coincided with nymphal epicuticle production) were observed during embryonic and larval development, respectively. HPLC-RIA analysis of two of these peaks revealed that they are composed of principally 20E along with some E. Ecdysteroid esters appear to be present in the freshly laid eggs. Whether they play a role in early embryonic development is unknown. However, they do not appear to be reused by the embryo and do not appear to be hydrolyzed for the production of ecdysteroids during the shortening of the germ band and larval and nymphal epicuticle production. Thus, the production of ecdysteroid peaks may be by *de novo* synthesis although it is not known if all the steps from the conversion of cholesterol take place in the embryo. *In vitro* studies with [^{3}H]-E demonstrated that embryos 2 days old and older possess the enzymes necessary for the conversion of E to 20E. Production of polar products occurs during peak hydroxylation and during the endogenous peak and this pathway acts as an inactivation pathway. The enzymes needed for conjugation of [^{3}H]-E with esters are present throughout larval and embryonic development. This finding suggests that conjugation is a detoxification pathway active at all times.

In contrast to *O. moubata* eggs, *A. hebraeum* eggs contain free E and 20E at oviposition. Their function in the embryo is unknown but they may play a role in meiotic reinitiation, embryonic cuticle production or act as a feeding deterrent against predators. No peaks of ecdysteroids are found during embryonic development but this may be due to poor synchronization of the embryos and not a lack of ecdysteroids produced. Endogenous ES titers may decrease during the first 15 to 20 days of development and these ES are probably conjugated to esterase-hydrolyzable conjugates. This is corroborated by the metabolism of incorporated [^{3}H]-E and [^{3}H]-20E during embryonic development. These tritiated compounds were conjugated to form apolar esters between days 15 and 30 and, in addition to being conjugated, they were converted to the 3α epimers of E and 20E. Whether conjugation or epimerization takes place first is not known. These conjugates and conjugated epimers did not appear to be rehydrolyzed during embryonic development. *In vitro* studies demonstrated that the embryos probably convert E to 3αE via the formation of 3-dehydroecdysone. In addition, 40-day-old embryos appear to possess the enzymes necessary for the conversion of E to 20E. Although no peaks of ecdysteroids were detected during larval epicuticle production, it is probable that 20E stimulates its formation and that the 20E is produced by *de novo* synthesis.

Freshly laid *B. microplus* eggs contain the ecdysteroid conjugates, the C-22 esters of E (Crosby *et al.* 1986). Analysis of a tritiated form of these ecdysteroid esters during embryonic development demonstrated that most of these esters were converted to ecdysteroid-26-oic acids or conjugates of these acids by day 10 and appeared to remain in this form until hatching on day 21.

PREFACE

My decision to study the embryonic development was a logical step considering the history of endocrinological studies already carried out in this laboratory. The first description of molting hormones in ticks was done in the laboratory by P. Diehl in collaboration with J.-P. Delbecque of the University of Dijon (1978). They found ecdysone and 20-hydroxyecdysone in the nymphs of the tick *A. hebraeum*, thus the first chemical analysis of molting hormones in ticks. Then from Diehl's group came two papers in which a peak of ecdysteroids was correlated with the initiation of cuticle development in two tick species, *O. moubata* (Germond *et al.* 1982) and *A. hebraeum* (Diehl *et al.* 1982b). These ecdysteroid peaks were composed of ecdysone and 20-hydroxyecdysone; thus, it appeared that ecdysteroids had a function in ticks similar to that in insects. Other studies were done in which the metabolic fate of the radiolabelled ecdysone was investigated in the nymphs of *O. moubata* (Bouvier *et al.* 1982) and also *in vitro* culture with various tissues of this nymph (Vuillème, unpublished--see Connat 1987). With the arrival of J.-L. Connat in the laboratory, studies were carried further to the females of *O. moubata* and *A. hebraeum*. These studies involved topical applications of ecdysteroids, juvenile hormones, the presence and identification of ecdysteroids during oogenesis, and metabolic studies with radiolabelled compounds. Upon my arrival in Switzerland, it only seemed natural that the next life stage, the embryos, should be studied. This dissertation presents my results on the "Presence and metabolism of ecdysteroids during embryonic development of the argasid tick *Ornithodoros moubata* and the ixodid tick *Amblyomma hebraeum*: A comparative study." We attempted to correlate ecdysteroid titers with cuticle deposition and carried out metabolic studies with tritiated ecdysone to discern the fate of the embryonic ecdysteroids. These studies are covered by Appendices I-VI. Appendix VII covers preliminary studies with *B. microplus* embryos.

I. Cuticle deposition and ecdysteroid titers during embryonic and larval development of the argasid tick *Ornithodoros moubata* (Murray, 1877, *sensu* Walton, 1962) (Ixodoidea: Argasidae). E.M. Dotson, J.-L. Connat and P.A. Diehl. *Gen. Comp. Endocrinol.* 82 (1991): 386-400.

II. Apolar conjugates are not used as a storage form of molting hormone in the argasid tick *Ornithodoros moubata*. J.-L. Connat, E.M. Dotson and P.A. Diehl. *Arch. Insect Biochem. Physiol.* 9 (1988): 221-235.

III. Metabolism of [^{3}H]-ecdysone in embryos and larvae of the tick *Ornithodoros moubata*. E.M. Dotson, J.-L. Connat and P.A. Diehl. *Arch. Insect Biochem. Physiol.* (1993), in press.

IV. Metabolism of ecdysteroids in the female tick *Amblyomma hebraeum* (Ixodoidea, Ixodidae): Accumulation of free ecdysone and 20-hydroxyecdysone in the eggs. J.-L. Connat, E.M. Dotson and P.A. Diehl. *J. Comp. Physiol. B.* 157 (1987): 689-699.

V. Ecdysteroid titer and metabolism during embryogenesis of the ixodid tick *Amblyomma hebraeum* (Koch). E.M. Dotson, J.-L. Connat and P.A. Diehl, submitted to *Comp. Biochem. Physiol.*

VI. *In vitro* metabolism of [^{3}H]-ecdysone in embryos of the ixodid tick *Amblyomma hebraeum*: The presence of 3-dehydroecdysone and 3-epiecdysone. E.M. Dotson, J.-L. Connat, P.A. Diehl and R. Lafont, submitted to *Exp. Appl. Acarol.*

VII. Preliminary studies of [^{3}H]-ecdysone metabolism in *Boophilus microplus* embryos. E.M. Dotson, J.-L. Connat and P.A. Diehl, submitted to *Exp. Appl. Acarol.*

In addition, I have coauthored other papers concerning ecdysteroids in ticks that were not examined by the jury of this thesis. They are listed below.

1. Chemistry, function and metabolism of tick ecdysteroids. P.A. Diehl, J.-L. Connat and E. Dotson. In: Sauer JR, Hair JA (eds) *Morphology, Physiology, and Behavioral Biology of Ticks*. (1986), Ellis Horwood, Chichester, pp 165-193.

2. Comparative investigations of the egg ecdysteroids of ticks using radioimmunoassay and metabolic studies. J.-L. Connat and E.M. Dotson. *J Insect Physiol* (1988) 34: 639-645

3. Epidermis as the source of ecdysone in an argasid tick. X.X. Zhu, J.H. Oliver, Jr., E.M. Dotson. *Proc. Natl. Acad. Sci. USA* (1991) 88: 3744-3747

4. Hormonal control of molting and reproduction in ticks. J.H. Oliver, Jr. and E.M. Dotson *Amer. Zool.* (1993), in press

Abbreviations used: E =Ecdysone (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one); 20E = 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one); [^{3}H]-E=[23,24^{3}H]-ecdysone; [^{3}H]-20E= [23,24^{3}H]-20-hydroxyecdysone; ES-ecdysteroids; AP=apolar products; AP1=apolar products 1; AP2=apolar products 2 (fatty acid esters of ES); PP=polar products; E'=3-epi-Ecdysone (2β,3α,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one); 20E'=3-epi-20-hydroxyecdysone (2β,3α,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one); RP-HPLC=reversed phase high performance liquid chromatography; RIA-radioimmunoassay; CI/D MS-chemical ionization/desorption mass spectrometry; TEM-transmission electron microscopy

Key Words: Tick, embryo, larva, *Amblyomma hebraeum, Boophilus microplus, Ornithodoros moubata*, cuticle development, ecdysone, 20-hydroxyecdysone, 3-epiecdysone, 3-epi-20-hydroxyecdysone, 3-dehydroecdysone, ecdysteroid metabolism, apolar conjugates, polar products

INTRODUCTION

Tick eggs are round to ellipsoid and vary in length from 0.45 mm (*Hyalomma truncatum*, Dipeolu 1982) to more than 1.0 mm (*Ornithodoros moubata*, Diehl *et al.* 1982; *Hyalomma dromedarii*, Kammah *et al.* 1982). The transparent egg shell facilitates microscopic observations of tick development with the whitish embryo in contrast against the dark brown yolk, which is a haemo-lipo-glyco-protein complex (Diehl *et al.* 1982a). Although several recent papers have described the embryonic development of two ixodid species (Dipeolu 1982, Kammah *et al.* 1982, Shiraishi *et al.* 1990), the most complete study of tick embryogenesis is that of the argasid tick *O. moubata* (Aeschlimann 1958). The tick egg is centrolecithal, and the mode of cleavage was originally thought to be superficial. However, transmission electron microscope studies of the developing *O. moubata* embryo demonstrated that cleavage is total (Fagotto *et al.* 1988). This also appears to be the case in the ixodid ticks *Amblyomma hebraeum* and *Boophilus microplus* (Dotson and Fagotto, unpublished).

Many of the more recent papers dealing with tick embryogenesis have been biochemical and physiological studies. Boctor and Kamel (1977) found that the total free amino acid content decreased, and the total protein content remained the same, as embryogenesis progressed in *Dermacentor andersoni* embryos. During embryogenesis of the tick *H. dromedarii*, the O_2 and CO_2 consumption increased and a high respiratory quotient was correlated with morphogenesis (Ahmed and Bassel 1984). The activity of isocitrate lyase, the first enzyme in the glyoxylate cycle (Kamel and Fahmy 1982), peaked around day 15 in this same tick embryo, whereas the DNA and RNA production increased and the total protein content remained the same during embryogenesis (Kamel *et al.* 1982). Fagotto (1990) did extensive studies on yolk degradation in the tick *O. moubata*. However, no study had been published on the hormonal control of embryonic development.

In the conclusion of Aeschlimann and Hess's paper (1983) concerning the current knowledge of acarine embryology, they expressed a need for further investigation in several subject areas: 1) role and evolution of vitellophage cells, 2) metamerization of the germ band, 3) mechanism of blastokinesis, 4) gnathosoma formation, 5) organogenesis, 6) embryonic envelopes, and 7) intrachorionic molts and the role of embryonic ecdysteroids (ES) in those molts. In their studies of subject area 1, Fagotto and coworkers (1988) found that vitellophages did not exist in *O. moubata*. The small cells which were supposed to be digesting yolk platelets (Aeschlimann 1958) were, in fact, not cells but the perinuclear cytoplasm and nucleus of huge-yolk filled blastomeres and macromeres. The subject of this thesis, which is concerned with subject areas 6 and 7, is the study of the embryonic ES (presence and metabolism) and their possible role in the control of embryonic molts in tick embryos from the argasid and ixodid tick families.

Roles of ecdysteroids

Ecdysteroids are polyhydroxylated steroids first discovered as molting hormones in *Bombyx mori* pupae. Since the discovery of ecdysone (E) and the more active ecdysteroid, 20-hydroxyecdysone (20E), thirty or more ES have been isolated from arthropods (Horn 1989). Ecdysteroid have been isolated from almost all arthropod classes (see reviews by Rees 1989, Spindler 1989) and many nonarthropod phyla (reviewed by Franke and Käuser 1989). In addition to stimulating the production of epicuticle, ES appear to play many roles in insect development, i.e. stimulating spermatogenesis (reveiwed by Dumser 1980, Raabe 1989, Sehnal 1989, Oliver and Dotson in press) and, in dipterans, vitellogenesis (Bownes 1989, Hagedorn 1985, 1989). They also are involved in oocyte and embryonic development (Lanot *et al.* 1989a, Dübendorfer 1989) including stimulation of production of embryonic cuticles (see Hoffman and Lagueux 1985 and next section).

The roles ES play during tick development appear similar to those in insects. Applications of exogenous ES have been shown to affect many systems, i.e. terminate larval diapause and stimulate supermolting (see reviews by Solomon *et al.* 1982 and Diehl *et al.* 1986). Ecdysone and 20E have been correlated with cuticle development in at least three tick species of two families (the argasid tick *O. moubata* [Germond *et al.* 1982] and the ixodid ticks *A. hebraeum* [Diehl *et al.* 1982b] and *Amblyomma variegatum* [Stauffer and Connat 1990]). Ecdysteroids may be involved with sex pheromone production (Dees *et al.* 1984a,

1985) and have been implicated in salivary gland degeneration of fed *A. hebraeum* females (Kaufman 1991). Ecdysteroids have been detected in the hemolymph of two ixodid females *A. hebraeum* (Connat *et al.* 1985) and *B. microplus* (Wigglesworth *et al.* 1985, Diehl *et al.* 1986) during vitellogenesis. Preliminary evidence indicates that a significant stimulation of fat body from *Dermacentor variabilis* females to produce vitellogenin, the precursor to the yolk protein vitellin, was achieved *in vitro* with a physiological dose of 20E (Coons pers. comm.). Testes of *D. variabilis* males respond to exogeous doses of 20E by increased DNA synthesis (Oliver 1986). HPLC-RIA analysis of extracts of *D. variabilis* feeding males demonstrated the presence of an RIA-positive material with the same retention time as 20E (Dees *et al.* 1984b, Oliver and Dotson in press). The freshly laid eggs of *O. moubata* (Connat and Dotson 1988) and *B. microplus* (Crosby *et al.* 1986, Connat and Dotson 1988) contain ecdysteroid conjugates, whereas those of *A. hebraeum* contain free E and 20E (Connat *et al.* 1985). Their role in development is unknown, but they were suggested to be involved in deposition of embryonic cuticles (Connat *et al.* 1984, 1985, Wigglesworth *et al.* 1985, see next two sections).

Embryonic cuticles in insects and crustaceans

Embryonic molting or the development of several embryonic cuticles before hatching appears to be a common occurrence in arthropods and, as is the case in most areas of research carried out on members of this phylum, more studies have been carried out on insects than other arthropod classes. The fine structure of these embryonic cuticles has been described in several insect species (*Blaberus craniifer*, Bullière 1973; *Calliphora erythrocephala*, Bordes-Alléaume and Sami 1987; *Carausius morosus*, Louvet 1974, Fournier 1985; *Leucophaea maderae*, Rinterknecht 1985, Rinterknecht and Matz 1977 & 1983; *Locusta migratoria*, Lagueux *et al.* 1979; *Oncopeltus fasciatus*, Dorn 1983; *Schistocerca gregaria*, Sbrenna 1974). In general (except for *B. craniifer* and *C. erythrocephala*) these cuticles consist of: 1) an embryonic membrane or first embryonic cuticle which is laid down just before the dorsal closure and which is composed of a thin, electron dense, occasionally trilaminar membrane, 2) a second embryonic cuticle which is the true embryonic cuticle forming after the dorsal closure and which is composed of a fine cuticulin layer, epicuticle, a subepicuticular layer and a lamellate procuticle (1-2 lamellae), and 3) a third cuticle consisting of cuticulin, epicuticle and procuticle (many lamellae) which is, in actuality, the larval cuticle.

Ultrastructural studies have also been carried out in crustaceans. Transmission electron microscopy (TEM) studies of the embryonic development of the crab *Carcinus maenas* (Goudeau and Lachaise 1983) and the isopod *Hemioniscus balani* (Goudeau 1976) demonstrated that 4 cuticle cycles take place during embryogenesis (4 cuticular secretions alternating with 3 apolyses). Five embryonic envelopes are secreted by the embryonic epidermal cells of the lobster *Homarus gammarus* (Goudeau *et al.* 1990). In three species of *Balanus* (Klepal and Barnes 1978) and in three species of Ostracods (Tetart 1970), two and three cuticles, respectively, are deposited during embryogenesis. Most of the cuticles consist of an electron dense epicuticle and a sometimes lamellate procuticle. The last cuticle deposited, that of the free living stage, is generally thicker and is a more complete cuticle.

Embryonic ecdysteroids in arthropods

Ecdysteroids have been found in the freshly laid eggs of many insects (see review by Hoffmann and Lagueux 1985) and crustaceans (Goudeau *et al.* 1990, Young *et al.* 1991, Okazaki and Chang 1991; see review by Spindler *et al.* 1984). In insects, the majority of these maternally derived ES are present in a conjugated "inactive" form, mainly phosphate esters at the C-22 position or C-3 position, C-22 nucleotides (see Hoffmann and Lagueux 1985) or C-22 fatty acid esters (Whiting and Dinan 1988). As in insects and crustaceans, ES have also been found in vitellogenic females of several tick species and, similarly to insects, these maternal ES may be incorporated into the developing oocytes. Depending upon the tick species, freshly laid eggs may contain 1) free ecdysone and 20-hydroxyecdysone (*A. hebraeum* [Connat *et al.* 1985] and *A. variegatum* [Connat and Dotson 1988]), 2) apolar conjugates of ES (*B. microplus* [Wigglesworth *et al.* 1985] and *O. moubata* [Connat *et al.* 1984]) or 3) a combination of free and conjugated ES (*Rhipicephalus appendiculatus* and *H. dromedarii* [Connat and Dotson 1988]). These apolar conjugates have the same retention times on reversed

phase HPLC as the fatty acid esters of 20-hydroxyecdysone described in *O. moubata* nymphs (Diehl *et al.* 1985) and have been identified in *B. microplus* as fatty acid esters of ecdysone (Crosby *et al.* 1986).

The purpose of these ES in the freshly laid eggs is unknown. The presence of one or more peaks of free ES has been demonstrated during the embryonic development of a few crustacean and many insect species and, in many cases, these peaks have been correlated with embryonic molts (crustacean--*Homarus*, Goudeau *et al.* 1990; insects--*Calliphora*, Bordes-Alléaume and Sami 1987; *Carausius*, Fournier 1985; *Sarcophaga*, Wentworth and Roberts 1984; see Hoffmann and Lagueux 1985 for review of earlier papers). In 1979 Lagueux and coworkers proposed that the origin of these embryonic ES peaks in young *Locusta* embryos resulted from hydrolysis of maternal ES conjugates. In addition, in *Drosophila*, conjugated ES are covalently bound to the yolk proteins and are probably released during embryonic development as digestion of the yolk progresses (Bownes 1988). Because apolar conjugates of ES, most likely fatty acid esters in the C-22 position, are incorporated into eggs during vitellogenesis of several tick species (*O. moubata*, Connat *et al.* 1984; *B. microplus*, Wigglesworth *et al.* 1985; *Ornithodoros parkeri*, *H. dromedarii*, *Ixodes ricinus*, and *R. appendiculatus*, Connat and Dotson 1988), it was hypothesized that these conjugates may also serve as an ES source for embryonic molts. In this study, several techniques were used to analyze the ultrastructure of the embryonic cuticles; to determine the nature, origin and fate of embryonic ES; and to study the enzyme systems used by embryos at different times of development.

RESULTS AND DISCUSSION

1. Cuticle deposition in *Ornithodoros moubata* and *Amblyomma hebraeum* embryos

1.1. Deposition of embryonic cuticles

Three embryonic cuticles are produced before the larval cuticle is deposited near the end of embryonic development of the argasid tick *O. moubata* (Appendix I) and the ixodid tick *A. hebraeum* (Appendix V). These cuticles are labelled A, B and C according to the terminology used in the embryos of *O. moubata* (Vogel 1975). The first of these cuticles, layer A, is a thin, electron dense layer around 10 nm thick; it has an occasionally trilaminar appearance. The second cuticle, labelled B, is more complex, consisting of three different parts. The outermost part is a very thin layer which may be similar to the outer epicuticle already described in ticks (Germond *et al.* 1982). This thin layer is separated from the dense layer by a very thin electron translucent space and the innermost part of B is composed of a fibrous or granular material. Cuticle C is composed of a thin electron dense layer, which is deposited by electron dense plaques at the ends of microvilli, and a fibrous inner layer. Electron dense balls of material are present in the fibrous layer of C or attached to its inner surface. They were originally thought to be vesicles filled with material to be added to the dense layer, but in *A. hebraeum* they are still present after larval cuticle formation is complete.

The formation of these embryonic layers takes place at comparable times of embryonic development in these two species. The duration of embryonic development of *A. hebraeum* (45-60 days) is about 5-6 times as long as that of *O. moubata* (approx. 10 days). In *A. hebraeum* deposition of A, B and C occurs 3-4, 8 and 28-30 days, respectively, after oviposition and in *O. moubata*, they form after 24-32 hrs, 48-56 hrs and 6 days of development. In both ticks, B appears between the formation of the germinal disk and the completion of the germ band. C forms after the contraction of germ band takes place.

To classify these embryonic layer as cuticles may be questionable, especially the single layer A. Layers B and C, although reduced, have a more cuticular-like appearance consisting of a thin epicuticle and fibrous procuticle. However, the fibrous layer may resemble molting fluid, but it remains attached to the dense layer of B when B pulls away from C during the fixation process. Also, no resorption of this layer or any other part of the cuticles was observed and exuviation does not take place. The timing of apolyses, if they truly occur, are difficult to determine because of the violent retraction of the egg shell when it is pierced during the fixation process. These cuticles may be a general feature of tick embryonic development. In addition to the two ticks described here, very similar cuticles have been observed in an ixodid tick of a different genus, *B. microplus* (Crosby *et al.* 1987 and Dotson, unpublished results).

Comparison of these cuticles at the ultrastructural level with those of other groups of the chelicerates is difficult because of the scarcity of literature. In a spider of the Lycosidae family, the "vitellin membrane" which forms within 30 minutes after oviposition may correspond to cuticle A of ticks (Kondo 1969). Several molts occur in the spider *Chiracanthium virescens* between the breaking of the egg membrane and the complete shedding of this membrane (Canard 1987). These molts were observed with scanning electron microscopy and it is difficult to compare these cuticles with those of ticks.

In crustaceans the embryonic cuticles varied in number from two to six (Tetart 1970, Goudeau 1976, Klepal and Barnes 1978, Goudeau and Lachaise 1983, Goudeau *et al.* 1990). The six envelopes found in *Carcinus maenas* have been studied extensively. The first four do not resemble those of ticks; only the structure of the fifth resembles that of B and C (Goudeau and Lachaise 1983).

In insects the number of embryonic envelopes (generally three) is less variable than that of crustaceans. The first very thin atypical cuticle resembles cuticle A found in ticks. However, in some insect embryos, it is not present (Bullière 1973, Bordes-Alléaume and Sami 1987) and in *C. morosus*, a fibrous layer of material is secreted between it and the second cuticle (Louvet 1974). The second insect cuticle consists of an epicuticle and one or two procuticle lamellae and is more cuticular than the tick cuticles B and C (Bullière 1973, Louvet 1974, Sbrenna 1974, Lagueux *et al.* 1979, Dorn 1983, Rinterknecht and Matz 1977, 1983,

Rinterknecht 1985). The third embryonic cuticle is, in actuality, the larval cuticle and will be compared with the larval cuticle of ticks (next section).

1.2. Deposition of the larval cuticle

Epicuticle formation of the larval cuticle near the end of embryonic development of *O. moubata* (Appendix I) and *A. hebraeum* (Appendix V) begins with the appearance of electron dense plaques on tips of microvilli followed by the deposition of the dense layer of the epicuticle. In *O. moubata* embryos, an amorphous procuticle is deposited until hatching and then a lamellate procuticle is laid down. In *A. hebraeum* embryos, an amorphous procuticle is first deposited beneath the epicuticle. Differing slightly from *O. moubata*, the "prehatching" procuticle of the alloscutum has a faintly lamellate appearance but it is not as distinct as the 8-10 lamellae of procuticle deposited after hatching. In the scutum, a nonlamellate procuticle is laid down until hatching. After hatching, the scutal procuticle becomes sclerotized but no more procuticle is deposited in this region.

We also observed the deposition of the nymphal cuticle at the end of larval development of *O. moubata*. Its formation and the larval cuticle formation is very similar to the process already described in the fifth instar nymph of this tick species (Germond *et al.* 1982) and nymphs of the ixodid species *A. hebraeum* (Diehl *et al.* 1982b) and *A. variegatum* (Stauffer and Connat 1990) as well as the mite *Tetranychus urticae* (Mothes-Wagner 1984). The larval tick cuticle, with its epicuticle and lamellate procuticle resembles that of the last embryonic cuticle (in actuality that of the first free-living stage) of the crab C. *maenas* (Goudeau and Lachaise 1983) and of several insect species (Bullière 1973, Louvet 1974, Sbrenna 1974, Lagueux *et al.* 1979, Dorn 1983, Rinterknecht and Matz 1977, 1983, Rinterknecht 1985) and does not differ from the general arthropod scheme (Richards 1951, Neville 1975, Hepburn 1985).

The terminology used for the different layers of the procuticle has been very confusing. Several authors (Richards 1951, Balashov 1972, Amosova 1983, Hackman 1982, Hackman and Filshie 1982) divided the procuticle into exo-, meso-, and endocuticle and the use of these terms depends on the amount of sclerotization. The exocuticle is sclerotized and is found only in the sclerotized region of the tick. The mesocuticle is partially sclerotized and the endocuticle is not tanned at all. The endocuticle of the alloscutum can be divided into outer nonlamellate layer which is laid down before ecdysis and the inner lamellate layer which is laid down after ecdysis.

Other authors (Nathanson 1967, 1970, Beadles *et al.* 1973, Beadle 1974, Diehl *et al.* 1982b, Stauffer and Connat 1990) have used exocuticle to refer to the outer procuticle deposited before ecdysis and endocuticle as the layer, generally lamellate, deposited after ecdysis. This use of these terms seems more logical based on the meaning of the prefixes exo- (Greek for without, used scientifically to mean outer) and endo- (Greek for within, used scientifically to mean inner [Webster's Dictionary, 1983]). If we use this reasoning in the terminology, the prehatching amorphous (or faintly lamellate) procuticle should be called the exocuticle whereas the lamellate procuticle deposited after hatching should be called endocuticle. The exo- and endocuticle have also been referred to as pre- and post-ecdysial procuticle, respectively (Diehl *et al.* 1982b, Germond *et al.* 1982, Stauffer and Connat 1990).

2. Endogenous ecdysteroids in *O. moubata* and *A. hebraeum* embryos

2.1. Ecdysteroid titers during embryonic and larval development of *O. moubata*

2.1.1. Ecdysteroid titers and cuticle production

Using RIA and two different antibodies (a polyclonal antibody that recognizes numerous ES [reviewed in Connat 1987] and a monoclonal antibody that recognizes principally 20E and Makisterone A [Connat and Dotson 1988]), we investigated the fluctuations of ES levels during embryonic and larval development (see Appendix I, figure 4a). During the first three days of development, when embryonic cuticles A and B are deposited, the free ES titers were low and corresponded to approximately 3 and 0.36 pg eq. 20E/embryo using the polyclonal and the monoclonal antibody, respectively. On the 4th day a small peak of RIA-positive material was noted. It varied from 2 to 14 pg eq. 20E/embryo with the polyclonal antibody and coincided with the shortening of the germ band. The variability in the

quantity of ES detected was probably due to poor synchronization of the embryos. Deposition of C occurred on day 6 when ES titers were low or rising. Another peak occurred on the 7th and 8th day of development. The titers of ES detected with the polyclonal antibody (which has a higher affinity for ecdysone (E)) began to rise on the 7th day (up to 23.3 pg eq. 20E/embryo) and remained high for two days, while the ES detected with the monoclonal antibody (which has a higher affinity for 20E) were high (as much as 10 pg eq. 20E/embryo) on the 8th day of embryonic development. This slight advance of the peak detected with the polyclonal antibody suggests that E was produced first near the end of the 7th day and was then converted to 20E on the 8th day. Larval epicuticle production was observed coincident with the peak of 20E on day 8. The titers decreased during procuticle deposition until the second day of larval development. They then began to increase again reaching 32 pg/larva (monoclonal antibody) and 63 pg/larva (polyclonal antibody) on the 3rd day. As with larval epicuticle production, the production of nymphal epicuticle coincided with the peak of RIA-positive material on the third day of larval development. Ecdysteroid titers sharply decreased thereafter to a basal level at the time of the molt to the nymph.

Analysis of the 25%, 60% and 100% methanol fractions (separated by reversed phase chromotagraphy using Waters RP C-18 SEP-PAK cartridges) of extracts from two embryonic (0 day and 8 day) and larval stages (1 day and 3 day) demonstrated that the majority of the RIA-positive material was in the 60% methanol fraction (Appendix I, Table 2). The 60% fractions of the day 8 embryo extract and the day 1 and day 3 larval extract contained mostly material which had retention times similar to those of 20E and some E. In the 8 day embryos the ratio between these two hormones was approximately 11 : 1. In the case of the 1 day and 3 day old larvae, the ratio between the 2 hormones varied and was 1.6 : 1 in young larvae and 4.2 : 1 during the RIA-positive peak on the 3rd day of larval development.

Ecdysteroids appear to play a role in the deposition of the larval cuticle and that of the first instar nymph of *O. moubata*. Because the majority of the individuals of the same age as those having high ES titers showed epicuticle deposition, a correlation between high titers and epicuticle deposition seems valid. These results are similar to those found in the fifth instar nymph of this species where a peak of ES was temporally correlated with epicuticle formation (Germond *et al.* 1982). In this peak the hormones present were principally 20E and E, as demonstrated with gas chromatography-mass fragmentography. Similarly, with RP-HPLC-RIA analysis, we have tentatively demonstrated that the peaks temporally correlated with epicuticle deposition during embryonic and larval development are composed principally of 20E along with smaller quantities of E. *In vitro* studies using [^{3}H]-E have shown that embryos appear to possess the enzymes to hydroxylate E to 20E and the highest percentages of metabolites hydroxylated occur around the ES peak (see section 3.1.2. and Appendix III). The production of E followed by 20-hydroxylation (as suggested by the slight advance of the ES peak detected using the polyclonal antibody with respect to that detected with the monoclonal one) is similar to the situation found in many insect larvae (see Smith 1985). However, the embryos of *O. moubata* tend to differ from embryos of *Locusta* where more E than 20E is present during ES peaks in embryonic development (Lagueux *et al.* 1979).

The question of whether ES play a role in the deposition of the three embryonic envelopes A, B and C of *O. moubata* remains unclear. Bordes-Alléaume and Sami (1987) suggested that ES did not play a role in the formation of the early embryonic cuticle in the dipteran *C. erythrocephala* since no free or conjugated ES were detected at that time. However, in *L. migratoria*, the formation of the serosal cuticle and apolysis before the deposition of the other three cuticles produced during embryonic development appear to be initiated by a peak of ES (Lagueux *et al.* 1979). In *O. moubata* if an increase in titers of ES initiates the formation of any of these envelopes (A, B or C), then perhaps small peaks of short duration were overlooked because of the pooling of 50 embryos for an extraction. Another hypothesis is that few ES receptors are present on the cell membrane and thus only very low ES titers are needed. More sensitive methods allowing dosage of single embryos are needed to solved these problems definitively.

2.1.2. Presence of conjugates

In view of the fact that fatty acid esters (apolar conjugates) have been described in freshly laid eggs of this species either by means of radiotracers (Connat *et al.* 1984) or RIA after hydrolysis of fractions less polar than E (Connat and Dotson 1988), we investigated the presence of these esters throughout embryonic development. In the early stages of development in the egg batch investigated (see Appendix I, figure. 4b), about 9 times as much RIA-positive material was released by esterase hydrolysis, corresponding to 22-25 pg eq. 20E per embryo. This quantity remained constant until the 7th day of development when the amount of hydrolysable material increased. This increase in RIA-positive material released by hydrolysis was approximately equal to the ES titer detected in this peak. The esterase-labile products remained around 45 pg eq. 20E/embryo or larva until just after the ES peak during larval development, where again the increase in hydrolysable products was a little more than the ES peak. The amount of RIA-positive material released thereafter remained about the same. In another series of extractions of embryos, the same general pattern was found. However, we noted in a second series of extractions of the larvae that a much greater level of esterase-labile ES conjugates was present (data not shown); this may only be reflecting the presence of a higher quantity of ES conjugates incorporated into the egg during vitellogenesis. This observation is corroborated by our investigation of the fate of conjugates which accumulated during vitellogenesis after injection of the radioactive hormones. No hydrolysis of the tritiated AP was observed (Appendix II, see section 3.1.1.).

The fact that the amount of RIA-positive material released by esterase hydrolysis increased with the appearance of each ES peak suggests that the endogenous ES peak is being synthesized *de novo* and is then inactivated by conjugation to form AP. Our deduction of *de novo* synthesis of ES in the embryos is valid if we have hydrolysed and detected all conjugates. Other unidentified conjugates not hydrolysable by known methods may also be present. Although we verified our hydrolysis technique with radiolabelled conjugates, perhaps longer incubation times would have released more conjugates as was demonstrated in the cricket *Acheta domesticus* (Whiting and Dinan 1988). Also, it is possible that conjugated forms of ES precursors are found in the freshly laid egg and their hydrolysis and conversion to E and 20E are the source of the ES peaks and subsequent conjugates. Nevertheless, *de novo* synthesis of ES has also been suggested in the embryos of the dipteran *C. erythrocephala* (Bordes-Alléaume and Sami 1987), the cockroach *Nauphoeta cinerea* (Lanzrein *et al.* 1984), the cricket *Gryllus bimaculatus* (Espig *et al.* 1989, Weidner *et al.* 1992) and the decapod *Macrobrachium rosenbergii* (Young *et al.* 1991).

The role of these AP remains unknown. Perhaps they play a physiological role during embryogenesis, but they appear to be inactivation products of both endogenous (Connat *et al.* 1984) and exogenous ES (Connat *et al.* 1986a). In this latter case, although large quantities are found in the midgut of females, small amounts are also incorporated into the eggs. The authors hypothesized that their presence in freshly laid eggs may only be "artifactual" where they are incorporated during vitellogenesis unspecifically bound to the lipophilic vitellogenins. This is in accordance with our finding that the quantity of endogenous AP found in the eggs is extremely variable (Connat and Dotson 1988).

In contrast to hydrolysis with esterase, hydrolysis with *Helix pomatia* juice throughout embryonic and larval development did not release significant amounts of RIA-positive material. These data suggest that conjugates similar to, e. g., the polar conjugates described in *Locusta* (see Hoffmann and Lagueux 1985), do not participate in the production of the embryonic and larval peaks.

2.2. Ecdysteroid titers in *A. hebraeum* embryos

In contrast to *O. moubata* embryos where a peak of ES was observed, no distinct peak of ecdysteroid immunoreactive material was detected when crude methanolic extracts of the various ages of embryos from 5 different series of *A. hebraeum* eggs were analyzed using radioimmunoassay (Appendix V, fig. 2a). The amount of immunoreactive material appeared to remain between 50-200 pg equivalents of E per mg. However, a few samples around the 12th day of development (following the production of embryonic layer B and the germinal disk) and around the 44-46th day (following the beginning of larval epicuticle production) contained as much as 350 pg/mg. Because a temporal correlation of high ES titers and cuticle

deposition has been made in nymphs of this species and in the embryos of *O. moubata* as well as in nymphs of other ticks species, it is probable that high titers of ES are needed for stimulation of, at least, larval cuticle production. The length of time necessary for the completion of embryonic development is variable for *A. hebraeum* and the development of these embryos is not well synchronized. A large number of eggs (20-30 mg) were extracted for each sample, and the combination of the "high" titers of some eggs and the lower ES titers of eggs at a slightly different stage of development may have obscured any peak.

Egg extracts from *A. hebraeum* embryos 0, 10, 20, 30, 40 and 48 days old were analyzed for ecdysteroid-immunoreactive material before and after hydrolysis with porcine liver esterase. The RIA-positive material decreased from 180 pg E per mg at oviposition to 110 pg 20 days later (Appendix V, Fig 2b). The amount of RIA-positive material remained about the same thereafter. Esterase hydrolysis did not release any RIA-positive material in the Day 0 and Day 10 samples. Esterase hydrolysis of extracts from embryos greater that 10 days old released RIA-positive material. However, the titer was only increased to that of the first day of development. It appears that between 10 and 20 days of development, the ecdysteroid immunoreactive material was conjugated to form esters not easily detected with RIA. The metabolism of tritiated E and 20E in the embryos to form AP1 and AP2 corroborates this idea (see Appendix V). Also, one must note that the esterase labile conjugates present at the end of development only increased the ecdysteroid RIA-positive material to the level of the freshly laid egg. If more ES are synthesized to stimulate larval cuticle production, then these newly synthesized ES do not appear to be inactivated by conjugation to form esterase labile conjugates. If the synthesized ES are produced in low quantities and are conjugated to form esters, the quantities are so low that they do not significantly change the amount of esterase-labile material present. This is unlike *O. moubata* embryos and larvae where, at the appearance of each ecdysteroid peak, an increase in the RIA-positive material releasable by esterase hydrolysis was noted.

The profile of ecdysteroid-immunoreactive material present during embryonic development of *A. hebraeum* is very different from that of *O. moubata*. In addition, although no distinct peak was detected during embryonic development of *A. hebraeum*, the ES titers in *A. hebraeum* are much higher than those in *O. moubata* embryos, even higher than the peaks detected. A situation similar to this has been noted in two crab species of the genus *Cancer* (Okazaki and Chang 1991). In embryos of *Cancer anthonyi*, in which no peak of ES is detected during development, ES titers, even at the lowest titer detected near the end of embryonic development, are higher than they are in embryos of *Cancer magister*, in which a peak of ecdysteroids was detected at the end of embryonic development. Perhaps the reason that no peaks of ES are detected at the end of embryonic development of *A. hebraeum* and *C. anthonyi* is that the ES production or release for a physiological event at the end of development, i.e., cuticle production, is masked by the high background of ES immunoreactive material.

3. Metabolism of [^{3}H]-ecdysteroids during embryonic development

Radiolabelled ES have been used extensively in the study of biosynthesis (see Rees 1985) and metabolism of ES (see Koolman and Karlson 1985, Lafont and Connat 1989). When used in metabolic studies, the radiolabel facilitates the detection of different metabolites and sometimes permits the quantification of relative abundance of metabolites with respect to others produced. When radiolabelled ES are injected into the whole animal, the rate at which the hormone is metabolized and the nature and relative abundance of the metabolites produced will vary depending on the physiological state of the animal and thus may reflect its physiological state. Also, the tissues that store and metabolize the hormone can be identified. More precise identification of these tissues can be made when separate tissues are cultured *in vitro* with the radiolabelled hormone. In addition, radiolabelled hormones are useful in characterizing enzymes involved in hormone production or metabolism.

However, there are limits to the data obtained with radiolabelled compounds. For example, because many hormones circulate in the hemolymph bound to a carrier protein, the injected unbound hormone may not be metabolized in the same manner as bound hormone. Only the incorporation of a remote radiolabelled precursor such as cholesterol would give the true picture of the natural metabolites. In cultures the tissues are separated from their natural environment and are without their nervous and humoral components and thus may not

metabolize the hormone in a normal manner. In addition, in cultures where embryos are broken open, certain tissues may be destroyed and, thus, the way the hormone is metabolized may be affected.

In ticks radiolabelled compounds have been used to study the metabolism of injected and ingested ES and to study the metabolism in various tissues (Connat *et al.* 1986b) and various developmental stages (Bouvier *et al.* 1982, see Connat 1987). Several possible sites (i.e. the midgut and the carcass) for the hydroxylation of E to 20E have been identified (Vuillème unpublished). In addition, investigations with radiolabelled compounds have lead to the discovery of a particularly efficient detoxification pathway—conjugation at the C-22 position to form apolar long chain fatty acid esters of ES (Diehl *et al.* 1985, Wigglesworth *et al.* 1985). This detoxification pathway appears to be present in all tick stages (see Connat 1987, Connat *et al.* 1988—Appendix II). Through the use of radiolabelled ES lacking an OH group at the C-22 position, it was found that *O. moubata*, at least, cannot inactivate such ES (Connat *et al.* 1986b). Two other possible inactivation pathways that have been discovered are the polar pathway and the formation of epimers (see Connat 1987).

Keeping in mind the advantages (and disadvantages) of radiolabelled hormones in metabolic studies, we investigated the metabolism of [^{3}H]-E and sometimes [^{3}H]-20E *in vivo* and *in vitro* in *O. moubata* embryos and larvae, and in *A. hebraeum* embryos. In addition, preliminary studies were done with *B. microplus* embryos. The results of these studies are summarized in Table 1. The *in vivo* studies followed the tritiated ES ([^{3}H]-E or [^{3}H]-20E) or ecdysteroid metabolites (polar products [PP] or apolar conjugates [AP]) found in eggs after vitellogenic females were injected with [^{3}H]-E or [^{3}H]-20E. In the *in vitro* studies, the metabolism of [^{3}H]-E cultured in TC199 medium with broken open embryos or larvae or embryo homogenates was investigated.

3.1. *Ornithodoros moubata*

3.1.1. Fate of incorporated conjugates in *O. moubata* embryos and larvae (Appendix II)

Freshly laid eggs from females injected with [^{3}H]-E or [^{3}H]-20E contained apolar conjugates (AP), mainly AP2 (75-85%) and smaller amounts of AP1 (12-25%). HPLC analysis of crude methanol extracts of several embryonic stages revealed that these products remained unchanged throughout embryonic development until the eclosion of the young larva 9-10 days after oviposition. During larval development, there was a conversion of AP2 to AP1, the percent of radiolabel attributed to AP1 increasing to 35% while the percent of AP2 decreased to 65%. These percentages remained unchanged in the freshly molted first instar nymphs. Esterase hydrolysis of AP from eggs or larvae of females injected with [^{3}H]-20E released only 20E. Esterase hydrolysis of AP from eggs and larvae of females injected with [^{3}H]-E yielded E and a small amount of 20E. No change was observed in the relative proportion of E and 20E released by hydrolysis in the stages investigated (Appendix II, Fig. 6).

Because no peaks of labelled free 20E nor a relative increase of 20E in the AP was observed during embryonic and larval development, the embryos and larvae do not appear to use these maternally derived conjugates. Special attention was paid to the 7th day of embryonic development which corresponded to high endogenous titers (20pg/embryo) of 20E. Since a detectable peak of 50 cpm is equivalent to 0.45 pg, the contribution of the radiolabelled AP in this case would need only to correspond to 1/444 of the ecdysteroid peak of 200 pg per 10 embryos (minimum number of eggs used for extraction). Contrary to what occurs to the polar conjugates found in the eggs of *L. migratoria* (Lagueux *et al.* 1979, Sall *et al.* 1983), or to that which has been suggested by hydrolysis experiments of ecdysteroid esters using homogenates of 15 day old *B. microplus* embryos (Wigglesworth *et al.* 1985), the AP of *O. moubata* are not hydrolysed during embryonic development.

These AP probably occur naturally in the embryos since it is possible to increase the quantity of RIA-positive material by hydrolysis with esterase (Connat *et al.* 1984, Connat and Dotson 1988, Dotson *et al.* 1991-Appendix I). However, the amount of esterase hydrolyzable material present is variable (Connat and Dotson 1988). Because the body fluids of vertebrates may contain ecdysteroid immunoreactive material either as a result of feeding on plants containing phytoecdysteroids (Simon and Koolman 1989) or as a result of a parasite infection, i.e., helminths (Franke and Käuser 1989, Simon and Koolman 1989), the

Table 1. Summary of [^{3}H]-ecdysteroid metabolites produced *in vivo* and *in vitro* by embryos or larvae of three tick species.

Species	Type of Experiment	Hormone Tested	Stage Tested	Conversion E to 20E	Formation of AP	Epimerization	Polar Products	Reference
Ornithodoros moubata embryos & larvae	*In vivo*	[^{3}H]-E &[^{3}H]-20E	Newly laid eggs - larvae-N1	No	Conversion of AP2 to AP1	No	No	Appendix II
O. moubata embryos	*In vitro* Hanging drop	[^{3}H]-E	Oocytes and each day of embryonic development	Yes, starts around Day 2	Yes, already present in freshly laid eggs	No	No	Appendix III
	In vitro Homogenate	[^{3}H]-E	Day 0 Day 0 (frozen) Day 6	No No Yes	Yes Yes Yes	No No No	No No No	Appendix III
O. moubata larvae	*In vitro* Hanging drop	[^{3}H]-E	Each day of larval development	Yes (amount depends on developmental stage)	Yes (amount depends on developmental stage)	No	Yes (unknown PP-A, B & C)	Appendix III
	In vitro Homogenate	[^{3}H]-E	Day 1	Yes (12 % of metabolites)	Yes (70% of metabolites)	No	No	Appendix III
Amblyomma hebraeum	*In vivo*	[^{3}H]-E	Freshly laid eggs to larvae	Yes	Yes (starts around 15th day of development)	Yes (present as apolar conjugates)	Yes (polar product 2')	Appendix V
	In vivo	[^{3}H]-20E	Freshly laid eggs to larvae	not applicable	Yes (starts around 15th day of development)	Yes (present as apolar conjugates)	Perhaps (very small quantities (3 %) of polar product 2	Appendix V
	In vitro Broken open	[^{3}H]-E	Day 0 Day 40	No Yes	?? (one peak fractions 106-110) ?? (one peak - fractions 106-110)	Yes Yes	Small quantities of many metabolites more polar than E	Appendix VI
	In vitro Homogenate	[^{3}H]-E	Every 10 days throughout development	No	?? (one peak - fractions 106-110)	Yes (all stages)	Small quantities of many metabolites more polar than E	Appendix VI
Boophilus microplus	*In vivo*	[^{3}H]-E	Freshly laid eggs, 10, 15, 21 days and larvae	No	AP2 to AP1 which are esters of PP	No	Yes (formed between 1st & 10th day of development, also released after hydrolysis of AP1)	Appendix VII
	In vitro Homogenate	[^{3}H]-E	only freshly laid eggs	Yes (2% of metabolites on RP-HPLC)	Yes	No	No (except one product slightly less polar than E)	Appendix VII

nutritional history of the female may contribute to this variability. To illustrate this, several stages of ticks were fed tritiated hormone and the eggs of the resulting females were analyzed. When second instar nymphs ingest [^{3}H]-E, 2.5% of the ingested radiolabel is recovered (as AP) in the eggs of the resulting females (Appendix II). If the female itself ingests the hormone, this proportion increased to 10.2% (Connat *et al.* 1986a). Thus, the frequency of which the females encounter ES and the concentration of these hormones in the blood meals throughout the life cycle may influence the proportion of AP accumulating in the eggs. Their incorporation into the eggs may be artifactual. The AP circulating in the hemolymph during vitellogenesis may be bound to the vitellogenins in a nonspecific manner and could thus be incorporated into the oocytes. In the early development of *O. moubata*, AP2 are converted to AP1 only after hatching when the intestine has fully developed and begins actively digesting the vitellus. This demonstrates that this transformation requires enzymes not present in the yolk but which are produced later by the midgut cells.

We conclude that in *O. moubata* embryos and larvae the AP are metabolites of an inactivation reaction and are not a source of conjugated hormone to be liberated during the molting cycle or embryonic development.

3.1.2. *In vitro* metabolism of [^{3}H]-ecdysone during embryonic and larval development of *O. moubata* (Appendix III)

When oocytes, embryos or larvae were broken or slit open in hanging drops of TC199 containing [^{3}H]-E, they were able to metabolize the hormone to one of the following products: 1) 20E, 2) apolar products, principally AP2, the fatty acid esters of E or 20E and 3) unknown polar products labelled A, B and C (see Fig. 2 of Appendix III). The rate at which [^{3}H]-E was metabolized depended upon the age of the embryos or larvae. Interestingly, the metabolic rate decreased when the endogenous titer began to increase and the rate increased again as the endogenous titers peaked (Fig. 1A). The decrease in metabolism did not appear to be due to a dilution of the specific activity by the endogenous hormone but was due to a slowing of the detoxification mechanism, AP production, during endogenous E production. Thus some regulation of the activity of the various enzymes appears to occur.

Hydroxylation of [^{3}H]-E to form 20E was first noted in 2 day old embryos. This is when the nucleolus forms in all cells (Fagotto *et al.* 1988). Thus, the "machinery" for enzyme synthesis is in place in all cells and may be the reason that 20-hydroxylation begins at this time. Also, during this period, the vitellus-filled macromeres are formed. They are the primary endoderm which will later help in the formation of the midgut epithelium. The formation of this tissue may be responsible for the initiation of 20-hydroxylation. All tissues of the fifth instar nymphs of *O. moubata* are able to hydroxylate [^{3}H]-E to form 20E *in vitro*; however, the midgut and the ventral carcass were the greatest producers of 20E (Vuillème, unpublished results). In nymphs of a closely related species, *O. parkeri*, the fat body appeared to be the site of ecdysone 20-hydroxylation (Zhu *et al.* 1991).

Ecdysone 20-hydroxylation was noted in cultures of all stages older than 2 days. The highest percentages of free 20E are produced one day before the endogenous peak occurs in the embryo and on the same day as the endogenous peak in the larvae (Fig. 1B). This resembles the situation in several insect species in which a peak of ecdysone 20-monooxygenase activity is associated with endogenous ES peaks. However, because we do not know how much 20E was metabolized to form the PP or conjugated to form AP (except in a few cases), we cannot be sure how much 20E was produced. By comparing the conjugated and free 20E in the few cultures of which we hydrolyzed the AP, we saw a reduction in the percentage of radiolabel converted to 20E. This suggests a reduction in 20-monooxygenase activity. In addition, by comparing the conjugated and free 20E in the cultures of 7 day old embryos and the 1 day old larvae, we found that the percentage of radiolabel corresponding to 20E was the same. However, only 30% was conjugated in the embryo cultures whereas 80% was conjugated in 1 day old larvae cultures. This suggests that production of AP may be involved in regulation of 20E titers.

AP production was observed in all stages of development, even oocytes, and was highest during periods of low endogenous titers (Fig. 1C). Because these conjugates are incorporated into the eggs of *O. moubata*, they were once thought to be storage products which would be hydrolyzed during development to release free ES (Connat *et al.* 1984).

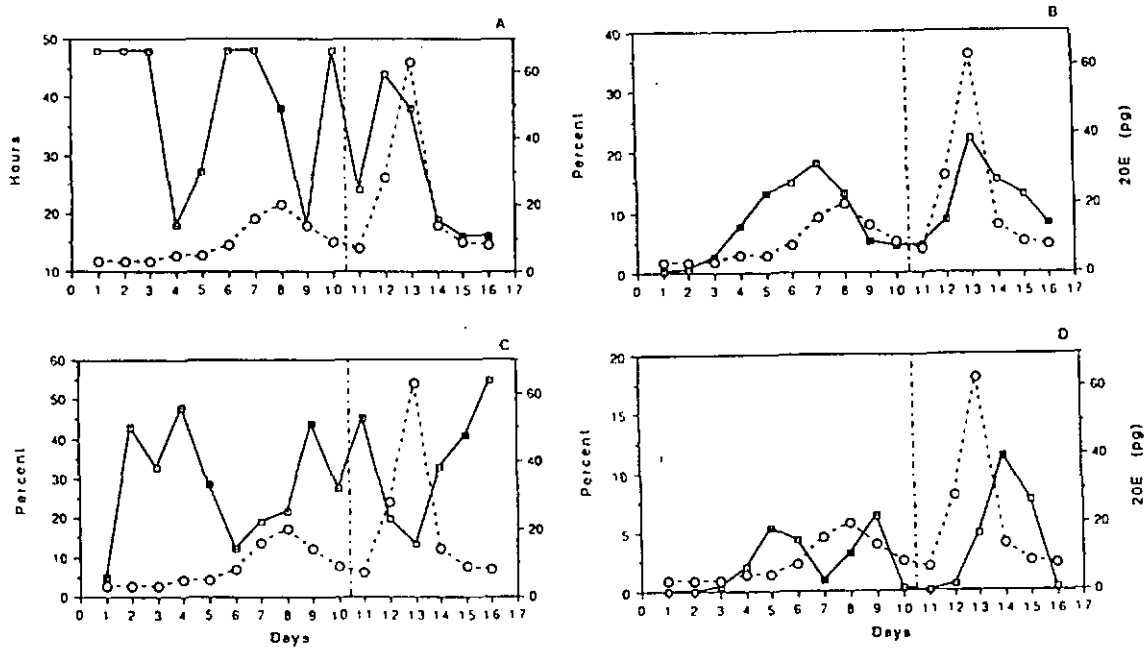

Figure 1. The endogenous ecdysteroid titer expressed in 20-hydroxyecdysone pg equivalents per tick (from Appendix I) (-o--o--o--o-, right axis) versus the half-life expressed in hours (A). In the graphs B, C and D, the endogenous ecdysteroid titer is plotted against the percent of radiolabel in the 24 hr culture corresponding to 20-hydroxyecdysone (B), apolar conjugates (C), and unknown polar products (D).

However, because the radiolabelled AP found in freshly laid eggs (see previous section) are not hydrolyzed during embryonic development, the endogenous AP appear to increase with each endogenous peak (see section 2.1.2.) and this apolar pathway is efficient in cultures of embryos or larvae with low ES titers, we conclude that they are detoxification products in the embryos and larvae of *O. moubata*.

The localization of this detoxification mechanism has not been completely defined, but it occurs in several tick organs (Vuillème, unpublished data). The midgut appears to be particularly efficient. The fact that young oocytes are able to conjugate the ES indicates that enzymes either are produced by the oocyte itself or are brought into the oocyte during vitellogenesis. The vitellus-filled macromeres, which constitute the young midgut, thus appear to contain their detoxification enzyme machinery.

The production of PP appears to be highest during periods of increasing or decreasing endogenous titers (Fig. 1D). Even, the first peak of PP production, which occurs in cultures of five day old embryos, appears to follow a not-always-evident peak of ES that occurs on the fourth day of development (see Appendix I). These data suggest that PP production occurs when endogenous titers should be decreasing and may represent an inactivation pathway. The nature of the polar products A, B and C is unknown; product B has retention times similar to 20,26-dihydroxyecdysone. More in depth studies are necessary to establish the significance of this pathway.

3.2. *Amblyomma hebraeum*

3.2.1. Fate of [^{3}H]-ecdysteroids or metabolites incorporated into *A. hebraeum* eggs

3.2.1.1. Fate of [^{3}H]-20-hydroxyecdysone and its metabolite

When [^{3}H]-20E is injected into *A. hebraeum* females at the beginning of oviposition, approximately 76% of the injected hormone is transferred to the eggs (see Appendix IV). More than 95% of the transferred radiolabel is free 20E (see Appendix V, Fig. 3a). The only other radiolabelled compound present in the freshly laid eggs is a compound more polar than 20E, which has the same retention time as product 2 described in females of this species (Appendix IV). HPLC analysis of extracts from various ages of embryos showed that the embryos appear to inactivate the hormone (Appendix V). By day 15 the embryos began

converting the 20E to AP2 which appeared to be gradually converted to AP1. By day 35 all of the 20E had been conjugated and the AP1 represents the major metabolite (70-75% of the radiolabel). Product 2 gradually increased to make up approximately 10% of the radiolabel by the end of development.

The most interesting finding in this study was the presence of epimers. Esterase hydrolysis of the AP released not only 20E (20%), but also a product which comigrates with the 3α epimer of 20E (80%). The proportion of these metabolites did not change after day 30.

3.2.1.2. Fate of [^{3}H]-ecdysone and its metabolites

When ecdysone was injected into engorged *A. hebraeum* females at the beginning of oviposition, four products were found in the eggs (Appendix IV and V). They corresponded to unmetabolized E, 20E, polar product 2' (polar product 2 is probably the 20-hydroxy homologue of this compound) and AP2. The latter were found only in eggs which were produced after 4-6 days of oviposition. As oviposition progressed, the proportion of radiolabel corresponding to E decreased as the other products increased in the freshly laid eggs (Appendix IV and V). Because the percentages of radiolabel corresponding to these products varied with each day of oviposition, it was necessary to determine the percentages of these compounds in the freshly laid eggs and compare that with older, developing embryos in order to calculate how the percentages of the different products had increased or decreased. Figure 6 of Appendix V is a composite of the percentages of the metabolites observed in the 15, 20 and 30 day embryos and the remainder of the graph was extrapolated from the other days observed. By day 15, the E had been reduced to slightly more than half and the 2' had doubled. The amount of radioactivity in 20E had only decreased by 4%. Most of the E appeared to be metabolized to 2' and some of the E and 20E had been conjugated to form AP2. The radioactive ecdysone is completely metabolized by day 30. The amount of radiolabel corresponding to 2' increased only while the E was being metabolized. This polar product is probably not the product of 20E metabolism since it is not produced when only 20E is incorporated into the eggs. Because product 2' has a retention time of 5 min instead of 15 min when H_2O is used in the place of Tris buffer as the eluant on HPLC, it appears to be ionic in nature. In addition, it is resistant to *Helix* and esterase hydrolysis.

Production of AP2 follows a similar pattern to that observed in the metabolism of [^{3}H]-20E in embryos from females injected with [^{3}H]-20E. The formation of these products is first observed in the 15 day cultures (8% of the radiolabel) and increases to 50% of the radiolabel in the 25 day culture (Appendix V, Fig. 5b). Thereafter the percentage of AP2 decreases to represent only 18% of the radiolabel at the end of development. The AP1 are first observed in the 20 day culture. These products increase as the AP2 are decreasing and reach 38% of the metabolites in the 45 day embryos.

Esterase hydrolysis of the AP1 and AP2 releases 20E and E and the 3α epimers of these ES (Appendix V, Fig. 5c). By comparing the percentages of radiolabel corresponding to polar product 2', 20E compounds (20E and its 3α epimer 20E') and the E compounds (E and its 3α epimer E') in the crude and esterase-treated extracts of 25 day old embryos with the freshly laid eggs, we see that E is converted to polar product 2' or conjugated to form AP1 and AP2 which may also be hydroxylated and/or epimerized. The order in which conjugation, hydroxylation and epimerization takes place is not known. Hydroxylation presumably takes place before conjugation, because the long chain fatty acid at C-22 would probably interfere with hydroxylation at C-20. The fact that no free epimer or 3-dehydro compound was detected suggests that perhaps epimerization may take place after conjugation. However, substrates for the ecdysone oxidase (enzyme of the first reaction of the epimerization process) of *Calliphora* requires a C-22 hydroxyl group (Koolman 1985).

This is the first time epimers have been found in the eggs of a tick. They do not appear to be present in *O. moubata* (Appendix II and III) or *B. microplus* embryos (Appendix VII). Preliminary evidence suggests the presence of epimers in *O. moubata* nymphs (Connat 1987). They have been shown to be present as conjugates at the end of oviposition of *A. hebraeum* females injected with [^{3}H]-E or [^{3}H]-20E. The role of the epimers in ticks is unknown. However, in insects ecdysteroid epimer formation is irreversible and represents an inactivation process (Lafont and Connat 1989). Thus, the [^{3}H]-E and [^{3}H]-20E incorporated

into *A. hebraeum* eggs appear to be doubly inactivated by conjugation and epimerization during development.

3.2.2. *In vitro* metabolism of [^{3}H]-ecdysone during embryonic development of *A. hebraeum* (Appendix VI)

When homogenates of various ages of *A. hebraeum* embryos are cultured in [^{3}H]-E, this hormone is metabolized mainly to the 3α epimer of E, 3-epiecdysone (E'). Other metabolites less polar than E included 3-dehydroecdysone (3DE) which was verified with CI/D mass spectrometry, an unknown group of products labelled Y which are slightly less polar than 3DE, and unknown product Z which had retention times similar to 22,25-dideoxyecdysone or 2,22,25-trideoxyecdysone. A small percentage (10% or less) of the metabolites were more polar than E.

Broken open freshly laid eggs produce the same metabolites as the homogenates except in different percentages. The E' is not the major metabolite. The 3DE and Y are in equal or greater percentages. In addition, the compounds more polar than E are more abundant. In the 40 day old broken open embryos, a metabolite which has retention times similar to that of 20E (8%) was present. It is likely that some of the smaller peaks following this compound correspond to 20E' and 3D20E.

Interestingly, when all the cultures are surcharged with E (3 ng/ml) to increase the ecdysteroid concentration of the medium to that of the freshly laid *A. hebraeum* eggs, most the [^{3}H]-E is metabolized. In the culture of the homogenized and broken open freshly laid eggs without a surcharge of E, 35 and 41%, respectively, of the [^{3}H]-E remained whereas in the surcharged cultures only 7% of the [^{3}H]-E was still present. The explanation for the increased metabolic rate is not known. In addition, in the surcharged 40 day broken open cultures, very little 20E is present. It is probable that the 20-hydroxylation is taking place, perhaps at the same rate as previously, but that the specific activity of the hormone is so reduced that the 20E is not detectable.

Unlike the homogenate cultures of freshly laid eggs of *O. moubata* and *B. microplus*, where AP production was observed, the cultures of the freshly laid eggs of *A. hebraeum* do not indicate the presence of the enzyme(s) for apolar ester formation. In addition, no AP appear to be present in the cultures of older embryos where we know that conjugate formation takes place *in vivo*.

3.3. *Boophilus microplus* (Appendix VII)

3.3.1. Fate of incorporated conjugates in *B. microplus* embryos

When *B. microplus* females are injected with [^{3}H]-E, 76 to 96% of the radiolabel is recovered in the eggs. Similarly to *O. moubata*, most of the radiolabel incorporated into the eggs appear to be AP2, which are most likely esters of E. Only a small percentage of label comigrates with AP1 and occasionally minute amounts comigrate with E. Esterase hydrolysis of AP in freshly laid eggs releases only E. Unlike *O. moubata* embryos, *B. microplus* embryos appear to metabolize the AP2. By day 10 most of the AP2 has been converted to AP1 (67% of radiolabel) and to polar products (20%) which are most likely ecdysteroid-26-oic acids (Crosby *et al.* 1987). Esterase hydrolysis of the AP releases mostly the polar products. This indicates that the AP1 of *B. microplus* are probably esters of ecdysteroic-26-acids and not of E as in *O. moubata*. The principal change that occurs in the metabolites thereafter is that the PP increased whereas the AP1 decreased.

These AP appear to occur naturally in *B. microplus* embryos. Esterase treatment of RP-18 HPLC fractions corresponding to AP2 released E (Connat and Dotson 1988), which is the major ecdysteroid during vitellogenesis of *B. microplus* females (Wigglesworth *et al.* 1985). The presence of AP in the eggs of *O. moubata* has been described as a possible artifactual incorporation (Appendix II), and this may be the case in *B. microplus*.

Whether these AP are a source of hormone for embryonic cuticles or development remains a question. Because they do not appear to be changed after day 10, they probably do not influence the latter part of embryonic development and larval cuticle production. However, during the first 10 days of development when the conversion to ecdysteroid-26-oic

acids and their conjugates occurs, the AP2 could be hydrolyzed to release free E which could influence the production of embryonic cuticles. The free ecdysteroid could then be inactivated by acid formation and conjugated to form esters. It is also possible that the AP2 are converted to AP1 by acid formation which would make the product doubly inactivated.

3.3.2. *In vitro* metabolism of [^{3}H]-ecdysone in cultures of freshly laid eggs of *B. microplus*

The homogenates of freshly laid eggs are able to produce metabolites with retention times similar to those of the apolar ester conjugates of E. This is similar to the situation in *O. moubata*. Since the apolar conjugates are found in the eggs of both species and both species are able to conjugate [^{3}H]-E very early in development, it is possible that free hormones are incorporated into the oocytes and the enzymes for conjugation convert the hormone to esters in the oocyte. In addition, an unknown product slightly more polar than E is also present in cultures of *B. microplus*.

CONCLUSIONS

The three embryonic cuticles produced by the argasid tick *O. moubata* are very similar in structure to those produced during early embryogenesis of the ixodid ticks *A. hebraeum* and *B. microplus*. The fact that they are present in all three of these distantly related ticks suggests that these embryonic cuticles may be a common feature in developing tick embryos. The production of the fourth embryonic cuticle, in actuality the larval cuticle, resembles that of other immature ticks and in *O. moubata* embryos, at least, production of this cuticle has been correlated with a peak of ES. Although no discernable peaks of ES were detected during larval cuticle production in *A. hebraeum* embryos because of lack of synchronization of development, it is probable that production of this cuticle is stimulated by ES in a manner similar to that in other immature ticks.

In contrast to the cuticles, the titer and metabolism of ES appear to differ among the embryos of these three species. *O. moubata* eggs contain maternally derived fatty acid ester conjugates of ES and no free ES when oviposited. During development they do not appear to use the conjugates as an ecdysteroid source for the peak of 20E and E produced during cuticle production. In fact, they appear to produce more of these conjugates at the appearance of each endogenous peak. Even developing oocytes possess the capability of conjugating the ES and conjugation appears particularly efficient during periods when ES titers should be low. This suggests that this metabolic pathway is an inactivation and detoxifaction mechanism. In addition, *O. moubata* embryos are capable of hydroxylating E to form 20E. This enzyme system appears efficient during periods when endogenous titers should be high. Because *O. moubata* embryos do not appear to reuse the maternal conjugates but produce more at each endogenous peak and because they are able to hydroxylate E to 20E, it appears that the embryos synthesize ES *de novo*. To inactivate these high levels of ES, the embryos appear to possess another pathway of inactivation in addition to the conjugation to form apolar esters. This is the production of unknown polar products, which was shown to be active during high or decreasing ecdysteroid titers.

B. microplus embryos, like *O. moubata* embryos, appear to contain maternally derived apolar ecdysteroid esters and possess the capability of forming these ester conjugates when they are oviposited. However, *B. microplus* appears to deal with the incorporated conjugates differently than *O. moubata*. In *B. microplus*, within the first 10 days of development, the apolar esters are converted to polar products which may be ecdysteroid-26-oic acids and esters of these acids. Whether these ES are a hormone source during embryonic development is unknown. If the ecdysteroid-26-oic acids are irreversible inactivation metabolites in ticks as they are in insects, they are not likely to be reutilized in this tick embryo.

The situation in *A. hebraeum* embryos differs greatly from that in *O. moubata* or *B. microplus*. Mostly free E and 20E are found in the newly laid eggs. During the first 15 days of development, some of the E is converted to 20E and an unknown polar product. Around day 15, the embryos begin to conjugate the free 20E and the remaining E to form apolar fatty acid esters. In addition, part of the 20E and E are converted to 3α epimers of these compounds, probably before conjugation. *In vitro* analyses revealed that the formation of epimers takes place via the formation of 3-dehydroecdysone. If the formation of the 3α epimers is an irreversible inactivation process, as it is in insects, then the ES are doubly inactivated in *A. hebraeum*.

The purpose of the free ES in the freshly laid eggs remains unknown. Perhaps they are involved in egg shell production of developing oocytes (Diehl *et al.* 1986), or stimulate meiotic reinitiation in the oocyte in a manner similar to that described in several insect species (Lanot 1987, 1988, 1989a,b). The free ES may play a role in the production of embryonic cuticles A and B since these cuticles are produced before the hormones are inactivated. Another possible role for these ES is to act as a feeding deterrent for predators in a manner similar to the allochemicals found in the eggs of certain plant feeding insects (Hinton 1981). ES concentrations as low as 10^{-5} *M* have been reported to discourage feeding in several insects (Ma Wei Chun 1972, Schoonhoven and Derksen-Koppers 1973). Because the incorporated free ES of *A. hebraeum* embryos appear to be inactivated before larval cuticle production, they probably are not involved in stimulating this process. If high ES titers are needed to stimulate production of this cuticle, then they are probably synthesized *de novo*.

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I

GENERAL AND COMPARATIVE ENDOCRINOLOGY 82, 386–400 (1991)

Cuticle Deposition and Ecdysteroid Titers during Embryonic and Larval Development of the Argasid Tick *Ornithodoros moubata* (Murray, 1877, *sensu* Walton, 1962) (Ixodoidea:Argasidae)

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Timing of embryonic and larval molts at the ultrastructural level and presence of ecdysteroids (ES) during embryonic and larval development of the argasid tick *Ornithodoros moubata* were studied. Embryonic "cuticles" A, B, and C were deposited 24–30 hr, 48–56 hr, and 6 days after oviposition, respectively. Deposition of the larval cuticulin layer started on Day 8 of embryonic development and procuticle deposition continued after hatching until apolysis of the larval cuticle 40 hr posthatch. Plaques of cuticulin formed on tips of microvilli 48–56 hr after hatching and procuticle was deposited until after ecdysis. Radioimmunoassay (RIA) was used to determine the ES titer in methanolic extracts of various ages of embryos and larvae. No peaks of RIA-positive material were detected during deposition of envelopes A, B, and C. However two peaks of ES were observed during embryonic development, one which coincided with the shortening of the germ band and a second which coincided with the deposition of the larval epicuticle on Day 8. During larval development, a peak of ES was observed on Day 3 (48–56 hr posthatch) and was correlated with nymphal epicuticle deposition. HPLC–RIA revealed that these last two peaks consisted mainly of 20-hydroxyecdysone together with a small quantity of ecdysone. Conjugated RIA positive material was present in freshly laid eggs and an augmentation of this esterase hydrolysable material was noted at the appearance of each ES peak. Thus the embryos did not appear to be hydrolyzing the maternal apolar conjugates to release ES during embryonic development; on the contrary, they appeared to be conjugating the newly synthesized hormones.

Embryonic molting or the development of several embryonic cuticles before hatching appears to be a common occurrence in arthropods and, as is the case in most areas of research carried out on members of this phylum, more studies have been carried out on insects than other arthropod classes. The fine structure of these embryonic cuticles has been described in several insect species (*Blaberus craniifer*, Bullière, 1973; *Calliphora erythrocephala*, Bordes-Alléaume and Sami, 1987; *Carausius morosus*, Louvet, 1974; Fournier, 1985; *Leucophaea maderae*, Rinterknecht, 1985; Rinterknecht and Matz, 1977, 1983; *Locusta migratoria*, Lagueux *et al.*, 1979; *Oncopeltus fasciatus*, Dorn, 1983; *Schistocerca gregaria*, Sbrenna, 1974). In general (except for *B. craniifer* and *C. erythrocephala*) these cuticles consisted of (1) an embryonic membrane or first embryonic cuticle which is laid down just before the dorsal closure and which is composed of a thin electron-dense, occasionally trilaminar membrane, (2) a second embryonic cuticle which is the true embryonic cuticle forming

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after the dorsal closure and which is composed of a fine cuticulin layer, epicuticle, a subepicuticular layer, and a lamellate procuticle (1–2 lamellae), and (3) a third cuticle consisting of cuticulin, epicuticle, and procuticle (many lamellae) which is in actuality the larval cuticle.

Ultrastructural studies have also been carried out on other arthropod groups. Transmission electron microscopic (TEM) studies of the embryonic development of the crab *Carcinus maenas* (Goudeau and Lachaise, 1983) and the isopod *Hemioniscus balani* (Goudeau, 1976) demonstrated that four cuticle cycles take place during embryogenesis (four cuticular secretions alternating with three apolyses). In three species of *Balanus* (Klepal and Barnes, 1978) and in three species of Ostracods (Tetart, 1970), two and three cuticles, respectively, are deposited during embryogenesis. Most of the cuticles consist of an electron-dense epicuticle and an inner sometimes lamellate procuticle. The last cuticle deposited, that of the free-living stage, is generally thicker and is a more complete cuticle. In the tick *Ornithodoros moubata*, three embryonic envelopes (two of which have structures similar to the embryonic cuticles in crustaceans) are formed before the deposition of the last embryonic cuticle (in reality the larval cuticle) (Vogel, 1975).

The deposition of these cuticles (in some cases all and in others only one or two) seems to be initiated by peaks of ecdysteroids (ES) in several insect species (for review see Hoffmann and Lagueux, 1985). In *C. maenas*, ponasterone A may be involved in the deposition of embryonic envelopes (Lachaise and Hoffmann, 1982).

In our study we reinvestigated the deposition of embryonic envelopes in *O. moubata* in order to determine whether peaks of ES were present during the formation of these embryonic and larval cuticles. Furthermore, we attempted to determine whether the ES present during embryonic development of this tick are a result of hydrolysis of maternal ES conjugates described in this species (Connat *et al.*, 1984, Connat and Dotson, 1988). Peaks of ES resulting from hydrolysis of maternal conjugates have been suggested during embryonic development of several insect species (For review see Hoffmann and Lagueux, 1985).

METHODS

Animals. The *O. moubata* (Murray, 1877; *sensu* Walton, 1962) females used in this study were from a strain that originated in Tanzania and has been kept in our institute for several years. In the laboratory the ticks were fed on defibrinated pig blood at 37° through a Parafilm membrane and were placed separately in cotton-plugged vials in the dark in an incubator at 27° and 30–40% relative humidity. Ten to twelve days after feeding, the females began ovipositing their batches of 50–200 spherical brown eggs (each ~1 mm diam) during an ovipositional period which lasted 10–15 days depending on how often the female was disturbed during this period.

Eggs were collected daily or every 8 hr depending on the experiment. To minimize handling of the eggs, which could disrupt embryonic development, instead of removing the eggs, each female was removed from her vial and placed in another. Approximately 10 days after the eggs were oviposited, the hexapod larvae hatched out. The nonfeeding larvae molted 5 days later, and the eight-legged first instar nymphs emerged.

Transmission electron microscopy. Embryos and larvae were submerged in 6.25% glutaraldehyde in 0.1 *M* Sörensen phosphate buffer, pH 7.4, containing 2% sucrose (Sabatini *et al.*, 1963). Because the egg shell and the cuticle do not permit penetration of the fixative, it was necessary to prick the eggs with a fine electrosharpened tungsten wire or to slit the larvae with a scalpel. The embryos and larvae remained in the fixative for at least 24 hr at 4°. The different stages were rinsed three times for 10 min with 0.2 *M* Sörensen phosphate buffer, pH 7.4, containing 5% sucrose and then held overnight at 4° in this solution. They were then postfixed for 2 hr at 4° with 1% OsO_4 in a 0.028 *M* veronal sodium–acetate buffer (Palade, 1952) containing 5% sucrose. After three 15-min rinses with Palade buffer containing 5% sucrose, the embryos and larvae were dehydrated with acetone and embedded in Spurr resin (Spurr, 1969). Semi-thin (750 nm) and thin sections (80 nm) were cut with a Sorvall MT2-B ultramicrotome. Semi-thin sections were stained with toluidine blue. Thin sections were contrasted in uranium acetate for 15 min and in lead citrate (Reynolds, 1963) for 30 min and then observed on a Philips EM 201.

Radioimmunoassay (RIA). The ES concentrations

were determined by using the RIA of De Reggi *et al.* (1975) by competition with [23,24-^{3}H]ecdysone when dialyzing against the polyclonal antibody (anti-20-hydroxyecdysone-succinyl-HSA serum; reference L. III, 16/10/74, (Hirn, 1978)) or by competition with [23,24-^{3}H]-20-hydroxyecdysone when dialyzing against the monoclonal antibody (EC-19 clone from Immunotech, Marseille-Luminy, France). The polyclonal antibody recognized ecdysone (E) about 3 times better than 20-hydroxyecdysone (20E) and about 100–150 times better than the esters of these hormones. The EC-19 is 30-fold more sensitive to 20E than the polyclonal antibody and recognized 20E approximately 12 times better than E. (For reference curves see Connat and Dotson, 1988). For convenience, all the present results are expressed in equivalents of 20E.

Extractions. The embryos and larvae were homogenized in 100% methanol, vortexed, sonicated for 3 min, and centrifuged for 10 min at 10,000 rpm at 4° to extract ES. The pellets were extracted again in 100% methanol. Depending on the volume of the combined supernatants, the volume of the extracts was reduced on a rotary film evaporator or dried directly under a flow of N_2 and resuspended in a known volume of methanol. RIA measurements were carried out on aliquots equivalent to 50 embryos or 25–50 larvae.

When it was necessary to extract ES from large quantities of eggs or larvae, the material was homogenized in 10 ml 100% methanol, vortexed, sonicated, and centrifuged for 15 min at 15,000 rpm at 4°. The pellet was reextracted with 5 ml methanol. The volume of the combined supernatants was dried on a rotary film evaporator, and the extract was removed from the balloon using 0.5 ml H_2O and then 2.3 ml methanol. This extract was dried under a N_2 flow and resuspended in methanol and then water, with the final solution being 25% methanol. The sample was then deposited on a SEP PAK cartridge.

Esterase hydrolysis. An aliquot equivalent to 25 embryos or larvae for each day of development was dried in a glass tube under a N_2 flow, then resuspended in 450 μl Tris buffer (pH 8.0, 0.10 *M*), vortexed, and sonicated for 3 min. Fifty microliters (50 IU) of esterase (EC 3.1.1.1, Boerhinger) was added and the solution was incubated overnight at 37°. The hydrolyzed extract was extracted in 9 ml methanol and centrifuged for 15 min at 15,000 rpm. The volume of the resulting supernatant was adjusted to 10 ml. An aliquot equivalent to 4 embryos or larvae was then dried under a N_2 flow and analyzed for ES using RIA (polyclonal antibody). Before carrying out the reaction, we verified this protocol by incubating the enzyme with radiolabeled apolar products from *Boophilus microplus* or *O. moubata* embryos and analyzed the products released on HPLC.

Hydrolysis with Helix juice. An aliquot equivalent to 25 embryos or larvae for each day of development was dried under a N_2 flow and resuspended in 100 μl 10 m*M* citrate buffer pH 5.5, vortexed, and sonicated for 3 min. Ten microliters of *Helix* juice (Merck 4114, twice dialyzed to remove much of the RIA-positive material) was added and then vortexed. The sample was incubated at 28° overnight. Two blanks (only citrate buffer) were also incubated with 10 μl *Helix* juice to estimate the traces of RIA positive material remaining in the enzyme solution. To stop the reaction, 1.4 ml methanol was added. The solution was vortexed, sonicated, and centrifuged. The supernatant was removed and dried. The extract was resuspended in 1.5 ml methanol and recentrifuged to remove more of the *Helix* enzymes which destroy the dialysis membrane. Two-thirds of the supernatant was dried under a N_2 flow and assayed for ES using RIA (polyclonal antibodies). Before carrying out the hydrolysis, we verified the protocol with radiolabeled phosphate conjugates found in the excrement of *Locusta* and analyzed the hydrolyzed material with HPLC.

High performance liquid chromatography. Samples in 80-μl 50% methanol were injected on a Kontron system with two 420 pumps piloted by an IBM-PC computer on a Merck Lichrosorb RP-18 (7 μm phase) column (25 cm × 4 mm) with a precolumn (4 cm × 4 mm) packed with RP-8 (40-μm phase). The eluting system was 50% methanol isocratic for 20 min and a methanolic purge for 5 min with a flow rate of 0.8 ml/min. Fractions were collected every minute and aliquots of each fraction or combination of fractions were assayed using RIA for ES.

RESULTS

Duration of Embryonic and Larval Development

In order to determine the duration of embryonic development, we collected eggs every 8 hr from six different females. Although the mean developmental time varied surprisingly between females (Table I), we considered the 486 eggs from these females as one population since, because of experimental needs, analyses would be carried out on eggs from several females (from different days of the ovipositional cycle). The average duration of embryonic development was 30.9 8-hr periods or 10.4 days (Fig. 1a). However, the standard deviation was quite large (2.2 8-hr periods) indicating that the population was not synchronized enough to justify carrying out experiments on every 8-hr of embryonic development. Eggs were collected and analyzed daily;

TABLE 1
MEAN DURATION OF EMBRYONIC DEVELOPMENT OF EGGS FROM SIX DIFFERENT FEMALES

	No. of eggs	Duration of development (8-hr periods)
♀ 1	140	29.2 ± 1.28
♀ 2	58	30.7 ± 1.55
♀ 3	121	30.7 ± 1.71
♀ 4	17	30.8 ± 0.81
♀ 5	49	31.1 ± 1.58
♀ 6	96	33.6 ± 1.71
Total	486	30.9 ± 2.20

47% hatched on the 10th day and 38% on the 11th day of development.

After hatching, the larva, a nonfeeding stage, continued to develop. The time needed for the larva to develop until the newly formed nymph withdraws its legs from the larval exuvia (tick has a gray rather than brown appearance) and the nymph breaks open the exuvia was 5 days (15 ± 1 SD 8-hr periods) and 5.5 days (16.6 ± 1.4 SD 8-hr periods), respectively (Fig. 1b and 1c). Since the standard deviation was smaller in these two cases and also since the developmental period was shorter, correlation analyses were carried out every 8 hr of larval development.

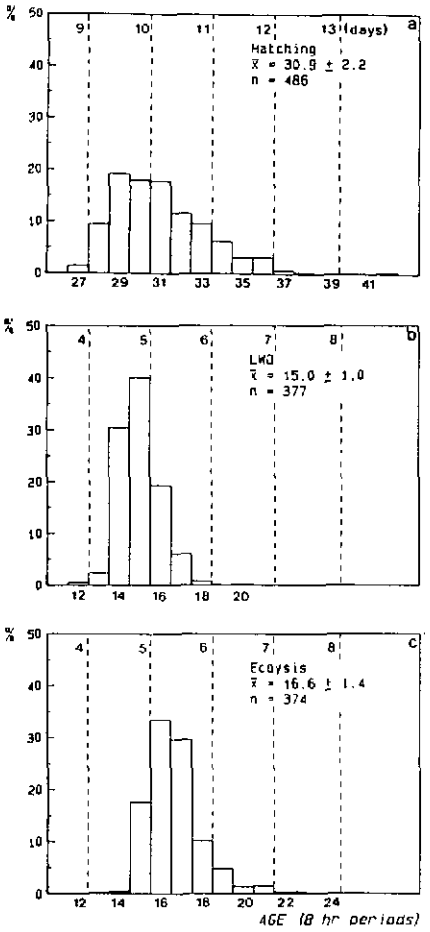
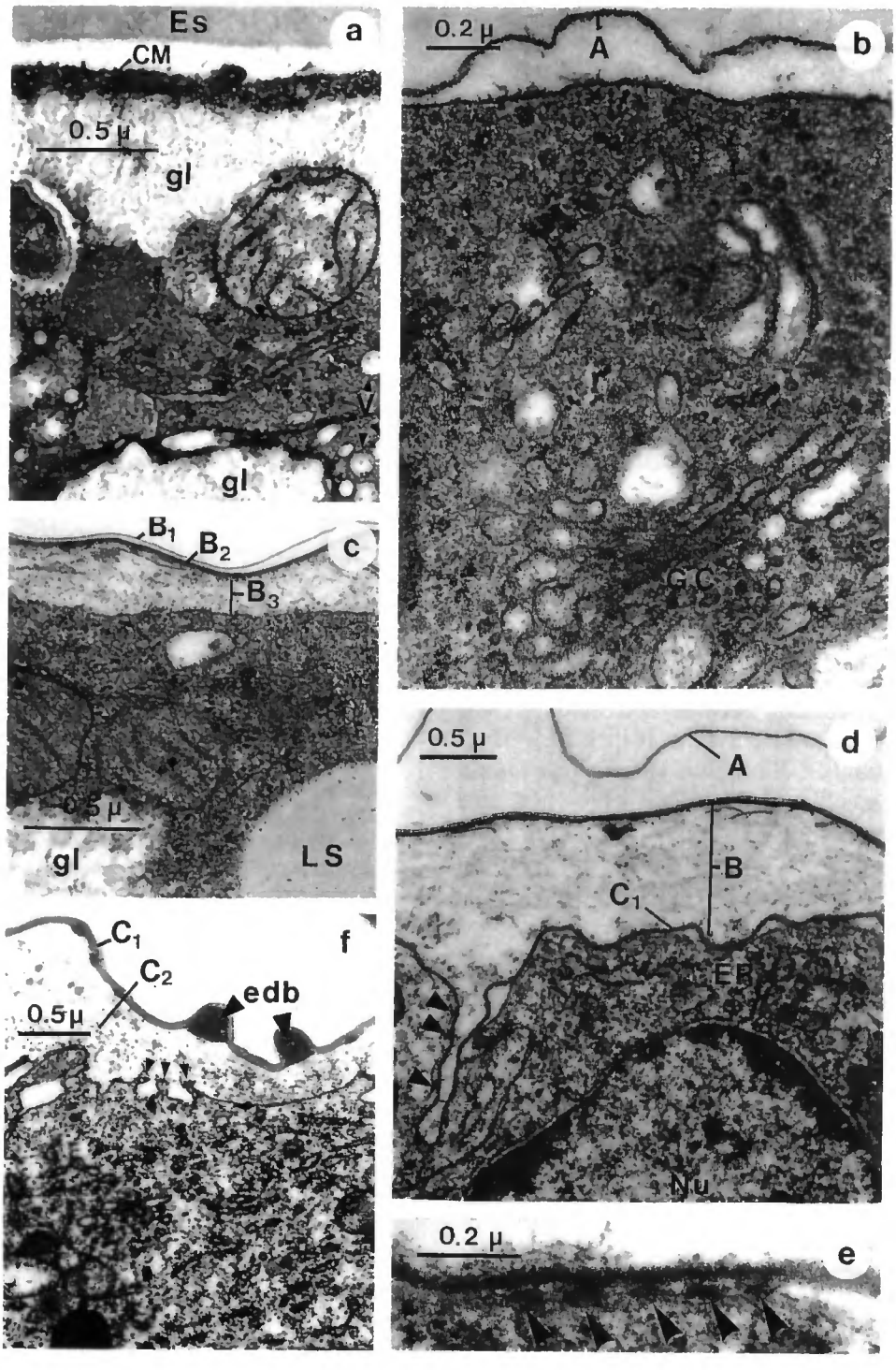


FIG. 1. Distribution of (a) age of embryos at the time of hatching, (b) age of larvae when the enclosed nymph withdraws its legs from the larval exuvia (LWD, leg withdrawal) (c) age when emerging nymph breaks open larval exuvia, expressed in 8-hr periods. Dotted lines represent time divisions expressed in days.

Deposition of Embryonic "Cuticles"

Observations of thin sections made every 8 hr during the first 56 hr of embryonic development demonstrated that the formation of the first envelope occurred between 24 and 32 hr after oviposition which coincided with the diploblastic stage of embryonic development (Fagotto *et al.*, 1988). The membrane, called envelope A by Vogel (1975), consisted of a "single" thin layer (7–10 nm) of electron-dense material and, at high magnifications, resembled the cell membrane. The "epidermal" cells just prior to (Fig. 2a) and after the appearance of envelope A (Fig. 2b) contained large glycogen-filled regions, lipid spheres, Golgi complexes, and multivesicular bodies and some exocytosis appeared to be occurring.

A second envelope corresponding to the B envelope described by Vogel (1975) was formed between 48 and 56 hr after oviposition, just before the formation of the germ band. This membrane consisted of a very thin outer layer (4-nm thick) which may be similar to the outer epicuticle already described in ticks (Germond *et al.*, 1982). This thin layer was separated from the inner dense layer (20 nm) by a very thin electron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translucent space. The innermost part of envelope B was a fibrous or granular material (Figs. 2c and 2d).

On the 6th day of development we observed the deposition of a third embryonic envelope, called C by Vogel (1975), which consisted of an electron-dense layer (22 nm) and a fibrous layer (200–700 nm). In Figs. 2d and 2e, electron-dense plaques were found on the microvilli or just above the cell membrane during the formation of the electron-dense layer. On the ventral surface of the embryo, the presence of electron-dense balls in the fibrous material and their addition to the inner surface of the dense layer of C were observed (Fig. 2f).

Deposition of Larval and Nymphal Cuticles

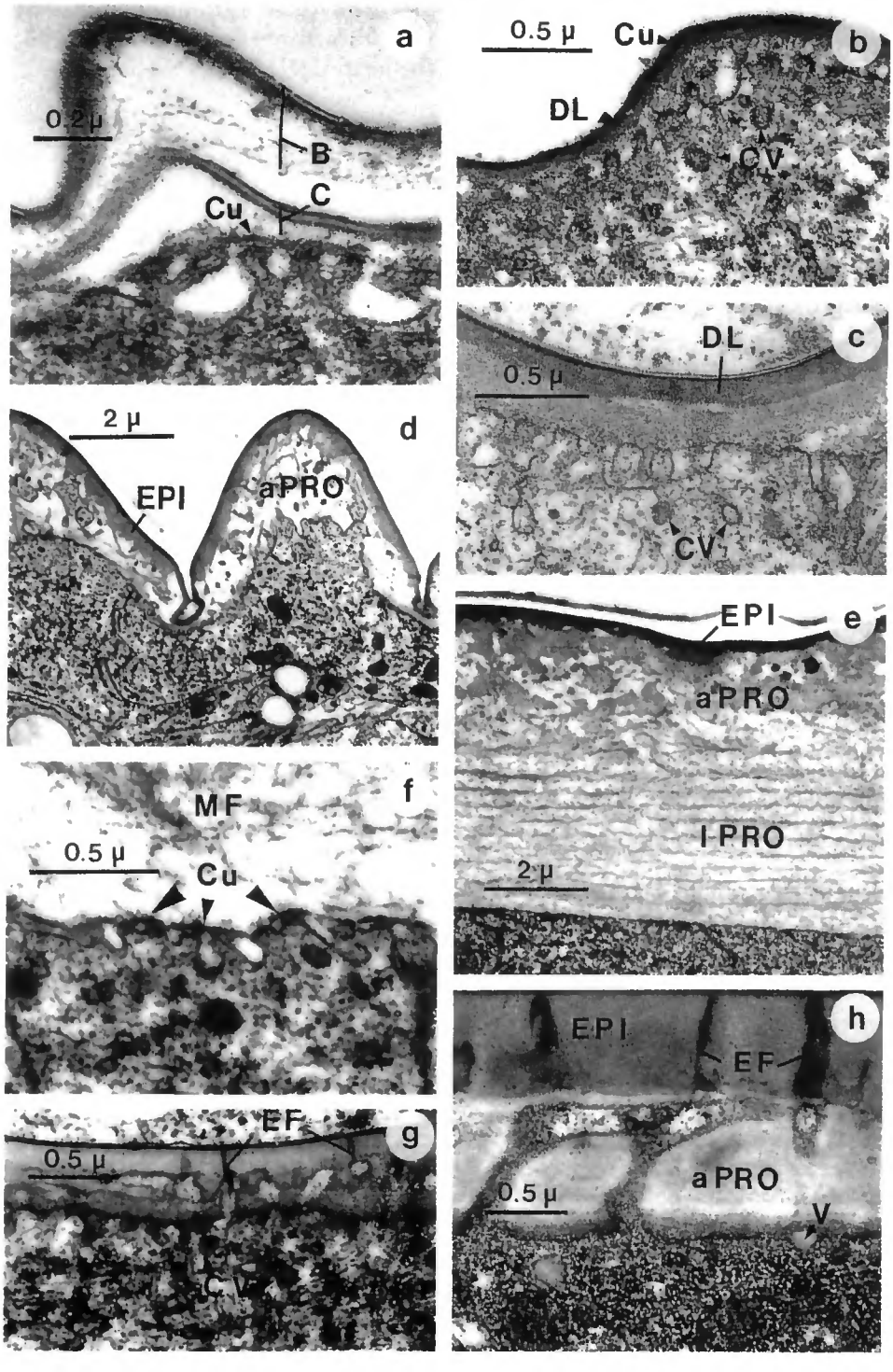
On the 8th day of embryonic development, larval epicuticle formation began with the deposition of the cuticulin layer by electron-dense plaques on the tips of microvilli (Fig. 3a) which was followed by the production of the electron-dense layer (Figs. 3b and 3e). Procuticle deposition occurred on the 9th and 10th days of development (Fig. 3d). Lamellate procuticle production did not start until after eclosion of the larva from the egg shell, but continued until 32–40 hr after hatching (Fig. 3e). Several hours later apolysis occurred. At 48–56 hr after hatching electron-dense plaques along with the small lengths of cuticulin were evident at the tips of microvilli (Fig. 3f). Epicuticle formation followed and procuticle formation continued until after the larva molted (Figs. 3g and 3h).

Ecdysteroids during Embryonic and Larval Development

Using RIA, we investigated the fluctuations of ES levels during embryonic and larval development. Since the weights of the embryos and larvae continually decreased throughout development, especially during the eclosion of the larva and the molt to the first instar nymph, we found it more convenient to express the results as picogram equivalents of 20-hydroxyecdysone (pg eq 20E) per individual rather than as pg eq 20E per mg of material. Extracts of at least 50 embryos or 25 larvae were necessary to detect significant fluctuations during development.

Figure 4a represents the average ES measured from two different series of pooled embryos and larvae determined with two RIAs: one RIA employed a polyclonal antibody and the other a monoclonal antibody. During the first three days of development the free ES titers were low and corresponded to approximately 3 pg eq 20E/embryo using the polyclonal antibody and only 0.36 pg with the monoclonal one. On the fourth day a slight rise of approximately 2 pg eq 20E in the ES titer was noted. In another series of extractions (data not

FIG. 2. (a) Embryo less than 24 hr old. No envelope present between cell membrane (CM) and egg shell (Es). Large glycogen fields (gl) are present along with small vesicles (V). Magnification, 34,650×). (b) Embryo 24 to 32 hr old. The "A" envelope is no longer attached to the cell membrane. GC, Golgi complex. (Magnification, 51,470×). (c) Embryo 56 to 64 hr old. The three layers of B are present: B_1, thin electron-dense layer; B_2, thicker electron-dense layer; and B_3, layer of fibrous material. gl, Glycogen; LS, lipid sphere. (Magnification, 33,110×) (Note: A is beyond the frame of the photograph.) (d) Embryo, sixth day after oviposition. Envelopes A and B are present and the first layer of C (C_1) is almost completely formed. Electron-dense plaques are present on cell membrane (▼). The endoplasmic reticulum (ER) appears continuous with nuclear envelope. Nu, nucleus (22,060×). (e) Embryo, sixth day after oviposition. Five electron-dense plaques (▼) are present on the cell membrane, just beneath envelope C. (Magnification, 73,530×). (f) Embryo, seventh day after oviposition. Balls of electron-dense material (edb) are being added to electron-dense layer of C (C_1). Electron-dense plaques are present on cell membrane (▼). C_2, fibrous material of envelope C (Magnification, 19,836×).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shown), this peak was more pronounced corresponding to approximately 14 pg eq 20E/embryo as detected with the polyclonal antibody. The reason this peak was not always apparent was probably due to poor synchronization of the embryos. Another peak occurred on the seventh and eighth day of development. Using the polyclonal antibody (which has a higher affinity for E) the titers of ES began to rise on the seventh day (up to 23.3 pg eq 20E/embryo) and lasted 2 days, while the ES detected with the monoclonal antibody (which has a higher affinity for 20E) were high (as much as 10 pg eq 20E/embryo) on the eighth day of embryonic development. This slight advance of the peak detected with the polyclonal antibody suggests that E was produced first near the end of the seventh day and was then converted to 20E on the eighth day. The titers decreased until the second day of larval development when they began to increase again reaching 32 pg/larva monoclonal and 63 pg/larva polyclonal antibody on the third day, and sharply decreased thereafter to a basal level at the time of the molt to the nymph.

To determine the nature of ecdysteroids present in various developmental stages a large number of freshly laid eggs (2025), 8-day-old embryos (850), 1-day-old larvae (1636), and 3-day-old larvae (476) were collected, extracted, and submitted to a simplified chromatography on SEP PAK. After having been deposited on the cartridge in 25% methanol in water, the 25% methanol fraction, 60% methanol fraction (containing free ES, Lafont *et al.*, 1982), and the 100% methanol fraction (which should contain apolar conjugates) were assayed with RIA. In each stage tested no significant amount of RIA positive material was present in the 25% methanol fraction (B/T > 0.610) (Table 2). The pure methanol fraction contained low but significant amounts of RIA positive material. In fact, hydrolysis with esterase increased the apparent quantity of ES (Table 2) suggesting the presence of conjugated ES. In the four stages studied the major part of the immunoreactivity was found in the 60% methanol fraction. We thus decided to analyze three of these fractions with HPLC/RIA. For the 8-day-old embryo, the RIA positive material comigrated with 20E or E on a reverse column (Fig. 5). The ratio (weight:weight) between these two hormones was approximately 11:1. In the case of the 1-day- and 3-day-old larvae, only fractions comigrating with 20E or E contained immunoreactivity. However, the ratio between the two hormones varied and was 1.6:1 in young larvae and 4.2:1 during the RIA positive peak on the 3rd day of larval development.

In view of the fact that fatty acid esters (apolar conjugates) have been described in freshly laid eggs of this species either by means of radiotracers (Connat *et al.*, 1984)

FIG. 3. (a) Embryo, eighth day after oviposition. The cuticulin (Cu) is being deposited. Electron-dense plaques are present at tips of microvilli. B, envelope B; C, envelope C (Magnification, 55,062×). (B) Embryo, eighth day after oviposition. The epicuticle formation is continuing. Cu, cuticulin; DL, dense layer; CV, coated vesicles (Magnification, 30,780×). (c) Embryo, ninth day after oviposition. Epicuticle formation is almost complete. DL, dense layer; CV, coated vesicles (Magnification, 33,110×). (d) Embryo, ninth day after oviposition. Procuticle formation is well advanced. EPI, epicuticle; aPRO, amorphous procuticle (Magnification, 7439×). (e) Larva, 40–48 hr after hatching, just before apolysis occurs. The amorphous procuticle (aPRO) and the laminate procuticle (lPRO) laid down before and after hatching, respectively, are visible, EPI, epicuticle (Magnification, 7439×). (f) Larva, 48–56 hr after hatching. Short lengths of cuticulin are visible at the tips of microvilli. Cu, cuticulin; MF, molting fluid (Magnification, 36,575×). (g) Larva, 72–80 hr after hatching. Epicuticle production is more advanced. EF, epicuticular filaments; CV, coated vesicles (Magnification, 24,880×). (h) Larva, 96–104 hr after hatching. Epicuticle (EPI) production is now complete and amorphous procuticle (aPRO) production is beginning. V, vesicle (Magnification, 24,111×).

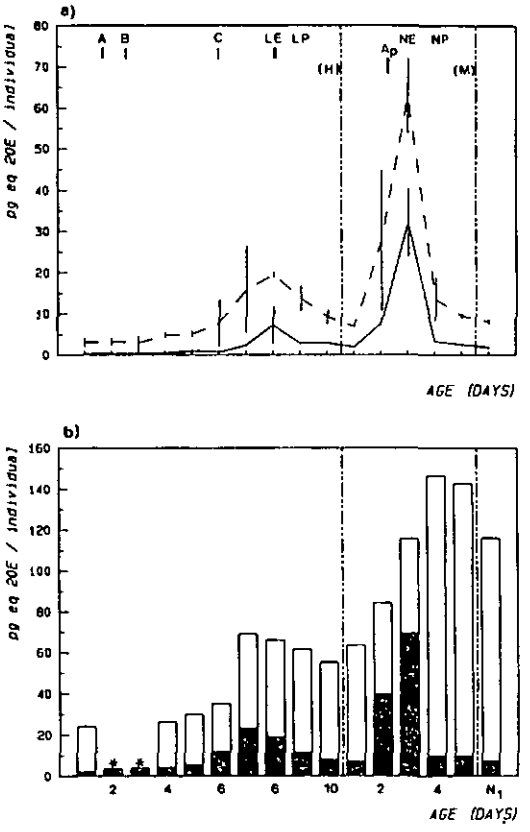

FIG. 4. (a) RIA measurements of ecdysteroid titers with a monoclonal(—) or a polyclonal (---) antibody (vertical bars represent range, $n = 2$.) H, hatching; M, molting. A, B, C, deposition of embryonic "cuticles" A, B, and C; LE, deposition of larval epicuticle; LP, deposition of larval procuticle; Ap, apolysis; NE, beginning of deposition of nymphal epicuticle; NP, beginning of deposition of nymphal procuticle. (b) RIA measurements of ecdysteroid titers with a polyclonal antibody before (■) and after (□) esterase hydrolysis of crude methanolic extracts. *Esterase not carried out.

or RIA after hydrolysis of fractions less polar than E (Connat and Dotson, 1988), we investigated the presence of these esters throughout embryonic development. In the early stages of development in the egg batch investigated (Fig. 4b) about nine times as much RIA positive material was released by esterase hydrolysis corresponding to 22–25 pg eq 20E/embryo. This quantity remained constant until the seventh day of development when the quantity of hydrolyzable material increased. This increase in RIA positive material released by hydrolysis was approximately equal to the quantity of ES detected in this peak. The esterase-labile products remained around 45 pg eq 20E/embryo or larva until just after the ES peak during larval development, where again the increase in hydrolyzable products was a little more than the ES peak. The quantity of RIA positive material released thereafter remained about the same. In another series of extractions of embryos the same general pattern was found. However, we noted in a second series of extractions of the larvae that a much greater quantity of esterase-labile ES conjugates was present (data not shown); this may only be reflecting the presence of a higher quantity of ES conjugates incorporated into the egg during vitellogenesis.

In contrast to hydrolysis with esterase, hydrolysis with *Helix pomatia* juice throughout embryonic and larval development did not release significant amounts of RIA positive material (data not shown).

Correlation of ES Titers and Cuticle Deposition

Finally, utilizing our knowledge of the "cuticular events" and the ES levels during embryonic development, we attempted to show a correlation between these developmental events. Embryos or larvae from the same egg batch were fixed and cut for TEM while others were extracted and assayed for ES using RIA. Nine to eleven embryos and five to six larvae were investigated for each stage of development. During the first 3 days of development, thus during deposition of embryonic envelopes A and B, no peaks were detected although measurements were also carried out on samples taken every 8 hours for the first 2 days. On the fourth day, the first small peak occurred. This coincided with, or just preceded, the shortening or contraction of the germ band. In 10 of 11 embryos fixed and

TABLE 2
DISTRIBUTION OF RIA-POSITIVE MATERIAL AMONG SEP PAK FRACTIONS OF EXTRACTS OF FOUR DIFFERENT DEVELOPMENT STAGES

Stage (No.)	Quantity assayed/5 ml	Methanolic Fractions of SEP PAK Purification							
		25%		60%		100%		E of 100%	
		B/T	ng/5 ml	B/T	ng/5 ml	B/T	ng/5 ml	B/T	ng/5 ml
Embryo									
First day (2025)	200 µl	0.612	nc	0.588	2.58	0.610	nc	0.459	5.58[a]
Eighth day (850)	150 µl	0.611	nc	0.424	15.27	0.576	4.07	0.403	9.42[a]
Larva									
First day (1636)	200 µl	0.611	nc	0.401	13.6	0.531	5.00	0.333	16.9[a]
Third day (476)	150 µl	0.622	nc	0.373	22.37	0.590	3.36	0.337	21.55[a]

Note. E of 100% is the 100% methanol fraction hydrolyzed with hog liver esterase; B/T, proportion of labeling bound to the antibody; nc, not calculable (B/T is not different than those obtained with blank samples).
[a] Only 100 µl of the 5 ml 100% methanol fraction was subjected to hydrolysis and assayed.

cut on the sixth day of embryonic development, envelope C was present or forming, and again as with the other embryonic envelopes, no ES peak was present. On the seventh day when ES levels began to rise, epicuticle formation of the future larval cuticle had not yet begun, and electron-dense balls of material could be seen within the fibrous layer attaching to the electron dense layer. On the day when the ES peaked in the embryo, epicuticle deposition had started in 9 of 11 embryos. Afterward, during the period of decreasing or low titers, procuticle was deposited.

After the larvae hatched out, lamellate procuticle deposition continued. Apolysis of this larval cuticle started around 40–48 hr and this corresponded to a rise in the free ES titers. The ES peak coincided with the formation of epicuticle 48–56 hr after hatching. Again during decreasing titers, procuticle was deposited (See Fig. 4a).

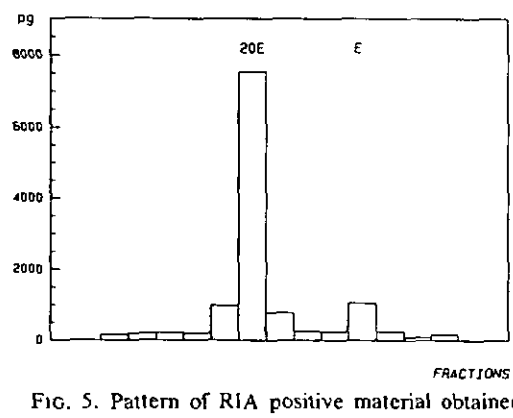

FIG. 5. Pattern of RIA positive material obtained after HPLC analysis of 60% methanol fraction of an extract of 8-day-old embryos (850) which had been partially purified with a RP-18 SEP PAK.

DISCUSSION

Structure of Embryonic Cuticles

In this study we observed the formation of three envelopes (A, B, and C) during the embryonic development of *O. moubata* which could correspond to "embryonic cuticles" already described in many species of insects and crustaceans. However, one can question whether the first envelope, consisting of only a very thin electron dense membrane, is a true cuticle. Envelopes B and C, although very reduced, have a more cuticular appearance, consisting of a thin epicuticle and fibrous procuticle. Apolysis, but not exuviation or reabsorption, of these "cuticles" occurs; however,

the timing of apolyses is difficult to determine because of the violent retraction of the egg shell when pierced during the fixation process. These "cuticles" seem to be a general feature of embryonic development in tick species. They have also been found in the embryos of the ixodid ticks *Amblyomma hebraeum* (Dotson *et al.*, in preparation) and *B. microplus* (Crosby *et al.*, 1987 and Dotson, unpublished results).

Literature on the ultrastructure of the embryonic cuticles of the Arachnida is very limited. Kondo (1969) described a "vitelline membrane" which develops approximately 30 min after oviposition of eggs into the egg cocoon of a spider of Lycosidae family; this membrane could correspond to the first embryonic envelope of *O. moubata*. A scanning electron microscope study of early stages of development of the spider *Chiracanthium virescens* indicated that molts occur within a short period after the breaking of the egg membrane before the young spider has completely shed the egg membrane (Canard, 1987). The cuticles of these embryonic-looking stages could correspond to the embryonic cuticles observed in *O. moubata*.

The crustaceans are better studied; however, there is such a variation in number (2–6) of embryonic envelopes formed (Tetart, 1970; Goudeau, 1976; Klepal and Barnes, 1978; Goudeau and Lachaise, 1983) that it is difficult to compare them with the embryonic cuticles of other orders. During embryonic development of *C. maenas* which has been extensively studied by Goudeau and Lachaise (1980a,b, 1983) six envelopes are formed. The first four envelopes (the first two of which compose the embryonic capsule) do not resemble those found in *O. moubata*. The fifth, which corresponds to the protozoea cuticle, has a structure very similar to the embryonic cuticles B and C of *O. moubata*. Finally, the sixth envelope, which is the cuticle of the freeliving zoea, has a normal cuticular structure resembling that of the fourth or larval envelope of *O. moubata*.

The number (generally three) of embryonic envelopes among insects appears more uniform than that found in crustaceans. At first a very thin atypical cuticle which resembles envelope A of *O. moubata* is formed. Then a true embryonic cuticle composed of an epicuticle and one or two procuticle lamellae is deposited. (This envelope, however, does not appear to be present in dipterans; Bordes-Alléaume and Sami, 1987). This second envelope is more cuticular in structure than the embryonic cuticles (envelopes B and C) of ticks, but is reduced in structure in comparison with the third embryonic (first larval) cuticle of insects (Bullière, 1973; Louvet, 1974; Sbrenna, 1974; Lagueux *et al.*, 1979; Dorn, 1983; Rinterknecht and Matz, 1977, 1983; Rinterknecht, 1985).

The formation of the larval and nymphal cuticles during embryonic and larval development of *O. moubata* begins with the appearance of dense plaques on the plasma membrane during epicuticle deposition followed by amorphous and lamellate procuticle formation. This is very similar to the process already described in the fifth instar nymph of this tick species (Germond *et al.*, 1982) as well as the larval cuticles of insects (Riddiford, 1985) and crustaceans (Spindler *et al.*, 1980).

Ecdysteroid Titers and Cuticle Deposition

On one hand, the data presented in this study strongly indicate that ES play a role in the deposition of the larval cuticle and that of the first instar nymph of *O. moubata*. Although RIA measurements were not carried out on the material observed with the TEM, the embryos or larvae used for histology were sampled in such a manner that they should reflect events in the material extracted. Since the majority of the individuals cut at the times of high ES

titers showed epicuticle deposition, a correlation between high titers and epicuticle deposition seems valid. Additional support for this hypothesis is that a peak of ES has been temporally correlated with epicuticle formation in the fifth instar nymph of this species (Germond *et al.*, 1982). In this peak, the hormones present were principally 20E and E, as demonstrated with gas chromatography–mass fragmentography. Similarly, with HPLC–RIA analysis, we have tentatively demonstrated that the peaks temporally correlated with epicuticle deposition during embryonic and larval development are composed principally of 20E along with smaller quantities of E. Furthermore, with the use of a monoclonal antibody which has a higher affinity for 20E, we demonstrated the same peaks as detected with the polyclonal antibody. If we compare the profiles of RIA positive material detected with the monoclonal and polyclonal antibody (Fig. 4a), we note a slight advance of the ES peak detected with the polyclonal antibody with respect to that detected with the monoclonal antibody. This suggests that perhaps E is produced and is then converted to 20E. *In vitro* studies using [^{3}H]E have shown that embryos possess the enzymes to hydroxylate E to 20E and the highest percentages of metabolites hydroxylated occur around the ES peak (Dotson *et al.*, in preparation). The production of E followed by 20-hydroxylation is similar to the situation found in many insect larvae (see Smith, 1985). However, the embryos of *O. moubata* tend to differ from embryos of *Locusta* where more E than 20E is present during ES peaks of embryonic development (Lagueux *et al.*, 1979).

The question of whether ES play a role in the deposition of the three embryonic envelopes A, B, and C of *O. moubata* remains unclear. Bordes-Alléaume and Sami (1987) suggested that ES did not play a role in the formation of the early embryonic cuticle in the dipteran *C. erythrocephala* since no free or conjugated ecdysteroids were detected at that time. However, in *L. migratoria*, the formation of the serosal cuticle and apolysis before the deposition of the other three cuticles produced during embryonic development appear to be initiated by a peak of ES (Lagueux *et al.*, 1979). In *O. moubata* if an increase in titers of ES initiates the formation of any of these envelopes (A, B, or C), then perhaps small peaks of short durations were overlooked because of the pooling of 50 embryos for an extraction. More sensitive methods allowing dosage of single embryos need to be developed before these problems can be definitively solved.

Origin of Ecdysteroids

Presence of ES in the form of apolar conjugates (AP) has been demonstrated in freshly laid eggs of *O. moubata* (Connat and Dotson, 1988). These conjugates identified in nymphs as esters of ES with fatty acids in the C22 position may accumulate in the eggs during vitellogenesis as has been demonstrated by injection of tritiated hormones into vitellogenic females (Connat *et al.*, 1984). Conjugates of this type have also been found in the freshly laid eggs of *B. microplus* (Wigglesworth *et al.*, 1985; Crosby *et al.*, 1986). It has been hypothesized in both tick species that the maternal ES conjugates are a source of ES which will be hydrolyzed during embryonic development for the initiation of embryonic cuticles or other processes. We tried to determine whether this is the case in *O. moubata* by measuring ES titer before and after esterase hydrolysis in extracts from each day of embryonic and larval development. No decrease in the quantity of material released was observed during the endogenous peaks of ES. On the contrary, it seems that at the appearance of each ES peak there is an increase in RIA positive material released by esterase which corresponds approximately

to the RIA positive material present in that peak. These data suggest that the endogenous ES peak is being synthesized *de novo* and then is inactivated by conjugation to form these AP. This observation is corroborated by our investigation of the fate of conjugates which accumulated during vitellogenesis after injection of the radioactive hormones. No hydrolysis of the tritiated AP was observed during development (Connat *et al.*, 1988).

Our deduction of *de novo* synthesis of ecdysteroids in the embryos is valid if we have hydrolyzed and detected all conjugates. Other unidentified conjugates not hydrolyzable by known methods may also be present. Although we verified our hydrolysis technique with radiolabeled conjugates, perhaps longer incubation times would have released more conjugates as was demonstrated in the cricket *Acheta domesticus* (Whiting and Dinan, 1988). Nevertheless, *de novo* synthesis of ecdysteroids has also been suggested in the embryos of *C. erythrocephala* (Bordes-Alléaume and Sami, 1987) and of *Nauphoeta cinerea* (Lanzrein *et al.*, 1984) and tentatively demonstrated with radiolabeled cholesterol in embryos of the cricket *Gryllus bimaculatus* (Espig *et al.*, 1989).

The role of these AP remains a question. Perhaps they play a physiological role during embryogenesis, but they appear to be inactivation products of both endogenous (Connat *et al.*, 1984) and exogenous ES (Connat *et al.*, 1986). In this latter case, although large quantities are found in the midgut of females, small amounts are also incorporated into the eggs. The authors hypothesized that their presence in freshly laid eggs may only be "artifactual" where they are incorporated during vitellogenesis unspecifically bound to the lipophilic vitellogenins. This is in accordance with our finding that the quantity of endogenous AP found in the eggs is extremely variable (Connat and Dotson, 1988).

Finally, no significant increase in RIA positive material was noted after hydrolysis with *Helix* juice, suggesting that no polar conjugates similar to those described, e.g., in *Locusta* (see Hoffmann and Lagueux, 1985) participate in the production of the embryonic and larval peaks.

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II

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Apolar Conjugates of Ecdysteroids Are Not Used as a Storage Form of Molting Hormone in the Argasid Tick *Ornithodoros moubata*

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Fifth (last) instar nymphs of the tick *Ornithodoros moubata* convert ingested 20-hydroxyecdysone (20E) to apolar conjugates AP2, which are then converted to the more polar conjugates AP1. Only small quantities of free hormone were transferred to the hemolymph and the carcass within the first 2 days after the blood meal. The proportion of radiolabel in these two compartments was highest at the time of the endogenous ecdysteroid peak; however, no traces of free [^{3}H]20E were detected. The conversion probably occurs principally in the intestinal cells. Eleven days after ingestion, 84% of the radiolabel is located in the digestive tract, mainly in the form of AP1 conjugates.

AP1 obtained in second instar nymphs fed with [^{3}H]ecdysone ([^{3}H]E) remain stable throughout the following nymphal instars. The ecdysteroid moiety of AP1 remained unchanged. The hydrolysis, although not complete, always yielded a peak comigrating with the reference E but never 20E or any other clearly distinct peaks that may have corresponded to metabolites of 20E. Less label per individual was present in adults, but its nature remained the same, viz., AP1 mainly located in the digestive tract. In females, 2.5% of the label was transferred to the progeny during the first ovipositional cycle.

Apolar products (mainly AP2) that accumulated in eggs of females injected with [^{3}H]E or [^{3}H]20E during vitellogenesis remained unchanged during the whole embryonic development. During the molting cycle of larvae, there was only a slight conversion of AP2 to AP1, but esterase hydrolysis of these products released the same percentages of E and 20E as in the freshly laid eggs.

We conclude that in this tick species apolar conjugates of ecdysteroids are inactivation metabolites that are not reutilized during the development of the animal. These metabolites are mainly retained in the tick, probably

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because of its peculiar blocked midgut. Several studies have shown that in other arthropod species (ticks, spiders, and insects), these apolar metabolites are excreted in the feces.

Key words: metabolism of ecdysteroids, development

INTRODUCTION

As in other arthropods, the molting cycle of ticks is triggered by ecdysteroids [1]. Fifth instar nymphs of the argasid tick *Ornithodoros moubata* molt 10 days after a blood meal, with an ecdysteroid peak composed essentially of 20-hydroxyecdysone occurring concomitantly with the beginning of cuticle synthesis (day 5–6) [2]. The injection of [^{3}H]ecdysone into nymphs at various time after feeding, indicated that two different metabolic pathways exist, depending upon the endogenous ecdysteroid titer [3]. When endogenous ecdysteroid levels are low, ecdysone is converted principally to apolar conjugates, AP*, while a pathway leading to the formation of polar products is prevalent during the ecdysteroid peak. Metabolic studies with radiotracers have demonstrated that when ecdysteroids are either injected or ingested, AP accumulate in intestinal cells and in the midgut lumen (Diehl, unpublished results; [4]). In the case of 20E ingestion, the first apolar products to appear (AP2) were identified as long chain fatty acid esters of 20E at the C-22 position [4]. These products were then converted to more polar conjugates, AP1, the structure of which is unknown. Both AP1 and AP2 conjugates are, however, hydrolyzable with esterase, liberating a mixture of free E and 20E. Because ecdysteroid fatty acid esters are less immunoreactive than their corresponding free ecdysteroids, it is possible to estimate these conjugates by performing RIA analysis on samples both before and after enzymatic hydrolysis. Thus we observed an increase in RIA-positive material after hydrolysis of tick extracts collected after the endogenous ecdysteroid peak. This amount, however, remained constant throughout the last part of the instar, indicating that conjugated ecdysteroids are stored in the animals. [5].

Additional studies have demonstrated that other tick life stages are capable of producing AP, as these products accumulate in the eggs of ticks that were injected with radiolabeled ecdysteroids as vitellogenic females [5–8]. The compounds that accumulate in eggs of the hard tick *Boophilus microplus* have been identified as the same long chain fatty acid esters of E (at C-22) as found in *O. moubata* [9].

Since the metabolic pathways leading to the production of E and 20E are unknown in ticks, we have investigated if AP conjugates in nymphs or in the eggs could serve as a source of molting hormone during the molting cycle or during embryonic development, respectively.

*Abbreviations: AP = apolar products (with reference to compounds that are appreciably less polar than ecdysone) previously described [7,11] and specified as AP1 and AP2 according to their retention times in reverse-phase HPLC; E = ecdysone; 20E = 20-hydroxyecdysone; RP = reverse phase.

MATERIALS AND METHODS

Animals

Animals were obtained from a colony of the soft tick *O. moubata* (Murray, 1877; sensu Walton, 1962) (Ixodoidea; Argasidae), maintained in the laboratory for 5 years, and fed through a parafilm membrane with defibrinated pig blood. Adults molting from fourth instar nymphs are generally males, and those molting from fifth instar nymphs are females. Nymphs and adults are fed 1–2 months after ecdysis.

Chemicals

Ecdysone, (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one) and 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one) were purchased from SIMES (Italy). Tritiated [23,24-^{3}H(N)]ecdysone (53.6 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Tritiated 20E was obtained by incubation of *Locusta* Malpighian tubules with [^{3}H]E and purification with HPLC.

Nutrition

Ten microliters of ethanol containing one of the two tritiated hormones were added to 5 ml fresh defibrinated blood and vortexed. The nymphs were then allowed to feed through a parafilm membrane. In argasid ticks, much of the water and salts from the ingested vertebrate blood are discharged from the body during feeding as a fluid excreted from a pair of slitlike apertures located between coxae I and II. This coxal fluid was collected during the blood meal with a Pasteur pipette, and the coxal fluid excreted the first 24 h following the blood meal was absorbed on filter paper. Second instar nymphs ingested approximately 20 μl blood, and fifth instar 100 μl.

Loading of Eggs With Apolar Conjugates

Freshly laid eggs from vitellogenic females injected with [^{3}H]E or [^{3}H]20E contained apolar conjugates [7]. One of these hormones (500,000 cpm) dissolved in 5 μl of TC 199 Seromed medium (Biochrom, Berlin, FRG) was injected with a glass needle into the hemocoel of females through the third coxal membrane 8 days after engorgement. Females were kept separate in cotton-plugged glass vials, and eggs were collected every day by removing the female. In order to obtain enough radiolabel in a sample for an RP-HPLC run, between 10 and 20 eggs were extracted for each day of development investigated.

Dissections

The cuticle of each tick was cut circumferentially and the dorsum lifted away. The ventral half was covered with TC-199 medium, and the intestine (and in the case of female the ovary) was (were) removed. The intestine was torn open and washed in new medium until the contents had been completely expelled. The ovary and the carcass were also rinsed several times in

new medium. These rinses were combined with the medium containing the intestine contents.

Hemolymph was collected with calibrated capillary tubes after puncturing the articulation membrane of several leggs.

Extractions

Ticks or tissues were homogenized in pure methanol and centrifuged (15 min 10,000 rpm). The supernatant was collected, and adjusted to a known volume of methanol, and an aliquot of each extract was counted to estimate the radiolabel content. A suitable amount of methanolic extract was evaporated to dryness under nitrogen, and then resuspended in 300 μl of 30% methanol for injection in HPLC.

HPLC Analysis

HPLC analyses were performed with a Perkin-Elmer series 3 chromatograph with an LC 55 variable-wavelength spectrophotometer set at 242 nm. E and 20E standards were coinjected with the different samples. For analysis of the metabolites, the reverse phase mode (Merck HIBAR column RT, 25 cm, i.d.4 mm, packed with Lichrosorb RP-18, 7 μm) was used with a gradient of methanol-Tris buffer (pH 7.5, 20 mM), as previously described [7,10].

Hydrolysis With Esterase

Hydrolysis of apolar conjugates was accomplished with 50 IU of pig liver esterase (E.C. 3.1.1.1) (Boehringer) in 600 μl 0.1 M borate buffer, pH 8. After an overnight incubation at 37°C, 300 μl of methanol were added. The sample was vortexed, centrifuged (10,000 rpm, 10 min), and directly injected into the HPLC.

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 as uncorrected cpm because only a small difference in quenching existed between the different HPLC fractions. However, to have a correct estimation of cpm in each organ after dissections, a quench curve was established. A linear correlation existed between the standard channel ratio and the counting yield estimated by addition of internal standards to the biological samples.

RESULTS

Fate of Ingested 20-Hydroxyecdysone in Fifth Instar Nymphs

Because of their larger size, fifth (last) instar tick nymphs were used to investigate the fate and distribution of ingested hormone in intestinal lumen, intestinal cells, hemolymph, and carcass (the remainder of the body), during the molting cycle. The animals were fed with blood supplemented with 10 μg/ml 20E (and 10^6 cpm/ml [^{3}H]20E).

As in a previous experiment performed with adult females [11], the ingested hormone was rapidly converted to AP2 corresponding to 20E esterified at C-22 with the common long chain fatty acids C16:0, C18:0, C18:1, and C18:2 [4]. The radiolabel in the lumen of the midgut slowly diminished within 6 days after the ingestion of the tritiated hormone (Fig. 1), and 20E was progressively replaced by AP2, and then AP1 (Fig. 2). Simultaneously, the radiolabel in the intestinal cells and carcass increased (Fig. 1). One hour after ingestion, 20E represented a high proportion of the radiolabel present in these tissues (Fig. 2), indicating a transfer of this hormone from the intestinal lumen to the carcass through the intestinal cells. 20E is rapidly conjugated to AP2, which are progressively converted to AP1.

In the hemolymph we observed that the radiolabel gradually increased to reach a maximum 6 days after ingestion (Table 1), coinciding with the endogenous ecdysteroid peak [2]. The amount of radiolabel in the hemolymph during the first day was insufficient for HPLC analysis. However, analysis of the samples from days 2 and 6 revealed only the presence of AP2. Thus no free [^{3}H]20E was circulating in the hemolymph during the endogenous ecdysteroid peak.

Eleven days after ingestion (viz., 2 days after ecdysis) the label in the hemolymph and in the carcass decreased (Fig. 1), while that in the intestinal cells and the intestinal lumen increased. At this time, the major part of the radiolabel (84.3%) is located in the digestive tract under the form of AP1 (92.2%) and AP2 (7.8%).

Hydrolysis of these AP principally yielded 20E, but also liberated an unidentified polar product having a retention time of 12 min, which repre-

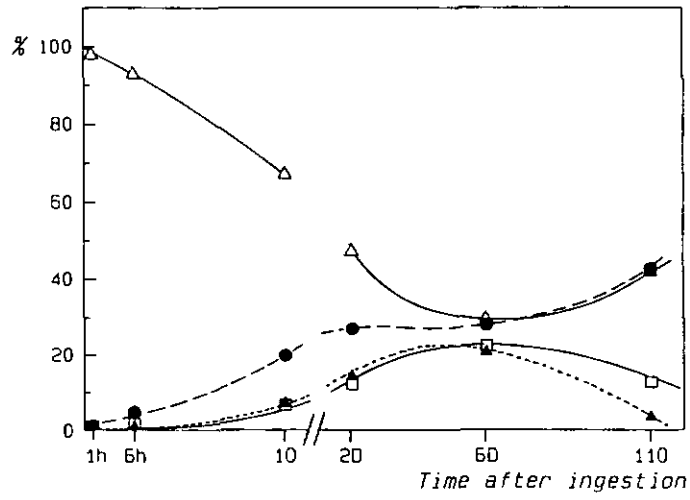
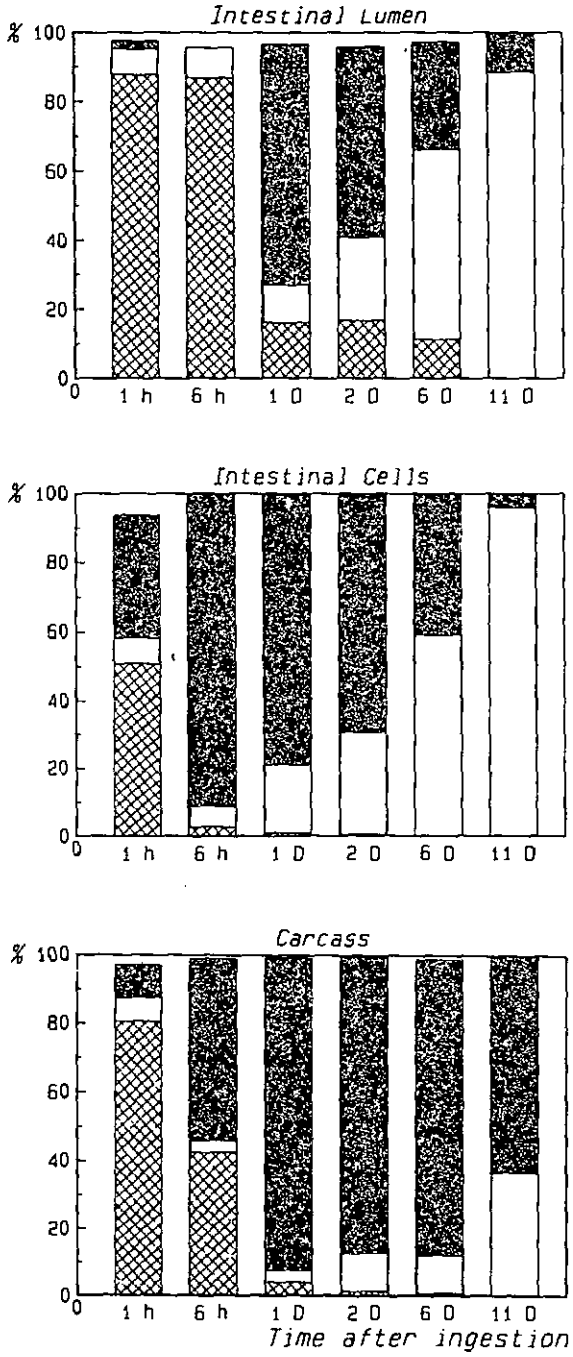


Fig. 1. Distribution of the radiolabel in *O. moubata* fifth instar nymphs after ingestion of blood containing 10 μg 20 E and 1,000,000 cpm [^{3}H]20E perml. Perentage of radiolabel from the whole animals contained in the intestinal lumen (△), in the intestinal cells (●), in the carcass (□), and in the hemolymph (▲). Hemolymphatic volume was approximated to 25 μl per nymph, and label content of the intestinal lumen was calculated by subtraction of remaining contaminant hemolymph. One hundred percent always corresponded tn approximately 190,000 cpm.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Intestinal Cells
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2 D
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Carcass
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60
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1 D
2 D
6 D
11 D
Time after ingestion

Figure 2

sented approximately 25% of the radiolabel (data not shown). In the hydrolysis experiments of carcass extracts, this peak was present in lower proportions (6.8%). This same peak has also been observed in adult *O. moubata* females [11].

Fate of Apolar Conjugates During Successive Molting Cycles

Several batches of second instar nymphs of *O. moubata* were fed blood containing 10^8 cpm of [^{3}H]E per ml. The nymphs were then washed with water and 70% ethanol in order to remove any label that possibly remained on the body surface. As previously described for fifth instar nymphs or adult females [11], within the first few hours after the blood meal, the ingested hormone was converted to the apolar esterase-labile conjugates AP2. In time, these AP2 are converted to the conjugates AP1 (Fig. 3A). Following ecdysis to the third instar, only these latter compounds are recovered (Fig. 3B). Although the AP1 are composed of several peaks, they are all esters of [^{3}H]E hydrolyzable with esterase (data not shown). We note that in this case no peak eluting at 12 min was present.

The third instar nymphs containing the radiolabeled apolar conjugates were then fed on blood without the added hormone, and ten nymphs were homogenized each day after feeding throughout the molting cycle. The radiolabel content of each group was analyzed by RP-HPLC. In a similar way, the remaining radiolabeled fourth instar nymphs were fed on blood without hormone, and the radiolabel pattern of each day of the molting cycle was monitored. In both cases, we observed that the radiolabel content of the nymphs did not change during the molting cycle. The pattern of four major peaks of AP1 followed by a minor one found in unfed third instar nymphs was observed throughout the two molting cycles (Fig. 3B and C). In order to ascertain that the apparent stability of AP1 did not hide rapid changes in their composition (e.g., liberation of E, conversion to 20E [for example], and then rapid reconjugation of this hormone), we analyzed extracts of fifth instar nymphs from each day of the next molting cycle, both by a direct RP-HPLC

TABLE 1. Evolution of the Concentration of Radiolabel in the Hemolymph of *O. moubata* Fifth Instar Nymphs After Ingestion of Blood Containing 10 μg Cold 20E and 10^6 cpm [^{3}H]20E per ml (Hemolymph from two nymphs was pooled for each time)

	Time					
	1 h	6 h	1 day	2 days	6 days	11 days
Hemolymph withdrawn (μl)	12.0	10.0	7.7	6.5	6.2	8.8
Radiolabel (cpm/μl)	11.3	61.5	533.8	858.6	2049.6	258.2

Fig. 2. Nature of the radiolabel content investigated with RP-HPLC, in the intestinal lumen, intestinal cells, and carcass of *O. moubata* fifth instar nymphs that had ingested blood containing 10 μg 20E and 1,000,000 cpm [^{3}H]20E per ml. (dark spotted columns), AP2; (open columns), AP1; (cross-hatched columns), 20E. The difference between the total of the bars and 100% corresponds to products different from these compounds.

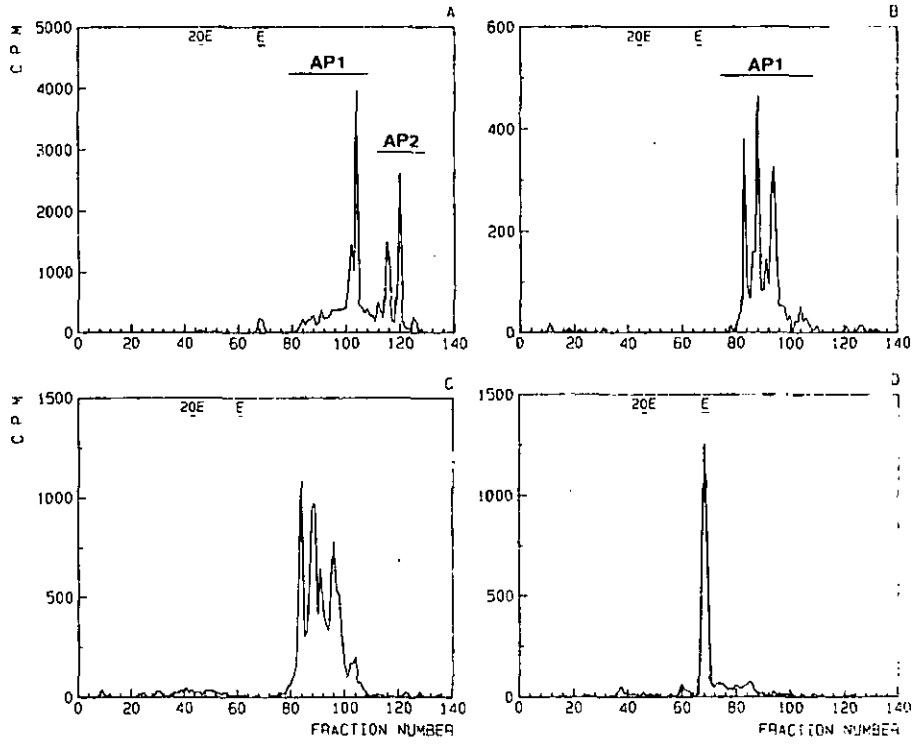

Fig. 3. Fate of [^{3}H]E ingested by *O. moubata* second instar nymphs. A: RP-HPLC radiochromatographic pattern 24 h after ingestion. B: Pattern in freshly ecdysed third instar nymphs and at each day of the third instar nymphs' molting cycle. C: Pattern during the fourth instar nymphs' molting cycle. D: Pattern after hydrolysis with pig liver esterase of the extract from freshly ecdysed fourth instar. E and 20E indicate the retention time of cold standards, coinjected with the sample.

analysis and by analysis after esterase hydrolysis of the AP1 (Fig. 4). The pattern after hydrolysis was always similar. The more polar component of AP1, which represented about 20% of the total radiolabel in the extracts, was partially esterase-resistant. After hydrolysis, this peak comprised 17.1% (SD = 4.4) of the radiolabel. Most of the remaining label (57.2%, SD = 8.4) corresponded to a peak that comigrated with E. No other significant radioactive peaks were liberated by the hydrolysis. However, radiolabel was found in the fractions having shorter retention times than E. The proportion of this diffusely dispersed polar material increased slightly during the molting cycle of fifth instar nymphs, from 2% at the beginning of this instar to 15% after the molt.

Fate of the Apolar Conjugates in Adults

The resulting adult males and females contained less radiolabel than was originally ingested by them as second instar nymphs, indicating that the radiolabel was lost during the molting cycles. Males appear to have lost more than females, as they contained 26–37% of the label ingested as second instar nymphs, while the females contained from 46% to 60%. The different exuviae

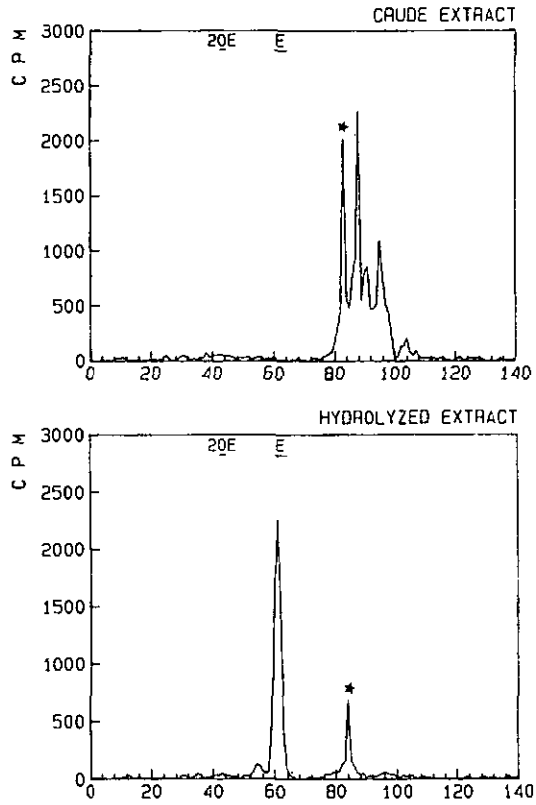

Fig. 4. HPLC pattern of labeled metabolites in fifth instar *O. moubata* fed [^{3}H]E as second instar nymphs. The pattern is similar to that of third and fourth instar nymphs and is similar at each day of the molting cycle. Hydrolysis with esterase revealed that the AP1 conjugates contained only E. ★, unhydrolysed AP1.

from the nymphs contained less than 1% of the total label in the animals. Neither the coxal fluids collected during each blood meal nor the wash of the vials in which the animals were kept, which contained the excreted guanine, had significant amount of radiolabel. We suspect that the loss of radioactivity could be due to the regurgitation of some of the intestinal content during the successive blood meals.

In males the radiolabel was principally located in the intestinal lumen (65.5%). The remaining radioactivity was in the carcass (13.3%) and the hemolymph (14.6%). Females were fed and mated, and their progeny collected. The extracts of all the first instar nymphs from three females contained only 69 cpm, corresponding to approximately 2.5% of the label present in the three females.

Fate of the Apolar Conjugates in Embryos and Larvae

Freshly laid eggs from females injected with [^{3}H]E contained apolar conjugates, mainly AP2 (74.7–84.8%) and smaller amounts of AP1 (12–24%) (Fig. 5A). Esterase hydrolysis of these AP, although incomplete, yielded compounds that co-eluted from the RP-18 column with cold 20E (6.1%) and E

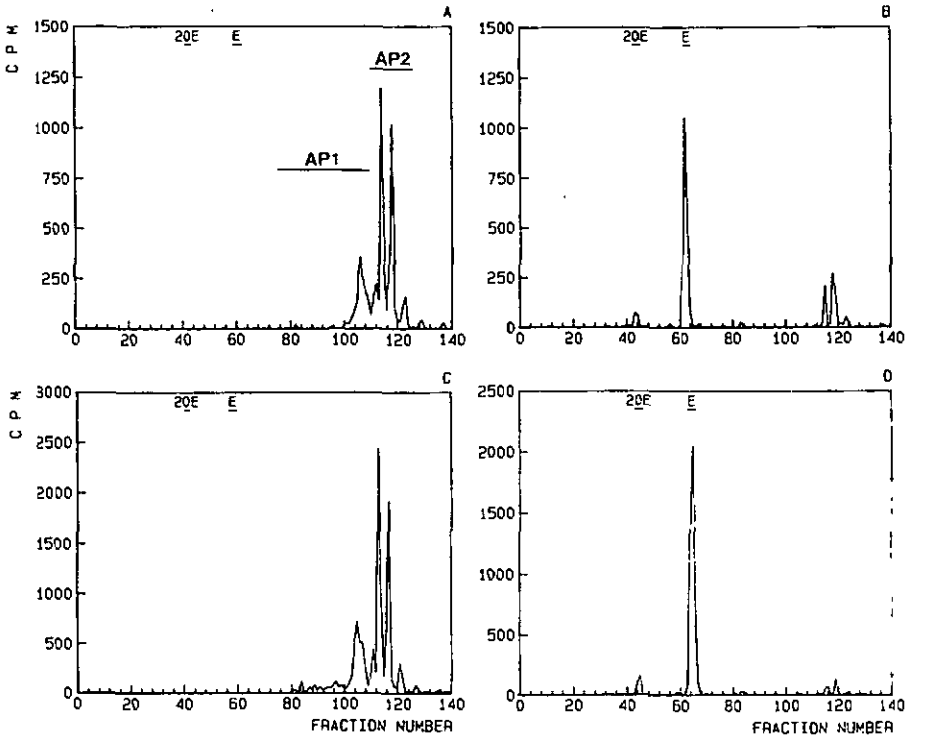

Fig. 5. Labeled apolar products in the freshly laid eggs and freshly ecdysed nymphs originating from *O. moubata* females injected with [^{3}H]E during vitellogenesis. A: RP-HPLC radiochromatogram of crude methanol extract of freshly laid eggs. B: RP-HPLC radiochromatogram of esterase hydrolysis products of crude methanol extract of freshly laid eggs. C: RP-HPLC radiochromatogram of crude methanol extract of freshly ecdysed first instar nymphs. D: RP-HPLC radiochromatogram of esterase hydrolysis products of crude methanol extract of freshly ecdysed first instar nymphs. E and 20E indicate retention times of cold standards, coinjected with the samples.

(84.2%) standards (Fig. 5B). No other products were liberated. HPLC analysis of crude methanol extracts and esterase-treated extracts of several embryonic stages revealed that these products remained unchanged throughout embryonic development until the eclosion of the larvae 9–10 days after oviposition (Fig. 6).

The larval stage of *O. moubata* is a nonfeeding stage living on the remainder of the vitellus [12]. After eclosion, it continues developing, forming a new

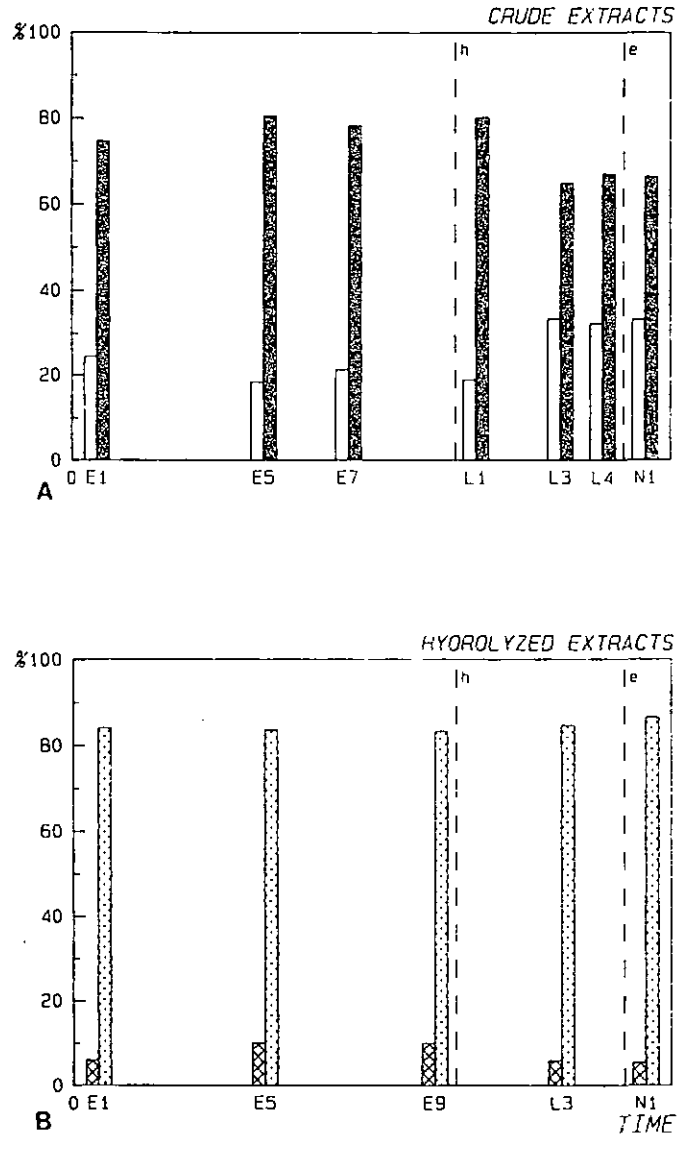

Fig. 6. Fate of the labeled apolar products contained in the freshly laid eggs of *O. moubata* females injected with [^{3}H]E during vitellogenesis. A: Crude extracts. Percentages of AP1 and AP2 in extracts of the 1st, 5th, and 7th day of embryonic development (E1, E5 and E7), 1st, 3rd, and 4th day of larval development (LI, L3, and L4), and freshly ecdysed first instar nymphs (N). (open columns), AP1; (heavy spotted columns), AP2. B: Hydrolyzed extracts. Percentages of radiolabel comigrating with 20E and E after esterase hydrolysis of extracts of the 1st, 5th, and 9th day of embryonic development (EI, E5 and E9), third day of larval development (L3), and freshly ecdysed first instar nymphs (N). (cross-hatched columns), 20E; (light spotted columns), E. Broken vertical line represents the hatching (h) of the larvae from the eggs or the ecdysis (e) of the first instar nymphs from the larvae.

nymphal cuticle, and molts 4–5 days later. During larval development there was a conversion of AP2 to AP1, and the percent of radiolabel attributed to AP1 increased to approximately 33%, while the percent of AP2 decreased to 65%. These percentages remained unchanged in the freshly molted first instar nymphs (Figs. 5C and 6). Esterase hydrolysis of apolar conjugates of larval and nymphal stages yielded approximately the same percentages of E and 20E as freshly laid eggs (Figs. 5D and 6).

Freshly laid eggs from three females injected with [^{3}H]20E contained AP1 (20.7%), AP2 (74.0%), and small amounts of unmetabolized 20E (2.6%). As with the eggs coming from females injected with [^{3}H]E, there was no apparent change of the metabolite pattern during embryonic development. Again, there appeared to be an increase of AP1 (33.4%) at the expense of AP2 (60.2%) during larval development. Esterase hydrolysis of these products yielded only 20E (data not shown).

DISCUSSION

Our results show that, in the different nymphal instars of *O. moubata*, ingested molting hormones, E or 20E, are converted to apolar conjugates AP1 via the formation of AP2. Although the intestinal cells are most probably responsible for the major conversion of the hormone to the conjugates, other tissues are capable of this conjugation, as demonstrated by in vitro incubation of Malpighian tubules, dorsal integument, or ventral carcass with [^{3}H]E (Vuillème, unpublished results, see in [6]). The possible pathways for 20E are shown in Figure 7. At the end of the first molting cycle after the ingestion, the hormones are principally located in the intestinal tract as AP1. The metabolite pattern remains unchanged during the successive molting cycles. No change in the composition of the ecdysteroid moiety of the apolar conjugates was found. If, for example, we had noted, in the case of E ingestion by second instar nymphs, the occurrence of free 20E or the release of 20E after esterase hydrolysis of the AP1 in the following instars, then we could hypothesize the reutilization of the apolar products during the molting cycle. In view of the specific activity of the tritiated E used, a detectable peak of 50 cpm is equivalent to 0.45 pg. Thus the contribution of the radiolabeled AP would only need to correspond to approximately 1/22,000 of the ecdysteroid peak in the fifth instar nymphs [2]. Since no apparent change in the AP1 occurs, it seems that the AP are inactivation products. These products are probably retained in the gut during the whole life cycle of this tick, as are most of the waste products of digestion, because the midgut does not communicate with the hindgut [13]. Although some regurgitation may take place, no excretion of the digestive waste products by the rectum occurs. In fact, in a closely related species, *Ornithodoros parkeri*, a part (25–76%) of the AP1 produced after ingestion of the tritiated hormones is excreted [10]. The exact chemical nature of these AP1 is not known, but, as demonstrated by hydrolysis with esterase, they remain esters. They are derived from the more apolar AP2 conjugates, which have been identified in the fifth instar nymphs of *O. moubata* by mass spectrometry as esters of 20E conjugated at the C-22 position with long chain fatty acids (C16:0, C18:0, C18:1 and C18:2) [4]. The

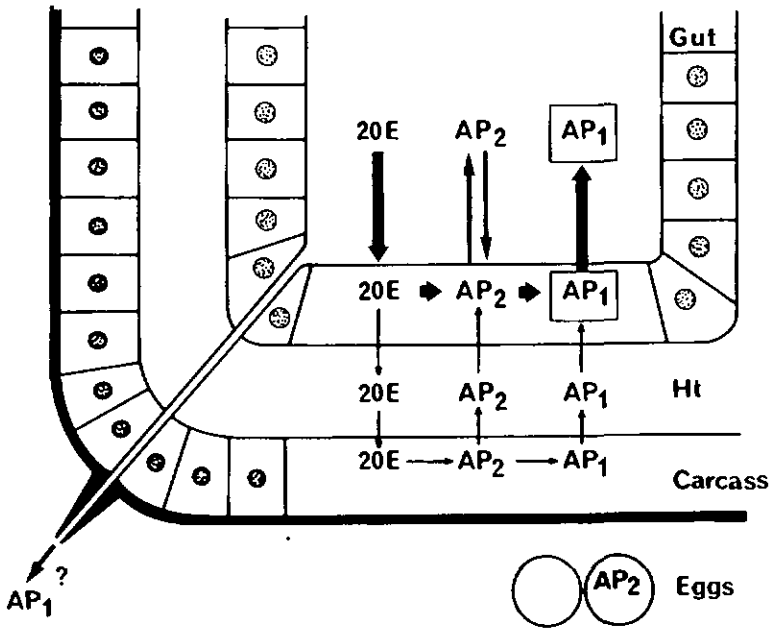

Fig. 7. Interpretative diagram of the fate of 20E ingested by nymphs of *O. moubata*. Within the first few hours after ingestion, part of the ingested 20E crosses the intestinal barrier and impregnates the peripheral tissues. These tissues can conjugate the hormone to AP2, and then to AP1. Afterwards, the conjugated hormone is slowly released and reabsorbed by the gut. The relative importance of the different pathway is represented by the width of the arrows. HL, hemolymph.

same products have recently been identified in feces of larval *Heliothis virescens* (Lepidoptera) [14] or *H. armigera* [15] after the animals had ingested large quantities of 20E. Although the exact chemical nature of the products is not known, apolar conjugates with retention times similar to the AP1 of *O. moubata* are produced by spiders ingesting ecdysteroids, and these apolar products are excreted in the feces [16]. We thus think that the normal fate of these apolar products is their elimination in the feces. *O. moubata* probably represents an exception, because of the peculiar morphological formation of its gut [13].

The other part of our study concerns the possible reutilization of the AP by the embryos during their development. Apolar products probably occur naturally in the eggs and embryos, since it is possible to increase the quantity of RIA-positive material by hydrolysis with esterase in ticks [5; Dotson et al., unpublished results] and in insects, e.g., *Periplaneta americana* [17] or *Drosophila melanogaster* [18]. However, the percent of increase by hydrolysis in ticks is variable. The nutritional history of the female may contribute to this variability. When second instar nymphs ingest [^{3}H]E, 2.5% of the ingested radiolabel is recovered in the eggs of the "resulting" females. If the adult females ingest the hormone, this proportion increases to 10.2% [11]. Thus the frequency at which the females encounter ecdysteroids and the concentration of these hormones in the blood meals throughout their life may influence the proportion of AP accumulating in the eggs. In the present

study, the [^{3}H]E was injected into the hemocoel to obtain a higher percentage of radiolabel in the eggs than that found after ingestion [7]. No peaks of radiolabeled free 20E or its metabolites were found during embryonic development. Special attention was paid to the 7th day of development, which corresponded to an endogenous peak (20 pg/embryo) of 20E [Dotson et al., unpublished results]. Since a detectable peak of 50 cpm is equivalent to 0.45 pg, the contribution of the radiolabeled AP in this case would only need to correspond to 1/444 of the ecdysteroid peak of 200 pg per ten embryos (minimum number of eggs used for extraction). Contrary to what occurs to the polar conjugates found in the eggs of *Locusta migratoria* [19,20], or to what has been suggested by the in vitro hydrolysis of ecdysteroid esters by homogenates of 15-day-old *B. microplus* embryos [8], the AP of *O. moubata* are not hydrolyzed during embryonic development. Their incorporation into the eggs may be artifactual. The AP circulating in the hemolymph during vitellogenesis may be bound to the vitellogenins in a nonspecific manner and could thus be incorporated into the oocytes. In the early development of *O. moubata*, AP2 are converted to AP1 only after hatching, when the intestine has fully developed and begins actively digesting the vitellus, demonstrating that this transformation requires enzymes not present in the yolk but produced later by the intestinal cells.

The intestinal cells appear to play an important role in the conversion of ecdysteroids to AP. An analogous conversion occurs in the gastric caecae of *L. migratoria*, where ingested ecdysteroids are acetylated at the C-3 position. This acetylation has been interpreted as an efficient inactivation mechanism [21]. We conclude that the tick AP conjugates are also metabolites of an inactivation reaction, occurring mainly in the intestinal cells, and not a source of conjugated hormone to be liberated during the molting cycle or embryonic development.

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Metabolism of [^{3}H]-Ecdysone in Embryos and Larvae of the Tick *Ornithodoros Moubata*

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The fate of [^{3}H]-ecdysone ([^{3}H]-E) was investigated in hanging drop cultures of embryos and larvae of the tick *Ornithodoros moubata* using HPLC. The hormone was metabolized more slowly during described periods of increasing endogenous ecdysteroid (ES) titers than during periods of low titers except for young embryos. Three different classes of metabolites were produced: 1) apolar products (AP) corresponding to C-22 fatty acid ester conjugates of E and, in some cases, of 20-hydroxyecdysone (20E), 2) unidentified polar products (PP) more polar than E, one peak of which had the same retention times as 20,26-dihydroxyecdysone, and finally, 3) 20E verified by comigration of cold standards on RP-18 and silica columns. Hydroxylation of E to 20E first became evident in cultures of 2 day old embryos and was present in all cultures of older animals. Highest production of free 20E occurred during increasing endogenous ES titers in embryos and during the ES peak in larvae. Conjugation of E to AP occurred in all stages investigated, but was more pronounced during periods of low endogenous ES titers, and may correspond to a detoxification mechanism. In contrast, PP were produced during high 20E production in embryos and during periods of high and decreasing endogenous titers in larvae. © 1993 Wiley-Liss, Inc.

Key words: Argasidae, apolar products, detoxification mechanism, embryogenesis, 20-hydroxyecdysone

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INTRODUCTION

The presence of one or more ES* peaks has been demonstrated during the embryonic development of many insect species and, in many cases, these peaks have been correlated with embryonic molts as in *Calliphora* [1], *Bombyx* [2], *Carausius* [3], *Sarcophaga* [4], and *Schistocerca* [5,6] (see Hoffmann and Lagueux [7] for review of earlier papers). In 1979 Lagueux and coworkers [8] proposed that the origin of these embryonic ES peaks in young *Locusta* embryos resulted from hydrolysis of maternal ES conjugates, mainly phosphate esters in the C-22 position. In the argasid tick *Ornithodoros moubata*, recent studies demonstrated that two ES peaks were found during embryogenesis, one of which coincided with epicuticle deposition of the last embryonic cuticle which also serves as the larval cuticle [9]. Since apolar conjugates of ES, most likely fatty acid esters in the C-22 position, are incorporated into eggs during vitellogenesis of several tick species (*O. moubata* [10]; *Boophilus microplus* [11]; *Ornithodoros parkeri, Hyalomma dromedarii, Ixodes ricinus,* and *Rhipicephalus appendiculatus* [12]), it was hypothesized that these conjugates may also serve as an ES source for embryonic molts. However, in *O. moubata* embryos, RIA analyses of crude and esterase-treated extracts of various ages of embryos and larvae revealed that, at the appearance of an ES peak, the quantity of conjugated material increased. This would not be expected if these conjugates were the source of ES for the peak [9]. In addition, the radiolabelled fatty acid esters found in the eggs from vitellogenic *O. moubata* females injected with [^{3}H]-E or [^{3}H]-20E were not hydrolysed during embryonic and larval development [13]. Thus the ES peaks during embryonic development of *O. moubata* did not appear to be of maternal origin.

During embryonic and larval development of this same tick species the ES titers measured with two different RIAs showed the occurrence of an E peak on the 7–8th day of embryonic development followed by hydroxylation to 20E [9]. HPLC-RIA analyses tentatively demonstrated that the ES peaks coinciding with the initiation of cuticle deposition on the 8th day of embryonic and 3rd day of larval development of *O. moubata* were composed principally of 20E along with a small quantity of E. We hypothesize that, as observed during postembryonic development of many insect species [14], the hemolymphatic 20E peak originates from conversion of E by the peripheral tissues.

In this study, we investigated the metabolism of [^{3}H]-E in vitro throughout embryonic and larval development to determine 1) whether these life stages are able to hydroxylate E to 20E and, if so, when they acquire this capacity and 2) whether qualitative or quantitative modification of the metabolites produced occurs throughout these developmental stages.

*Abbreviations used: AP = apolar products; E = ecdysone; ES = ecdysteroids; 20E = 20-hydroxyecdysone; PP = polar products; RH = relative humidity.

METHODS

Ticks

The *O. moubata* females came from a laboratory colony which has been maintained in our institute for several years. The ticks were fed on defibrinated pig blood at 37°C through a Parafilm membrane and were kept in the dark in cotton-plugged vials (27°C and 30–40% RH). Females oviposited approximately 10 days after feeding. Eggs were collected daily by removing the female from the vial and placing her in a different vial. This was done to minimize handling of the eggs which may disrupt development. Embryonic development took about 10 days, and the emerging hexapod larva, a nonfeeding stage, continued to develop, molting 5 days later to the first instar nymph. (For further information on development see [9].)

Chemicals

Ecdysone (2β,3β,14α,22R,25-pentahydroxy-5 β-cholest-7-en-6-one) and 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one) were obtained from SIMES (Milan, Italy). Labelled ecdysone ([23,24-^{3}H]-ecdysone, 53.6 Ci/mmol) was purchased from New England Nuclear Corporation (Boston, MA) and purified by HPLC when necessary [10].

Cultures

Because of the high internal pressure of *O. moubata* eggs and the small size (1 mm diameter) of the eggs and larvae, injection of [^{3}H]-E was difficult. Thus for each day of embryonic or larval development 10 embryos or larvae (or 21 ovocytes dissected from vitellogenic females) were broken or slit open and cultured in two hanging drops of 20 µl each of TC199 Seromed medium (Biochrom, Berlin, Germany) (osmolarity adjusted to 400 mOsmol with NaCl) containing 100,000 cpm [^{3}H]-E (53 Ci/mmol, thus a total 1.89 pmol or 875 pg). The culture medium also contained 200 U of penicillin/ml (G. Hoechst, Zurich, Switzerland) and 100 mg streptomycin/ml ("Streptothenat," Grunenthal, Germany). The embryos and larvae were incubated 6, 24, and 48 h at 27°C in a humidity chamber (small box with H_2O in the bottom) to prevent evaporation of the hanging drops. After incubation, the hanging drops and embryos or larvae were placed in a 1.5 ml centrifuge tube with washes (200 µl H_2O and 400 µl methanol) of the supporting microscope slide. This mixture was then ground with a teflon coated pestle and the pestle rinsed with 500 µl methanol. The homogenate was vortexed, sonicated for 3 min, and centrifuged for 10 min at 10,000*g*. The supernatant was removed and the pellet was resuspended with 500 µl methanol and recentrifuged as above. The supernatants were pooled and dried at 60°C under a N_2 flow and resuspended in 100 µl of methanol. Before injecting the sample on HPLC, 200 µl 20 mM Tris buffer, pH 7.5, were added.

High Performance Liquid Chromatography

Reverse-phase analyses were carried out with a Perkin Elmer Series 3 HPLC (Norwalk, CT) connected with a variable wavelength spectrophotometer set at 242 nm. The samples were separated on a Merck (Schweiz, AG, Zürich,

Switzerland) Lichrosorb RP-18 (7 µm phase) column (25 cm × 4 mm) with a precolumn (4 cm × 4 mm) packed with Merck RP-8 (40 µm phase), using a solvent gradient of methanol and 20 mM Tris buffer (pH 7.5) [10]. Fractions (30 s, 400 µl) were collected. Reference E and 20E were coinjected with the sample.

Normal phase HPLC analyses were carried out on a KONTRON system (KONTRON, Zürich, Switzerland) with a 420 pump connected with a variable wavelength spectrophotometer set at 242 nm. The samples were eluted with chloroform/isopropanol/water (100/25/1.25) at a flow rate of 2 ml/min on a silica column (Merck Lichrospher 5 µm, 25 cm × 4 mm). Fractions (30 s, 1 ml) were collected.

Liquid Scintillation Counting

An automatic liquid scintillation counter KONTRON MR 300 DPM and Riatron scintillation cocktail (KONTRON) were used. In the case of HPLC fractions results are expressed in noncorrected counts per minute since, under our conditions, only a small difference of quenching existed between the different HPLC fractions. The fractions from samples separated on the silica column were allowed to dry before adding the scintillation fluid.

Esterase

A pool of fractions corresponding to apolar products was dried under a N_2 flow, resuspended in 450 µl of Tris buffer (50 mM, pH 8), and sonicated 3 min. Porcine liver esterase (E.C. 3.1.1.1; Boehringer, Mannheim, Germany; 50 µl, 50 IU) was added and the mixture incubated overnight at 37°C. Methanol (250 µl) was added, the solution vortexed and centrifuged for 10 min at 10,000*g*, and the supernatant injected on RP-HPLC.

Estimation of the Biological Half-Life

To estimate the biological half-life of the [^{3}H]-E added to the cultures, the assumption was made that the cultures contained 100% of tritiated E at zero time; the percentage of E remaining in the cultures was plotted for the three incubation times (6, 24, and 48 h). From this curve we then interpolated the time when 50% of the radioactive hormone remained in the culture. Although this calculation did not take into account the endogenous ES levels, other calculations that include a correction for the endogenous ES of the injected hormone do not modify the general data pattern.

RESULTS

Metabolism of [^{3}H]-Ecdysone During Embryonic Development

Ovocytes and all embryonic stages investigated were capable of metabolizing the [^{3}H]-E (Fig. 1). In the different stages investigated, the production of one or more of three different types of metabolites was observed (Fig. 2). The first group corresponded to apolar products (AP) which eluted at retention times similar to those of labelled AP2 products isolated from the eggs of *O. moubata* [10] or *B. microplus* [11,15] females injected with [^{3}H]-E or [^{3}H]-20E

during vitellogenesis. These conjugates have been identified as esters of the common long chain fatty acids (palmitic, palmitoleic, stearic, oleic, and linoleic acids) at the C-22 position of ES in the eggs of *B. microplus* [16] and in fifth instar nymphs of *O. moubata* [17]. Esterase hydrolysis of these AP (Table 1) released both E and the second type of metabolite produced, 20E. This second metabolite comigrated with our reference 20E on both RP-18 and silica columns. The third group of metabolites produced were unidentified products more polar than E (labelled A, B, and C in Fig. 2b) and were collectively called polar products. Product B had the same retention time as the product tentatively identified as 20,26-dihydroxyecdysone in *O. moubata* fifth instar nymphs [18]. The relative proportion of each of these three classes of metabolites depended upon the age of the embryos incubated (Fig. 1). The biological half-life (a measure of the rate of metabolism) of the hormone (which is given in each upper right-hand corner of the diagrams in Fig. 1) varied greatly during the development of the embryos.

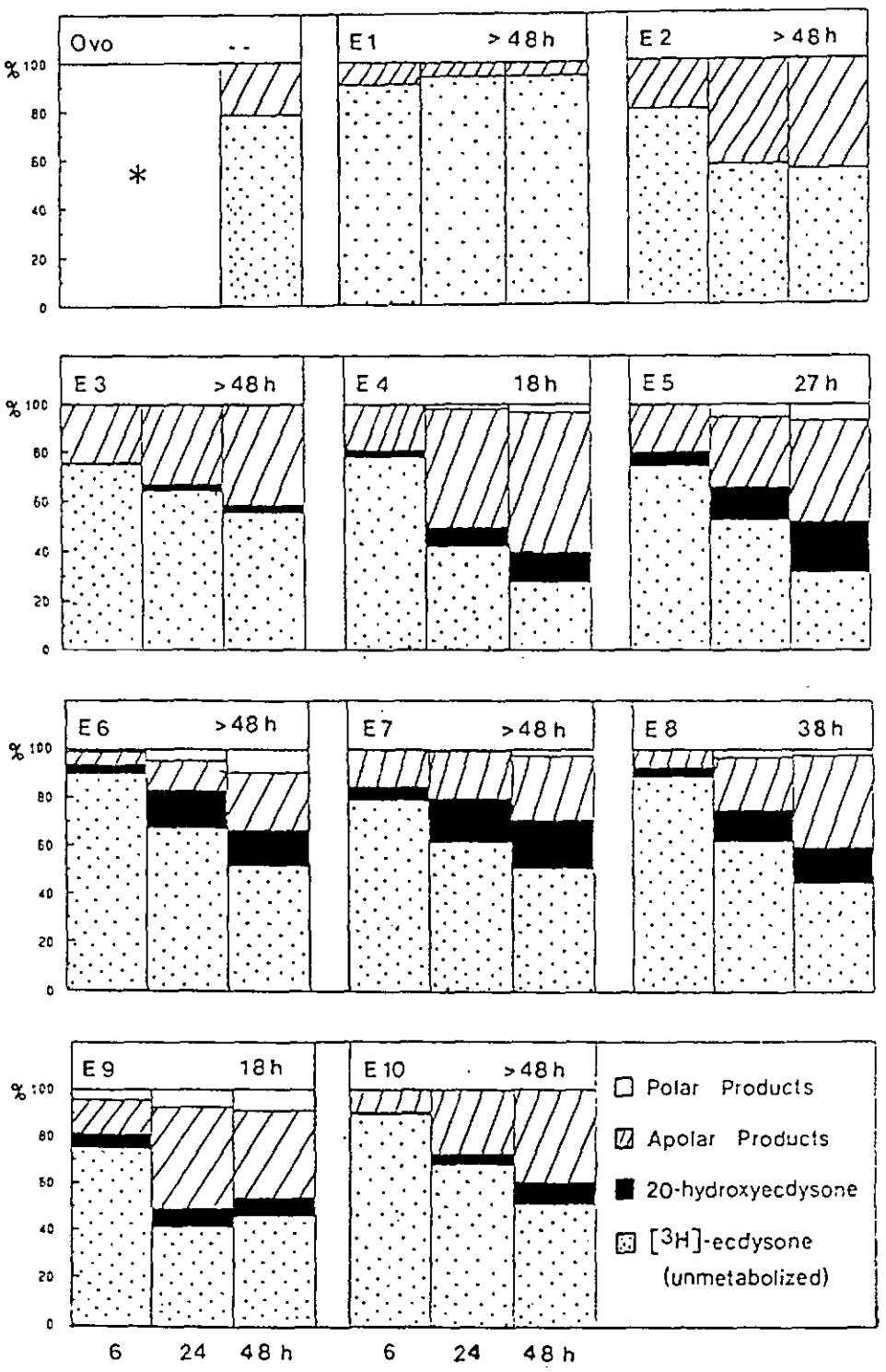
Metabolism of [^{3}H]-Ecdysone During Larval Development

All larval stages examined metabolized [^{3}H]-E although at rates which depended upon the age of the larvae incubated (Fig. 3). The same three types of metabolites (20E, AP, and PP; Fig. 2c) observed in the embryo cultures were produced; however, the AP included the AP1 subclass of AP which consists of unidentified esters of E first described in *O. moubata* females [10]. Esterase hydrolysis of all AP released products which comigrated with E and 20E (Table 1). The percentage of radiolabel corresponding to the different metabolites also depended upon the age of the larvae cultured.

DISCUSSION

Rate of Metabolism of the Tritiated Hormone

Our results demonstrate that *O. moubata* embryos and larvae metabolize [^{3}H]-E at a rate and in a pattern of metabolites which is dependent on the developmental stage. Except for young embryos, the shortest biological half-lives for [^{3}H]-E appear to coincide with low or decreasing titers of free endogenous ecdysteroids [9]. During the first 3 days of embryonic development the longer half-life could be due to low concentrations of enzymatic systems. Long half-lives were observed on days 6–7 and on the 2nd day of larval development. These decreases in metabolic rate may be associated with increasing endogenous ecdysteroid titers described in Dotson et al. [9] and could be due to a slowing of the detoxification mechanism (AP formation) during the endogenous production of E. The dilution of the tritiated hormone in the culture by an increase in endogenous ecdysteroids that would decrease the specific activity of the [^{3}H]-E did not seem to be responsible for this variation. On the last day of embryonic development, the half-life again increases, perhaps due to a decrease in metabolic activities along with the process of hatching.


Ovo
E1 >48h
E2 >48h
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E5 27h
E6 >48h
E7 >48h
E8 38h
E9 18h
E10 >48h
%
100
80
60
40
20
0
6
24
48 h
Polar Products
Apolar Products
20-hydroxyecdysone
[3H]-ecdysone
(unmetabolized)

Figure 1.

Occurrence of 20-Hydroxylation During Embryonic and Larval Development

Although not visible in Figure 1, hydroxylation of the [^{3}H]-E in the embryos begins a round day 2. In another 48 h culture of 2 day old embryos where more embryos were cultured, 2.3% of the metabolites corresponded to 20E (data not shown). This period in embryonic development follows the formation of the vitellus-filled macromeres, also known as the primary endoderm, which perhaps participate in the formation of the midgut epithelium later in development, and the formation of the nucleolus, which is responsible for ribosome production in all cells [19]. Whether or not only the primary endoderm or all cells of the embryo, which have just acquired the "machinery" for protein (thus enzyme) synthesis, are responsible for the initiation of 20-hydroxylation is speculative. In fifth instar nymphs of this species, all tissues incubated in vitro with [^{3}H]-E were capable of 20-hydroxylation. Nevertheless, the midgut along with the ventral carcass were the greatest producers of 20E (which may indicate that the fat body or other tissue is involved in 20-hydroxylation) while the Malpighian tubules and dorsal carcass hydroxylated only very minute quantities of the tritiated E (Vuilléme, unpublished results). In nymphs of a closely related tick, *Ornithodoros parkeri*, the fat body appeared to be the site of 20-hydroxylation [20]. Embryos of *Locusta* were able to hydroxylate the [^{3}H]-E on the 3rd day of development preceding production of an endogenous 20E peak [8]. In the postembryonic stages of many insects, this reaction appears to take place in fat body and Malpighian tubules (see [14,21]).

In *O. moubata* after the 2nd day of embryonic development, once the embryo has gained the capability of ecdysone 20-hydroxylation, this enzyme system remains functional throughout embryonic and larval development. However, the activity of this system fluctuates depending on the age of the tick. In cultures of 7 day old embryos, and 3 day old larvae, on the day just preceding the embryonic endogenous peak and on the day of the larval endogenous peak, respectively [9], the percentage of free 20E reaches its highest proportion of metabolites. In several insect species, a peak of ecdysone 20-monooxygenase activity has been associated with the endogenous ES peak [14]. Whether peak periods of monooxygenase activity exist in the embryos and larvae of *O. moubata* is difficult to say since conjugation of 20E was also taking place and we did not investigate PP or AP of all stages. Analyses of the products released after esterase hydrolysis of AP from the 7th day of embryonic development and on the 1st day of larval development revealed that 30% of the [^{3}H]-E was converted to 20E. However, at the 7th day of embryonic development only a third of the 20E is conjugated whereas on the 1st day of

Fig. 1. Bar representation of the distribution of the radiolabel in the 6, 24, and 48 h cultures of *O. moubata* embryos broken open in TC199 containing 100,000 cpm [3H]-ecdysone. The numbers in the upper left corner and in the upper right corner of each diagram are the age of the embryo (E1–E10) in days and the apparent half-life of the hormone in hours, respectively. The percentage of the radiolabel corresponding to unidentified polar products (□), to apolar products less than polar than ecdysone and most likely long chain fatty acid esters at C-22 position of ecdysone and 20-hydroxyecdysone (■), to a product comigrating with 20-hydroxyecdysone (▨), and to unmetabolized ecdysone (▩). *, Not investigated.

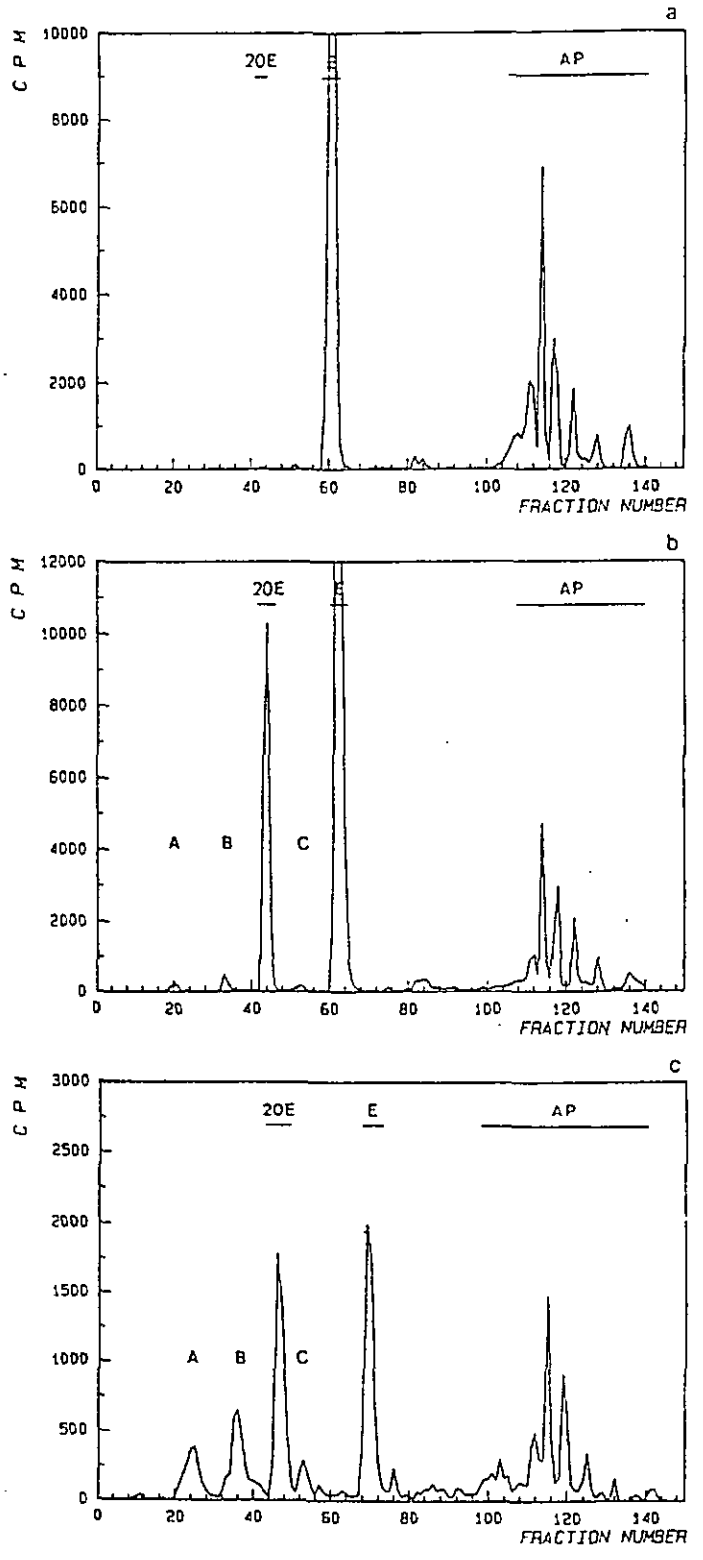
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Figure 2.

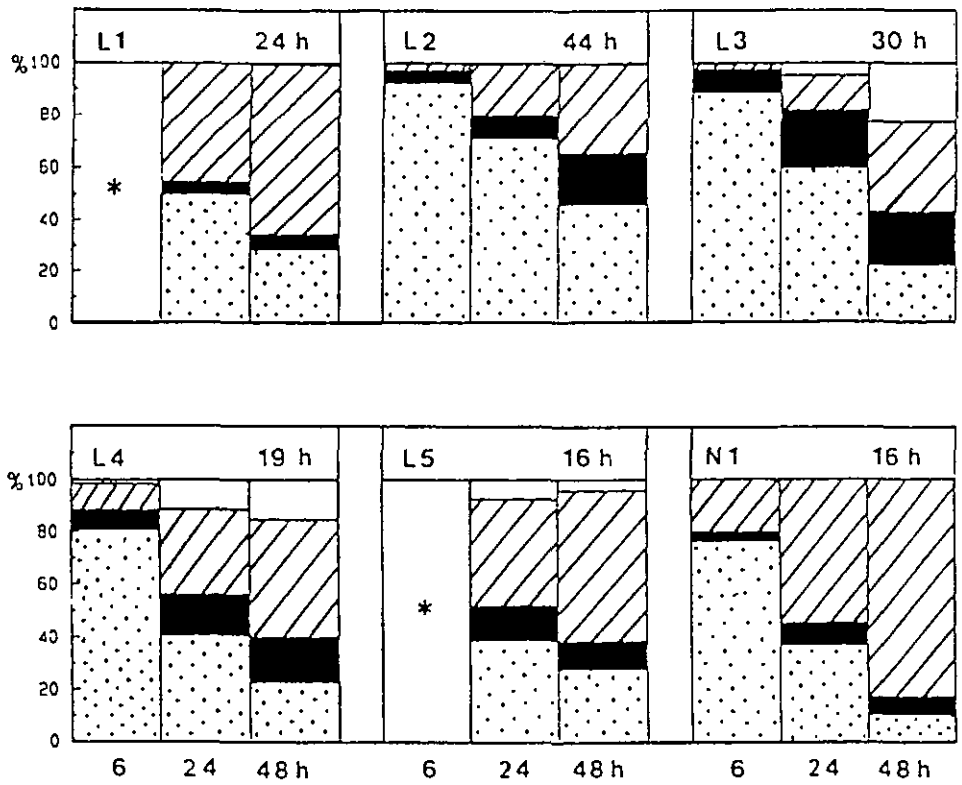

Fig. 3. Bar representations of the distribution of the radiolabel in the 6, 24, and 48 h cultures of *O. moubata* larvae slit open in TC199 containing 100,000 cpm [3H]-ecdysone. The numbers in the upper left corner and in the upper right corner of each diagram are the age of the larvae in days (L1–L5) and first instar nymphs (N) and the apparent half-life of the hormone in hours, respectively. The percentage of the radiolabel corresponding to unidentified polar products (□), to apolar products less polar than E and most likely long chain fatty acid esters at C-22 position of ecdysone and 20-hydroxecdysone (▨), to a product comigrating with 20-hydroxecydsone (■), and to unmetabolized ecdysone (▩). *, Not investigated.

larval development 80% was conjugated (Table 1). If we assume that 20-hydroxylation of E is taking place before and not after conjugation (this is most likely the case since the long chain fatty acid at position C-22 probably inhibits recognition of the C-20 site), the above information suggests that inactivation by conjugation plays an important role in regulating the free 20E titer during this period. However, a reduction in ecdysone 20-monooxygenase activity seems to occur at the end of larval development, where only 21% of the original [^{3}H]-E was hydroxylated to 20E (Table 1). One-half and two-thirds of the 20E were found in conjugated form in the cultures of the 5th day of larval development and of the first instar nymph, respectively.

Fig. 2. RP-18 HPLC radiochromatograms of hanging drop cultures of 10 *O. moubata* embryos or larvae broken open in TC199 containing [^{3}H]-ecdysone. E and 20E, retention times of cold ecdysone and 20-hydroxyecdysone standards, respectively; AP, apolar products; A, B, C, unidentified polar products. a: 48 h culture of day 2 embryos (E2). b: 48 h culture of day 7 embryos (E7). c: 48 h culture of day 3 larvae (L3).

TABLE 1. Percent of Radiolabel Corresponding to [^{3}H]-E and Its Metabolites in Hanging Drop Cultures of *O. moubata* Embryos and Larvae Incubated for 48h*

				AP	
Stage	E	PP	20E	E_c	$20E_c$
E7	51.1	2.6	20.0	17.9	8.6
L1	28.4	0.9	5.8	38.9	24.3
L5	28.0	4.3	10.4	45.7	11.6
N1	10.2	—	6.9	67.2	14.3

*E, unmetabolized [^{3}H]-ecdysone; PP, unidentified products more polar than ecdysone but not comigrating with 20E; 20E, products comigrating with 20-hydroxyecdysone on reversed and normal phase HPLC; AP, apolar products which are less polar than E and are most likely long chain fatty acid esters of E or 20E; E_c and $20E_c$, products comigrating with E and 20-hydroxyecdysone after esterase hydrolysis of AP; E7, culture of 7 day old embryos; L1 and L2, cultures of 1 day and 5 day old larvae; N1, culture of 1 day old nymphs.

The fact that the ecdysone 20-monooxygenase system is always operational after its "installation" and not limited to short periods associated with an ES peak suggests that perhaps factors other than an increase or decrease in monooxygenase activity may be involved in the regulation of 20E titers. These factors include regulation of production of conjugates such as AP, as suggested above, and the production of other metabolites such as PP.

Other Metabolic Pathways Observed During Embryonic and Larval Development

Production of AP was observed in all stages cultured including ovocytes. According to their retention times on RP-HPLC and the products of their hydrolysis, they seem to correspond to fatty acid esters of ES described in *B. microplus* eggs [16] and in *O. moubata* fifth instar nymphs [17].

The role of these AP is unclear. Connat et al. [10] and Wigglesworth et al. [11] suggested that these products which accumulate in freshly laid eggs when vitellogenic females are injected with [^{3}H]-E or [^{3}H]-20E may act as storage products which are a source of ES during embryonic development. Preliminary evidence indicates that *B. microplus* embryos metabolize the radiolabelled AP to ecdysteroid-26-oic acids [22] which correspond to inactivation products in insects [23]. On the other hand, in *O. moubata* embryos, the radiolabelled AP are not hydrolysed during embryonic or larval development [13]. On the contrary, they are endogenously produced during embryonic and larval development as shown by RIA analysis of extracts before and after esterase hydrolysis throughout embryonic and larval development. An esterase-labile substance increased at the appearance of each ES peak [9]. The agreement between our previous results demonstrating conjugation of the endogenous ES produced during embryonic development and the present data, indicating that the apolar pathway is particularly efficient at times when endogenous free ES levels must be low, and the fact that these conjugates are not used as a hormonal source during development [13] lead us to conclude that AP represent products of a mechanism of detoxification of ecdysteroids.

This detoxification mechanism is present and active in all the developmental stages of *O. moubata* from young ovocytes to nymphs [18] and adults [10,13].

It inactivates both endogenous [18] and exogenous [13,24] ES in the diet. Localization of this enzymatic system has not been completely defined but it is present in numerous tick organs: ovaries [10], Malpighian tubules, intestine, and dorsal and ventral carcass (Vuillème, unpublished results). The midgut appears to be the organ most active in conjugation, especially when ES are in the lumen. The fact that young ovocytes are able to conjugate the ES indicates that enzymes are brought with vitellus into the embryos. The vitellus filled macromeres, which constitute the young intestine, thus appear to contain their detoxification enzyme machinery. It is possible that, in this case, no regulation of the system exists and that all ES accessible to the apex of the intestinal cells are detoxified.

Another pathway of metabolism, the production of polar products, was observed during embryonic and larval development of *O. moubata*. The identity of these products is unknown. Product B appears to comigrate with 20,26-dihydroxyecdysone, the presence of which has also been suggested during [^{3}H]-E metabolism in *O. moubata* fifth instar nymphs [18], but products A and C do not correspond to any PP found in nymphs or in females either directly or after esterase hydrolysis [10,24]. As previously described in nymphs, the PP appear to be most actively produced during high or decreasing endogenous ES titers [9]. The regulation of the enzymatic activity may be based on substrate concentration. More in-depth studies are necessary to establish the significance of this apparent inactivation pathway.

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IV

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Metabolism of ecdysteroids in the female tick *Amblyomma hebraeum* (Ixodoidea, Ixodidae): accumulation of free ecdysone and 20-hydroxyecdysone in the eggs

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Summary [^{3}H]-20-hydroxyecdysone ([^{3}H]-20E) injected into *Amblyomma hebraeum* females 7 days before the beginning of oviposition, *viz.* at the beginning of vitellogenesis, was converted to 3 polar peaks of unknown nature called 1, 2 and 3, and to apolar conjugates AP1, AP2 and AP3. AP2 have the same retention times as the esters of 20E with long chain fatty acids described in *Ornithodoros moubata* (Diehl et al. 1985). However, principally unmetabolized 20E was incorporated into the ovaries, and 16% of the injected labelling was recovered in the eggs, ¾ being free 20E. When 20E was injected during oviposition, it was not converted to the polar products but only to the apolar products. At this time, 76% of the total radiolabel injected accumulated in the egg-batch, principally in the form of free unmetabolized 20E.

After injection of [^{3}H]-ecdysone ([^{3}H]-E), the three polar metabolites 1′, 2′ and 3′, probably 20-deoxy homologues of 1, 2 and 3 described above were always produced irrespective of the time of injection. In addition, E was metabolized to 20E and to the apolar conjugates AP1, AP2, and AP3. E, 20E and peak 2′ were incorporated into the ovary within the first day after injection. These 3 compounds were found in freshly laid eggs in variable proportions, the quantity of E decreasing with time while 20E and peak 2′ increased. At the end of oviposition, ca. 60% of the injected radiolabel had been incorporated into the eggs. Apolar products and polar metabolites accumulating in the body were apparently not used as a source of free hormone for the eggs.

Our results with tritiated ecdysteroids confirm our data concerning endogenous ecdysteroids of the eggs of *A. hebraeum* (Connat et al. 1985). This species, in contrast to 2 other female ticks, *Ornithodoros moubata* and *Boophilus microplus*, incorporates free E and 20E instead of ecdysteroid conjugates into its eggs. The role of these free ecdysteroids remains to be elucidated.

Abbreviations: *ES* ecdysteroids; *E* ecdysone; *20E* 20-hydroxyecdysone; *HPLC* high-performance liquid chromatography; *RP-18* reverse phase with C18 aliphatic chains grafted on the silica; *RIA* radio-immunoassay

* Part of this work was realized for the PhD thesis of E.M. Dotson

Introduction

Ecdysteroids (ES) are polyhydroxylated steroids that seem to occur in all arthropods; in addition, they have been recently detected in many other invertebrate phyla (for review, see Downer and Laufer 1983; Hoffmann and Porchet 1984). In insects and crustaceans, which are the most studied groups, they have a variety of regulatory roles controlling the major developmental processes: molting and reproduction. However, in species belonging to other groups where ES have been demonstrated, only a tentative correlation with molting processes has been proposed. In ticks, the presence of ecdysone (E) and 20-hydroxyecdysone (20E) was demonstrated in the nymphs of *A. hebraeum* by Delbecque et al. (1978a); then correlations between hormonal titers and cuticular deposition in this tick were described (Diehl et al. 1982).

Recent papers have focussed on the endogenous levels of ES and on the metabolism of these hormones in female ticks. As these animals do not molt during the adult stage, we suppose that ES are most likely used in reproduction. In the hard tick *Boophilus microplus*, a peak of free E occurred just prior to complete engorgement and detachment. Then the titres of E decreased to a very low value, since, as demonstrated with injected [^{3}H]-E, the free

hormone was conjugated to esterase labile apolar conjugates. These apolar products produced by the female were found in newly laid eggs (Wigglesworth et al. 1985). This situation is similar to that previously observed in the female of the soft tick *Ornithodoros moubata* (Connat et al. 1984). At that time in the respective species, the authors had hypothesized that these apolar conjugates could be a storage form of a hormone which could be used during embryogenesis, as suggested earlier in *Locusta migratoria* which accumulates polar conjugates in its eggs (Sall et al. 1983).

Interestingly, a quite different situation has been observed in the female hard tick *A. hebraeum*. In this species, only low amounts of conjugated ES were found. The ecdysteroid levels, measured by radioimmunoassay (RIA) increased continuously in the animal during the single gonotrophic cycle. About 350 ng 20E equivalents, distributed in the carcass, hemolymph, and ovary were found in each female prior to oviposition. These ES, identified by mass-fragmentometry of silylated HPLC fractions, corresponded to free 20E and E and were incorporated into the eggs (Connat et al. 1985). A quite similar situation seems to exist in female *Dermacentor variabilis*. RIA of crude extracts showed a great increase of immunoreactive material during engorgement (45 fold) on day 7 post attachment. Then, the ecdysteroid levels continued to rise during vitellogenesis to reach 19 ng 20E equivalents per tick (Dees et al. 1984). RIA coupled to HPLC analysis showed that part of the RIA-positive material comigrated with 20E. Apparent amounts of ES incorporated into the eggs were lower than in *A. hebraeum*, but hydrolysis experiments were not performed. Thus, the quantity of polar or apolar ecdysteroid conjugates present in the freshly laid eggs of *D. variabilis* is not known. No metabolic studies were undertaken in this species. In any case, it appears that at least 2 different strategies are used in reproduction in the Acarina. Some species incorporate apolar conjugates into the eggs, and others free hormones.

In this study we report the results of metabolic investigations with tritiated E and 20E in *A. hebraeum* females. Our data are in agreement with the endogenous incorporation of massive quantities of free hormones into the freshly laid eggs.

Materials and methods

Animals. Amblyomma hebraeum (Koch) females were generously supplied by CIBA-GEIGY (CRA St. Aubin, Switzerland) a few days post-drop after engorgement on young bovines. They were kept isolated in the dark at 28 °C and under 97% RH. The preovipositional period lasts approximately 12 days and the females oviposited for 20 to 30 days. More information about the biology and physiology of vitellogenesis of this species may be found in Norval (1977) and Connat et al. (1985).

Chemicals. Ecdysone (2β, 3β, 14α, 22R, 25-pentahydroxy-5β-cholest-7-en-6-one) and 20-hydroxyecdysone (2β, 3β, 14α, 20R, 25-hexahydroxy-5β-cholest-7-en-6-one) were purchased from SIMES (Italy). [23, 24-^{3}H(N)]-ecdysone (sp. act. 53.6 Ci/mmol) was purchased from New England Nuclear Corporation and when necessary was purified by high performance liquid chromotography (HPLC) before use. Tritiated 20-hydroxy-ecdysone was obtained by incubation of [^{3}H]-ecdysone with Malpighian tubules from 5th instar larvae of *Locusta migratoria*, then purified by HPLC.

Injections. 500000 cpm of [^{3}H]-ecdysone or [^{3}H]-20-hydroxy-ecdysone dissolved in 5 µl TC 199 were injected into each of the 59 and 29 females used, respectively. The injections were performed either with glass needles made from microcapillaries (in this case the needles were very sharp, and it was possible to pierce the intestine), or with a Hamilton 5 µl microsyringe (in this case, it was possible to see the metal needle under the dorsal cuticle and to assure injection into the hemocoel). After deliberate injection either into the intestine or into the hemocoel it was not possible to distinguish a difference in the metabolic pattern observed. Only the speed of metabolism seemed to be affected.

Dissections. Females were dissected under TC 199. A circular cut was made along the lateral cuticular margins, then the dorsum was slowly lifted with forceps. The intestine, ovary, oviducts, or other organs were carefully removed and rinsed twice in fresh TC 199 (5 min).

Extractions. The dissected organs, eggs, or whole females were homogenized in pure methanol, and then sonicated. After centrifugation (15 min, 10000 rpm), the supernatant was collected and evaporated to dryness with a rotary film evaporator (Rotavapor, Büchi). The sample was resuspended in 10 ml pure methanol, and aliquots were counted to estimate radioactivity. By this single extraction we recovered 75 to 85% of the injected label.

High performance liquid chromatography (HPLC). HPLC analyses were done with a Perkin Elmer Series 3 Chromatograph or a Kontron system with 2 pumps 420 controlled by a IBM-PC computer. The samples were separated on a Merck Lichrosorb RP-18 (7 µm phase) column (25 cm × 4 mm) with a precolumn (4 cm × 4 mm) packed with Merck RP-8 40 µm phase. A solvent gradient with methanol and Tris buffer pH 7.5 (20 m*M*) was generally used: 30–45% methanol (10 min), isocratic at 45% (15 min), 45% to 100% (20 min), purge. 100% methanol (20 min). In complementary experiments, other solvents (acetate buffer 50 m*M*, pH 3.5, or water with 1% trifluoroacetic acid, pH 2.5) with acidic pH were used. Effluents were monitored at 242 nm.

A suitable amount of the methanolic extract corresponding to 25000 to 35000 cpm was evaporated to dryness under N_2. Then the samples were resuspended in pure methanol by vortexing and sonication. Two volumes of Tris-buffer were added to the methanol, and the sample was vortexed again. This protocol ensured recovery of the apolar products which can be adsorbed on the plastic tube walls during the evaporation step. Finally, unlabelled ecdysone and 20-hydroxyecdysone standards were added to the sample in order to detect the comigration of the labelled hormones with the UV peaks of the standards. Direct phase HPLC using a silica column (Merck Lichrospher 5 µm, 25 cm × 4 mm) was employed with chloroform/isopropanol/

water = 125/25/1.25. To detect comigration of RP-HPLC fractions corresponding to 20E with internal standards, the flow rate was set at 2 ml/min (30 sec fractions), and to detect comigration of AP2 fractions with AP2 from *O. moubata*, at 0.8 ml/min (12 sec fractions).

Enzymatic hydrolysis. Hydrolysis of apolar conjugates was accomplished with 50 µl (50 IU) of pig liver esterase (E.C. 3.1.1.1) (Boehringer) added to the sample dissolved in 250 µl of 0.1 *M* borate buffer, pH 8, and overnight incubation at 37°C.

Hydrolysis of polar conjugates was realized with 30 µl *Helix* juice (IBF, France) added to the sample dissolved in 270 µl of 0.1 *M* citrate buffer, pH 5.2. Incubation was 4 h at room temperature. (This protocol for the hydrolysis of phosphate conjugates was verified on extracts of *Locusta* feces). In both cases of hydrolysis, after incubation 150 µl methanol was added to each sample. Then the tubes were centrifuged (15 min, 10000 rpm), and the supernatants were injected directly on HPLC.

Liquid scintillation counting. We used a KONTRON MR 300 DPM automatic liquid scintillation counter and Riatron scintillation cocktail. In the case of HPLC fractions, results are expressed in noncorrected cpm since under our conditions, only a small difference of quenching existed between the different HPLC fractions. However, since the counting efficiency was approximately 30% and since a correct estimate of the cpm in each organ after dissection was required, quench curves were established for the different extracts. The cpm were corrected using the linear correlation between the Standards Channel Ratio (SCR) and the counting yield estimated by addition of internal standards.

Results

Fate of injected [^{3}H]-20-hydroxyecdysone in Amblyomma hebraeum females

We investigated the fate of injected [^{3}H]-20-hydroxyecdysone ([^{3}H]-20E) in engorged females at the beginning of vitellogenesis approximately one week before the beginning of oviposition. The ovaries were dissected out 6 h and 2, 4 and 8 days after injection and were analysed separately from the rest of the animal. Within the first 6 h, the major compounds appearing in the body (without the ovary) were a polar peak eluting at fraction 20 (and called 2) and a group of apolar products AP2 migrating like those found in the soft tick *Ornithodoros moubata* when analysed with RP-HPLC under the same chromatographic conditions (Fig. 1 A) as well as when analysed with a silica column (data not shown). These 2 classes of products (2 and AP2) each represented approximately 10% of the total label in the body after 6 h of metabolism. Two days after the injection, 20E was completely metabolized and product 2 became prominent, constituting 30% of the radiolabel in the carcasses (Fig. 1 B). This compound was not hydrolysed by *Helix* juice and was nonionic as shown by elution under different pH conditions. Two other minor polar peaks (1 and 3) increased in proportion only

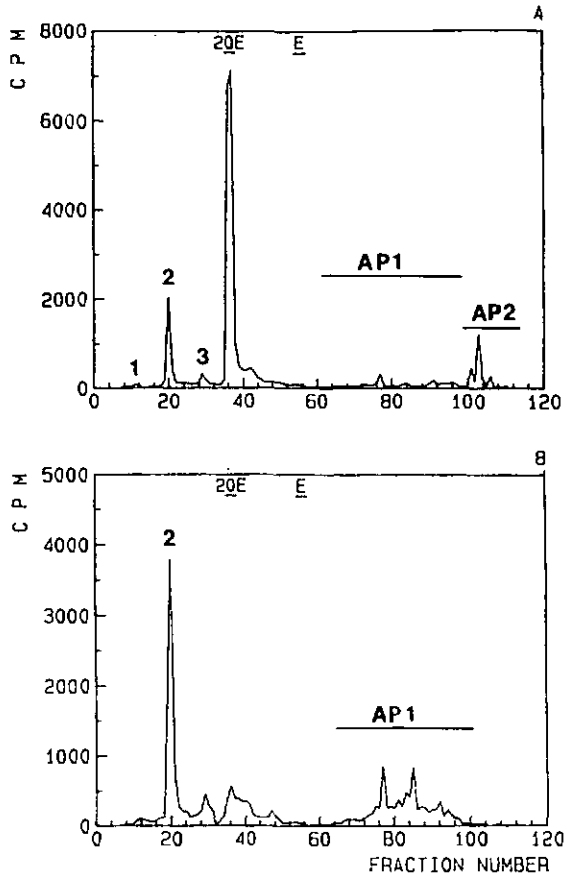

Fig. 1 A, B. Reverse-phase (RP-18) HPLC radiochromatograms of an extract of the body (without ovary) of an *A. hebraeum* female after injection with 500000 cpm [^{3}H]-20-hydroxyecdysone 7 days prior to the beginning of oviposition. A pattern 6 h after injection. B pattern 2 days after injection. AP1 and AP2 correspond to retention times of apolar conjugates previously described in *Ornithodoros moubata*. E and 20E indicate the retention times of authentic ecdysone and 20-hydroxyecdysone, respectively, coinjected with the sample

slightly within the first 2 days. As previously observed in *O. moubata*, AP2 disappeared and AP1 increased in abundance. Eight days after injection they represented 60% of the radiolabel in the carcasses and as demonstrated by esterase hydrolysis, represented conjugates of 20E (39%) and of a compound having the same retention time as the 3-epimer of 20E (61%) (data not shown).

During the first two days after injection, the ovaries contained only approximately 3% of the total radiolabel in the females. This radioactivity corresponded to 20E (40%) and AP (55%) being mainly AP2. After 4 days the proportion of the label contained in the ovary reached 16% in the form of 2 (4.7%), 20E (37.7%), AP1 (26.6%) and AP2 (22.8%). After 8 days, when the first eggs were laid, the amount of label present in the ovary decreased to 4.3%.

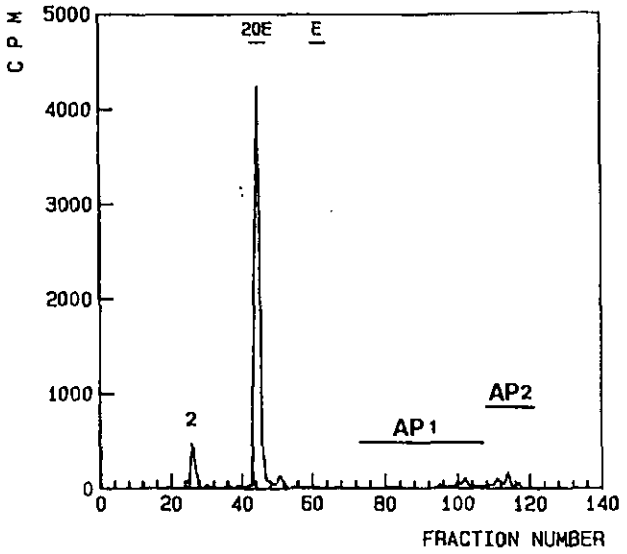

Fig. 2. Reverse-phase (RP-18) HPLC radiochromatogram of an extract of freshly laid eggs oviposited by an *A. hebraeum* female injected with 500000 cpm of [^{3}H]-20-hydroxyecdysone 7 days prior to the beginning of oviposition

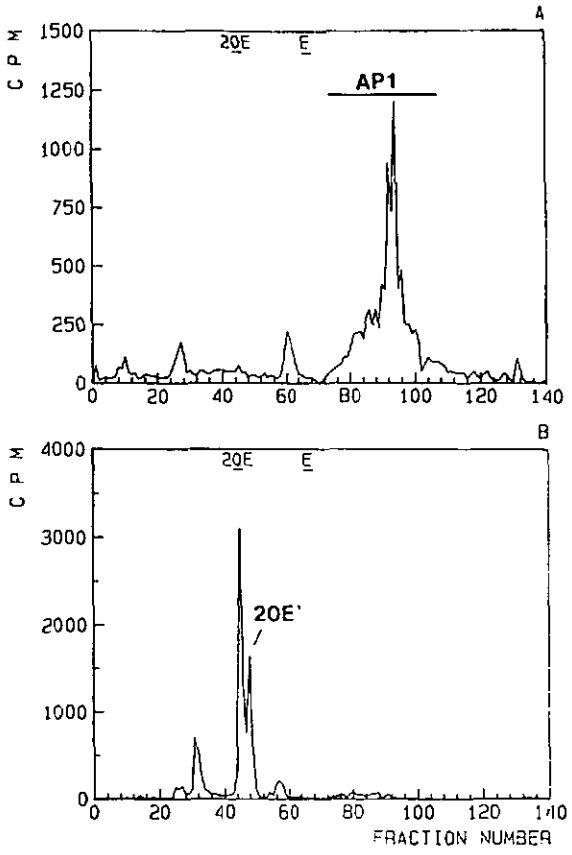

Fig. 3 A, B. Reverse-phase (RP-18) HPLC radiochromatograms of a whole body extract of an *A. hebraeum* female at the end of oviposition after having been injected with 500000 cpm [^{3}H]-20-hydroxyecdysone 7 days before the beginning of oviposition. A pattern of crude extract. B pattern of the same extract after esterase hydrolysis. 20E' corresponds to the retention time of the 3-epimer of 20E

At this time, however, only 10% remained as 20E, the remainder being principally apolar products (AP1: 35%; AP2: 50%). The fact that the proportion of AP increased in the ovary although the radioactivity decreased, indicated that AP were maintained in the ovary while 20E was incorporated into the eggs. This was corroborated by the fact that the RP-HPLC profile of the radioactivity of the eggbatch laid by the females showed mainly 20E (74.8%) and peak 2 (7%) (Fig. 2). RP-HPLC fractions corresponding to 20E were collected and injected on a silica column. The label comigrated with authentic unlabelled 20E. Thus, although the eggs were oviposited 8 days and more after injection, they contained almost exclusively unmetabolized 20E. At the end of oviposition, in whole body extracts of females, the remaining label was mostly in the form of AP1 (67.8%) (Fig. 3A). AP2 was present in negligible amounts (6.4%). The remaining label appeared between fractions 1 and 70 showing 2 distinct peaks at fractions 26 (4%) and 60 (6%). Hydrolysis of the crude extract with esterase mainly liberated a peak which comigrated with 20E followed 2 min later (4 fractions) by a sister peak (20E') which might correspond to the 3-epimer of 20E (Fig. 3B). Three other peaks of lesser importance were noted at fractions 26, 32 and 57.

In a second series of experiments, we investigated the fate of injected [^{3}H]-20E in females at the beginning of oviposition. The ovaries were dissected out 6, 24 and 48 h after injection. We observed that, in contrast to the first group of experiments, the injected hormone was not metabolized to the polar products 1, 2 and 3 but only to the apolar products. We first noted in the carcasses the occurrence of the characteristic group AP2 which was eluted from the RP-HPLC column in the same fractions 111, 114 and 117 as the linoleic, palmitic and oleic, and stearic acid esters, respectively, of 20E in *O. moubata* (Connat et al. 1984, 1986a; Diehl et al. 1985). On a silica column, AP2 from *A. hebraeum* also comigrated with AP2 from *O. moubata*. However, we also observed the presence of another group of apolar products (AP3) which were eluted after AP2. Later, a third group appeared which was less apolar than the AP2 and which had retention times corresponding to the AP1 described in *O. moubata*. All these products were labile to hydrolysis with esterase and yielded exclusively 20E (Fig. 4B). Thus we could suppose that at least AP2 corresponded to esters of 20E with long chain fatty acids, as demonstrated in *O. moubata* (Diehl et al. 1985). The other apolar

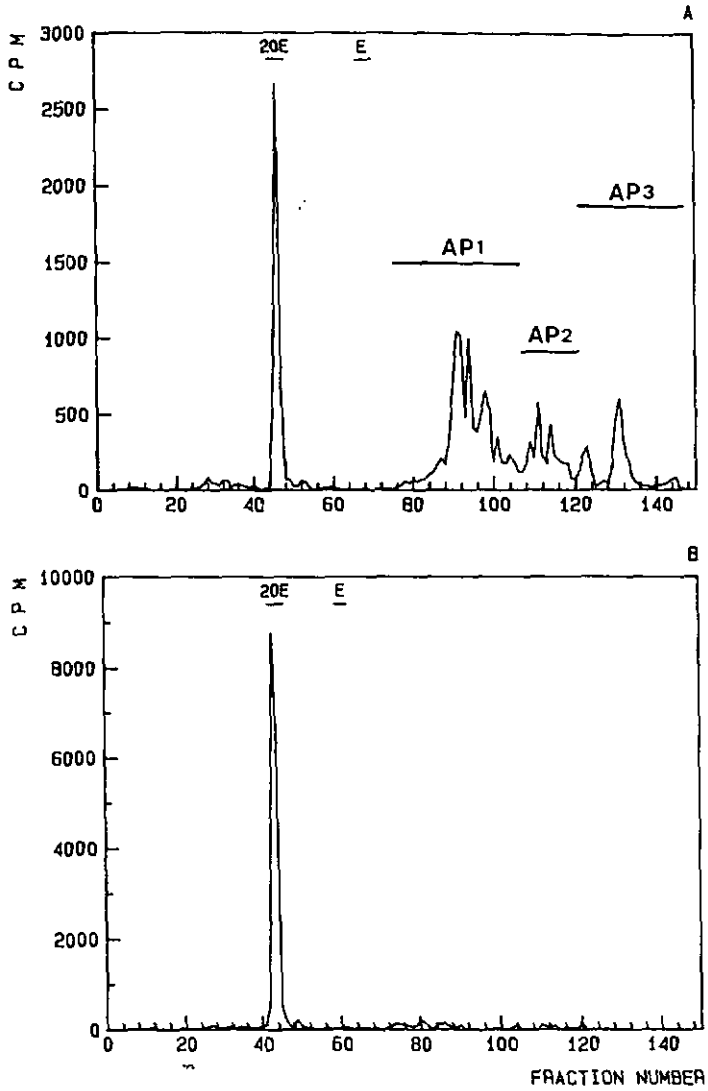

Fig. 4A, B. Reverse-phase (RP-18) HPLC radiochromatogram of a whole body extract of an *A. hebraeum* female 2 days after the injection of 500000 cpm [^{3}H]-20-hydroxyecdysone at the beginning of oviposition. A pattern of crude extract. B pattern of the same extract after hydrolysis with esterase. The apolar products have been completely hydrolyzed, yielding exclusively 20E

products presumably were also esters because they were hydrolysable. 48 h after injection, these apolar products represented about 75% of the radioactivity in the body, while the remaining cpm corresponded to unmetabolized 20E.

In different ovary extracts, a major peak was found with the same retention time as 20E and corresponding to 70 to 99% of the total label in the ovary. This peak comigrated again with unlabelled 20E injected on a silica column. Sometimes we observed in addition a small peak comigrating with E under reverse-phase conditions and which might correspond to 20E-3-acetate (20E-3A). 48 h after injection, 19 to 27% of the injected hormone had been transferred to the ovaries and the newly laid eggs. The maximum amount of radioactivity contained in the freshly laid eggs (expressed as cpm/mg eggs) was found 3 to 4 days after injection. At this time we recovered 2000 to 3000 cpm/mg fresh eggs. Then the radioactivity decreased during the following days but was still noticeable 10 days after injection (10 to 70 cpm/mg). HPLC analysis revealed that 20E always represented more than 90% of the total label in the freshly laid eggs, even 7 days after injection. The remaining cpm corresponded either to the small peak of (probable) 20E-3A also found in the ovaries, or to the unidentified polar product 2 with a retention time corresponding to fraction 26 (compound eluted after 13 min).

At the end of oviposition approximately 76% (SD = ± 25; $n = 8$ females) of the injected 20E was transferred to the eggs. The radioactivity remaining in the ovaries was very low (0.4 to 1.6% of the total label in the females). In addition, 72 to 80% of the label contained in the females was found in the midgut lumen under the form of AP1. This radioactivity was in fact underestimated since during dissection the intestine was often pierced, and some of the contents lost. In the dissection medium which contained the hemolymph, radioactivity fluctuated between 13 and 24% of the label in the body (1.5 to 2% of the total label) although it was contaminated with midgut content. The Malpighian tubules and rectal ampullae did not contain a significant amount of label (always less than 0.5%). This agreed with the fact that we never found significant amounts of label in the feces. The carcass (integument, muscles, nervous system and salivary glands) contained 1.3 to 3.3% of the label. Esterase treatment of the radioactive products (corresponding mostly to AP1) present in the females at the end of oviposition (Fig. 5A), yielded only one peak corresponding to 20E (Fig. 5B) similar to the previous finding in an earlier stage of metabolism. This indicated that no further transformation of 20E occurred after it was conjugated.

Fate of injected [^{3}H]-ecdysone in A. hebraeum females

The pattern of metabolites observed in *A. hebraeum* females after injection of [^{3}H]-E at the beginning of oviposition was more complex than that after [^{3}H]-20E injection. Fig. 6 shows the different metabolites occurring in a whole body extract of a female 24 h after injection. As previously observed after injection of 20E, the apolar products AP1 and AP2 were present, followed by 2 other more apolar peaks. Their hydrolysis with esterase within two days after

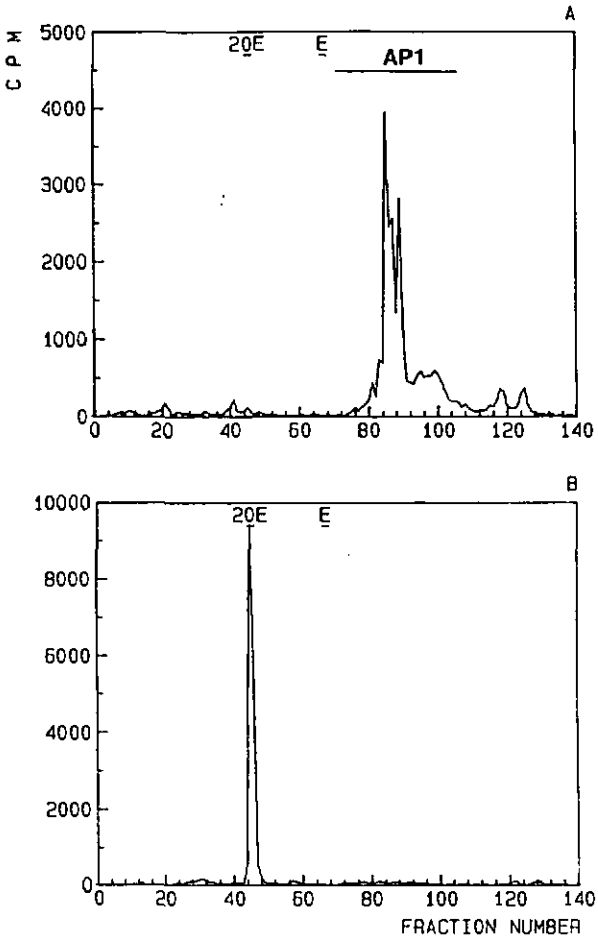

Fig. 5 A, B. Reverse-phase (RP-18) HPLC radiochromatograms of a whole body extract from an *A. hebraeum* female at the end of oviposition which had been injected with 500000 cpm of [^{3}H]-20-hydroxyecdysone at the beginning of oviposition. A pattern of crude extract. B pattern of the same extract after esterase hydrolysis. In contrast to Fig. 3, no 20E' is released after hydrolysis

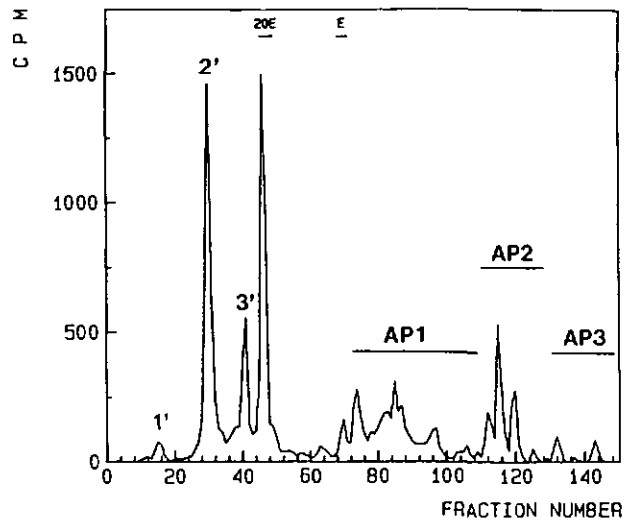

Fig. 6. Reverse-phase (RP-18) HPLC radiochromatogram of a whole body extract of an *A. hebraeum* female 24 h after the injection of 500000 cpm [^{3}H]-ecdysone at the beginning of oviposition

injection yielded principally E. 59% of the purified AP2 comigrated with reference ecdysone-22-palmitate on a silica column. In addition to these apolar conjugates, we also noticed a labelled peak comigrating with authentic 20E on both RP-18 and silica columns, indicating a conversion of E to 20E. However, in addition to these compounds, 3 peaks more polar than 20E were found. They were called peaks 1', 2' and 3' because they were slightly less polar than 1, 2 and 3 and thus appeared to be their 20-deoxy homologues. These peaks were resistant to esterase and *Helix* juice, indicating that they probably correspond to free ES. The major peak among these ES more polar than 20E, was always the peak 2'. This product appeared very rapidly, within the first 6 h after injection. Its retention time was very close to that of ecdysonic acid but did not correspond to this hormone since its retention time at different pH's did not change, thus indicating that this metabolite was nonionic. In addition, this product did not comigrate with any other polar ecdysteroid standards available (26-hydroxyecdysone, 20,26-dihydroxyecdysone, integristerone, abutasterone, and polypodine B) in our laboratory. However, the retention time of peak 3' did shift under different pH conditions. In some experiments, traces of the compound 2 coming from the newly metabolized 20E were present, eluting just before 2'. It was surprising, however, in contrast to the injection of [^{3}H]-20E, that here product 2 represented only a minor pathway although large quantities of E had been converted to 20E.

In the ovaries we observed a rapid accumulation of the labelling which represented approximately 50% 24 h after injection. In these organs, in contrast to the carcasses, apolar products were present only in small quantities. The major part of the radioactivity corresponded to peak 2', 20E and E. However, the proportions of these compounds varied with time (Table 1). The proportion of E decreased and that of the product 2' increased. As previously observed after [^{3}H]-20E injection, the freshly laid eggs contained large amounts of radioactivity. Its quantity (cpm/mg) followed the same pattern as that previously described for [^{3}H]-20E with a maximum content 3 days after injection. HPLC analysis of the freshly laid eggs on every day during oviposition showed that the [^{3}H]-E content of the eggs decreased (Table 2) while simultaneously, as observed in the ovary, peaks 2' and 20E increased. 5 to 7 days after injection only 3.6% of the radioactivity corresponded to E. We must also

Table 1. Proportions (expressed in % of the total radioactivity in the organ) represented by peak 2', 20-hydroxyecdysone (*20E*), ecdysone (*E*) and apolar products in the ovary at different times after injection of [^{3}H]-ecdysone into the female

Time after injection	Peak 2'	20E	E	Apolar products
1 h	1.2	10.5	71.8	14.1
6 h	2.3	27.5	29.6	27
24 h	6.9	59	24	8.4
48 h	13.8	48	13.8	17.6

Table 2. Proportions (expressed in % of the total radioactivity recovered in the egg extracts) of peak 2', 20-hydroxyecdysone (*20E*), ecdysone (*E*) and apolar products present in eggs oviposited on each day after injection of vitellogenic females with [^{3}H]-E

Time after beginning of oviposition	Peak 2'	20E	E	Apolar products
1 day	26.5	9.8	60.1	0
2 days	11.8	33.4	51.6	0
3 days	12.6	48.2	32.1	0
4 days	19.1	47.2	19.0	9.0
5–7 days	20.5	52.7	3.6	15.5

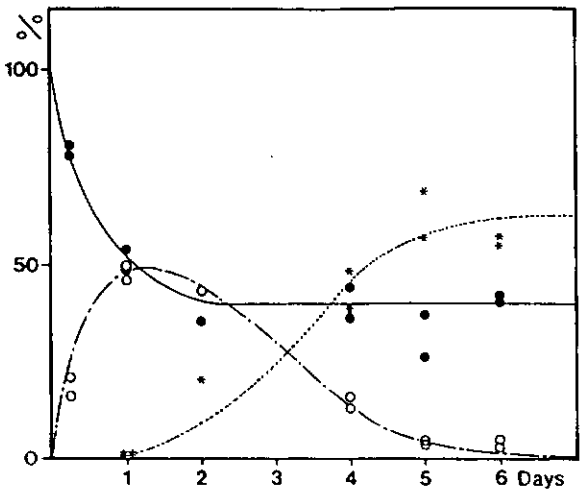

Fig. 7. Proportion of the radioactivity found in the ovary (—○—), the carcass without the ovary (—●—), and the freshly laid eggs (---*---) after injection of 500000 cpm [^{3}H]-ecdysone into *A. hebraeum* females at the beginning of oviposition. Generally, two females were used for each time investigated. Results are expressed in % of the total radiolabel recovered in the 3 compartments and in the feces of the animals. However, only minute quantities were recovered in the feces, and they may correspond to contamination of the vial after injection of the females

note that at this time, apolar products were present in noticeable amounts in the eggs.

At the end of oviposition, 59% (SD = ± 13.6, $n = 9$ females) of the injected label had been transferred to the eggs. However, depending on the animals, the label remaining in the females after oviposition, which had mainly accumulated in the intestinal lumen, was recovered in the form of AP1, or in the form of peak 2'. These different situations may be reflecting different sites of injection of the hormone.

In order to determine the origin of the free E and 20E which accumulated in the freshly laid eggs, we studied the repartition and the nature of the radiolabel in the ovary, the body without the ovary, and the freshly laid eggs at different times after a single injection of [^{3}H]-E at the beginning of oviposition. Fig. 7 shows that within the first day after injection, the radiolabel decreased in the body while it increased in the ovary. After 24 h, approximately one half of the radioactivity remained in the carcass while the other half had accumulated in the ovary. Then the proportion of the radiolabel in the carcass remained constant, indicating that no further incorporation into the ovary occurred. On the other hand, the fraction contained in the ovary slowly decreased during oviposition while the remainder of the radioactivity was found in the freshly laid eggs. We thus can deduce that the free hormones found in the eggs e.g. 6 days after injection come from a hormone stock probably incorporated into the vitellogenic ovocytes within the first day after injection. We can therefore exclude the possibility of an uptake and hydrolysis of apolar products AP1 or of a transformation of the polar product 2' which corresponded to the major part of the radioactivity in the carcasses at this time.

We also investigated the fate of [^{3}H]-E injected 7 days prior to the beginning of oviposition. Only 12% of the injected label was incorporated into the freshly laid eggs. This radioactivity was, however, mainly composed of 20E (40.3% of the total cpm), the remaining cpm representing a polar compound with retention time corresponding to fraction 20 (compound eluted between 9 and 11 min) (6.6%), and apolar products AP1 and AP2. These latter products were hydrolysable with esterase and yielded E, 20E and polar product 2'. Each of these peaks was flanked by another product which could correspond to their 3-epimer form. No free E or 20E remained in the carcasses at the end of oviposition, shortly before the death of the females. The radioactivity corresponded mainly to apolar products AP1 (62%), AP2 (7%), and to a compound with the retention time of peak 2' (11.5%).

Hydrolysis of these extracts with esterase yielded E, 20E and polar peak 2' accompanied by their 'sister' peak which could correspond to their 3-epimers.

Discussion

Our study with *Amblyomma hebraeum* females demonstrates that in this tick the metabolism of injected ecdysone (E) and 20-hydroxyecdysone (20E) may follow two different pathways, one leading to apolar conjugates and the other to unidentified polar metabolites.

The apolar pathway

Females of *A. hebraeum* are capable of conjugating injected E and 20E to apolar metabolites having similar retention times on both reverse-phase and silica columns as the AP2 of *Ornithodoros moubata*. Thus, they most likely correspond to the same compounds, namely long chain fatty acid esters (C16:0, palmitic; C18:0, stearic; C18:1, oleic; C18:2, linoleic) conjugated to the C22 position of the injected hormones (Diehl et al. 1985). Conjugation with common fatty acids seems to be very widespread. It occurs in the tick *Boophilus microplus* with C16:0, C16:1 (palmitoleic), C18:0, C18:1 and C18:2 as demonstrated by GC/MS analysis coupled to positive ion FAB mass spectrometry and p.m.r. spectrometry (Crosby et al. 1986). This type of apolar conjugate has also been found in insects. Esters of E at the C22 position with C16:0, C18:0 and C18:1 are produced in adult females of *Gryllus bimaculatus* (Hoffmann et al. 1985) and the same products as the AP2 of *O. moubata viz.* esters of 20E with C16:0, C18:0, C18:1 and C18:2 have been chemically identified in the frass of larval *Heliothis virescens* (Kubo et al. 1987). In fact, these apolar conjugates probably occur in numerous arthropods as suggested by reverse-phase HPLC profiles after injection of [^{3}H]-E (Connat and Diehl 1986) and conjugation seems to occur with all ES bearing an hydroxy group in the C22 position (Connat et al. 1986a).

We have noted in female *A. hebraeum* the presence of conjugates more apolar than the AP2 referred to as AP3. These products could correspond to esters of ES with longer chain fatty acids or to ES esterified with fatty acids at 2 or more positions.

The apolar conjugates AP2 and AP3 are further metabolized to less apolar conjugates AP1 which have similar retention times on RP-18 as the AP1 previously described in *O. moubata*. The nature of these compounds, which remain esters of the injected hormone as demonstrated by hydrolysis with pig liver esterase, is presently under investigation.

The role of these apolar products in *A. hebraeum* has not been elucidated. In two other tick species, *O. moubata* and *B. microplus*, the AP2 accumulate in the ovaries and are then incorporated into the eggs. This is not the case in *A. hebraeum*. Only very small quantities of AP were found in the ovaries at the end of oviposition; in contrast, in vitro studies have demonstrated that the ovary itself is capable of massively converting E to AP2, and these latter products remain in the ovary (Connat et al. 1986b). We suggest that this discrepancy between in vivo and in vitro studies may be due to the rapid binding of a large part of the injected hormone to vitellogenins and its incorporation into the ovary. Thus, the hormone remains protected and is not metabolized. Such a binding of free hormones has already been demonstrated in the ixodid tick *Rhipicephalus appendiculatus* (Whitehead et al. 1986). The small amount of AP2 found in the ovary could be due to the metabolism of unbound ES incorporated into young oocytes. We think that only the young oocytes are responsible for this conversion or incorporation of AP2 since AP2 are found in the ovary within the first 6 h after injection and are not released into the freshly laid eggs until 4 days after injection, corresponding to the time required for oocyte maturation. However, we could not clearly demonstrate this hypothesis by dissections of the ovary because of the location of the young ovocytes in the longitudinal groove along the length of ovary. In fact, previous in vitro studies have demonstrated that all tick organs are probably capable of converting E to AP2 (Connat et al. 1986b). This metabolism could correspond to a detoxification of the E which is artifactually distributed to various tissues after injection.

The polar pathway

When [^{3}H]-20E and [^{3}H]-E are injected into *A. hebraeum* females before active vitellogenesis, they are both rapidly converted to 3 unidentified polar ES. This is a new pathway for female ticks. Females of the two tick species studied thus far, the argasid *O. moubata* and the ixodid *B. microplus*, do not appear to convert injected ES to these polar products. The only other polar compounds described in ticks are products accumulating in conjugated form in the intestinal cells of *O. moubata* females after ingestion of tritiated ES (Connat et al. 1986a), and the polar metabolites found in *O. moubata* nymphs (Bouvier et al. 1982). These polar

compounds in *A. hebraeum* females do not correspond to any of these previously described polar products nor to any of the free ES available.

The three polar metabolites produced after injection of [^{3}H]-20E are slightly more polar than the respective polar peaks produced after injection of [^{3}H]-E. These metabolites of [^{3}H]-20E are probably homologous to those of [^{3}H]-E, only differing by an additional hydroxy group at the C20 position. However, in the case of injected [^{3}H]-E, none (except for trace amounts of product 2) of the polar products of [^{3}H]-20E is produced, although E is massively converted to 20E. This indicates that the 20E derived from the metabolism of [^{3}H]-E is not metabolized in the same manner as that injected into the animal. This could be due to a different localization of the hormone in the body or to the protective binding of the hormone to a carrier protein as suggested previously, allowing uptake by the ovary. Also, this may indicate that the production of these metabolites is artifactual and that these products are not naturally present or to be found only in minute quantities. The nature of the most abundant of these compounds, 2 and 2′, is currently under investigation. They probably do not correspond to the recently described β-D-glucoside conjugate of 26-hydroxyecdysone considering that the compounds 2 and 2′ produced after injection of 20E and E, respectively, are not hydrolysable and have retention times too close to correspond to 20,26-dihydroxyecdysone-β-D-glucoside and 26-hydroxyecdysone-β-D-glucoside (Warren et al. 1986).

Accumulation of free ecdysone and 20-hydroxyecdysone in the eggs.

Although the ovary of *A. hebraeum* females incubated with tritiated E in vitro, produces very large quantities of 3-epimers (Connat et al. 1986b), in vivo no epimers are incorporated into the ovary or eggs. This situation differs from those encountered e.g. in *Schistocerca* (Isaac et al. 1981) or in *Manduca* (Kaplanis et al. 1980) in which 3-epi-2-deoxy compounds or 3-epi-20-hydroxyecdysone, respectively, are present in the freshly laid eggs. Epimers are, however, produced in vivo at the end of the life of the *A. hebraeum* female and are recovered in conjugated form.

One of the major findings of our study is the accumulation of free radiolabelled E and 20E in the eggs of *A. hebraeum* females although the injected hormones were rapidly metabolized in the body to other polar and apolar metabolites. This is in agreement with our previous results which demonstrated the natural accumulation of RIA-positive compounds identified as E and 20E in the eggs (Connat et al. 1985). Preliminary studies with females of a closely related species, *Amblyomma variegatum*, show that the same pathways are used to metabolize [^{3}H]-E, and as in *A. hebraeum*, tritiated free E and 20E accumulate in the eggs. *A. hebraeum* females and perhaps all females of the genus *Amblyomma* thus differ in yet another respect from two other tick females studied (*O. moubata*; Connat et al. 1984; *B. microplus*, Wigglesworth et al. 1985). However, we have hypothesized that in *O. moubata*, the incorporation of AP2 in the eggs could be artifactual (Connat et al. 1986a). The uptake of free hormones by the ovary of *A. hebraeum* is of short duration. It lasts only for the first 2 days after injection when the hormones have not yet been completely metabolized. The AP2 do not appear to be hydrolyzed to free hormones and thus are not a source of hormone for the ovary.

In view of the present data and of our previous in vitro results (Connat et al. 1986b), we hypothesize that the ovary itself does not play an important role in the conversion of E to 20E. It probably incorporates the hormones and their metabolites produced in the body. Thus one day after the injection of E, E itself is the major compound incorporated. Thereafter the proportion of 20E present in the freshly laid eggs increases daily as a function of the rate of E metabolism. However, the accumulation of free hormone in the ovary does not seem to be due to a simple diffusion as observed in cultured ovaries of *Bombyx mori* (Ogiso and Ohnishi 1984) or the integument of the crayfish (Daig and Spindler 1983a, b). In fact, only very small quantities of 2, 2′ and AP are incorporated into the ovary although they become rapidly dominant in the environment of this organ. The ovary appears to selectively take up E and 20E, indicating an active transport as observed in the cultured fat body of *Sarcophaga peregrina* (Natori and Ohtaki 1976). This transport possibly takes place as a result of hormones bound to vitellogenins and subsequently incorporated into the oldest oocytes.

The role of ES in freshly laid eggs remains unknown. The most classical situation is that of the incorporation of maternal ES conjugated with phosphates. In *Locusta migratoria*, the conjugates are incorporated bound to vitellogenins (Lagueux et al. 1981), and it has been suggested in this species that these ES conjugates are hydrolyzed during embryonic development to provoke embryonic molts (Lagueux et al. 1984; Hoffmann and Lagueux 1985). In the lepidopteran, *Bombyx mori*, the majority of maternal ES in freshly laid eggs is also

present in conjugated form. In this species, although fluctuations in titres of ES conjugates occur during embryonic development, no correlation between embryonic molts and hydrolysis of conjugates could be made (Ohnishi 1986). Thus it appears that the role of the maternal ES conjugates in insects has not yet been completely elucidated.

In *A. hebraeum* eggs, in contrast to most insect species, the majority of the incorporated maternal ES are in free form. The same situation seems to prevail in the closely related species *A. variegatum* (Connat and Dotson, submitted for publication). In fact, such a phenomenon appears to be rare. Of other arthropod species studied, it occurs in the queen of the termite species *Macrotermes subhyalinus* (*michaelseni*) and *Macrotermes bellicosus* (Delbecque et al. 1978b) and in the cockroaches *Nauphoeta cinerea* (Zhu et al. 1983) and *Blaberus craniifer* (Bullière et al. 1979) which all belong to the primitive Blattopteroidea. The accumulation of free hormones in some species is quite astonishing since, according to the aforementioned hypothesis, the presence of these unconjugated hormones – even bound to proteins as demonstrated in *Rhipicephalus appendiculatus* (Whitehead et al. 1986) – could disturb normal embryonic development by acting too early on the target cells as the yolk is digested. In *A. hebraeum* embryos, these incorporated tritiated free hormones appear to be conjugated to AP2 early during development (Dotson, in preparation).

A common feature of termites and *Amblyomma*, which both incorporate free ES, is that they both lay very large quantities of eggs which develop slowly, and thus are exposed to predators over a long period of time. It may be that in the eggs of these animals the large quantities of free E and 20E act as a feeding deterrent in a similar way to allochemicals found in the eggs of certain plant-feeding insects (Hinton 1981). In addition, it is possible that the same substances may play several roles. For instance, lycopsamine from the plant *Helistropium stendneri* is a feeding attractant for the male of the African monarch butterfly. This compound is then used as a mating pheromone and is sexually transmitted to the female. Finally, the same product is recovered in high quantities in the eggs where it plays a defensive role against predators (Meinwald, Third cycle lectures, Neuchâtel Switzerland). ES from plants have been reported to be feeding deterrents for *Pieris brassicae* at concentrations as low as $10^{-5}M$ (Ma Wei Chun 1972) and in several insects at $10^{-4}M$ (Schoonhoven and Derksen-Koppers 1973). Although concentrations of this magnitude generally do not occur naturally in plants, ES could constitute a qualitative defense directed against nonadapted phytophagous insects (see review by Bergamasco and Horn 1983). It would be interesting to consider this possible additional role of ES in the eggs, especially in view of the fact the most commonly attacked eggs are those from different locusts (Hinton 1981) and consequently those containing high concentrations of 'inactive' ES conjugates.

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Ecdysteroid Titer and Metabolism during Embryogenesis of the Ixodid Tick *Amblyomma hebraeum* (Koch)

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ABSTRACT

Free 20-hydroxyecdysone (20E) and ecdysone (E) have been found to be the major ecdysteroids (ES) present in freshly laid eggs of the tick *Amblyomma hebraeum* (Connat *et al.* 1985). We investigated the ES content in embryos with RIA and the ultrastructure of the embryonic cuticles throughout embryonic development, the duration of which ranged from 48 to 60 days at 28°C. The quantity of RIA positive material varied between 50 and 200 pg ecdysone equivalents/mg (pg E eq./mg). No significant peaks were detected, although four embryonic cuticles were deposited. However, 4 extracts around the 40th day of development contained more than 300 pg E eq./mg, which coincided approximately with the deposition of the fourth embryonic (in actuality the larval) cuticle. Due to the poor synchronization of embryonic development of this species, it is possible that a more significant peak of ES was "overlooked." Esterase hydrolysis of extracts at the beginning of development released no or very little RIA-positive material, but in extracts of older embryos, the amount of immunoreactivity doubled after hydrolysis, indicating that ES had been conjugated.

In a second study, we monitored the fate of labelled 20E, E and polar products 2 and 2' contained in freshly laid eggs of females injected with [^{3}H]-20E or [^{3}H]-E during vitellogenesis. Around the 15th day of development, the embryo starts conjugating E and 20E to apolar products (AP) having similar retention times on RP-HPLC as the fatty acid esters conjugated at the C-22 position of ES which have been described in other species. Free E is also converted to polar product 2'. No free ES are present from day 30 to the end of embryonic development. Esterase hydrolysis of the AP releases 20E and E and, in higher percentages, products which comigrate on RP-HPLC with 3-epi-20E and 3-epi-E.

The free 20E and E of maternal origin present in freshly laid eggs appear to be doubly inactivated. The role of these ES remains to be elucidated.

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INTRODUCTION

Knowledge of the endocrinology of ticks has greatly increased in the last fifteen years (see Diehl *et al.* 1986, Connat 1987). Although physiological effects of exogenous applications of ecdysteroids and other studies had given evidence for hormonal control of development (see review by Solomon *et al.* 1982), the first ecdysteroids identified in ticks were ecdysone (E) and 20-hydroxyecdysone (20E) in nymphs of the ixodid tick *Amblyomma hebraeum* (Delbecque *et al.* 1978). Subsequently, high titers of these hormones were correlated with cuticle deposition in this nymph (Diehl *et al.* 1982) and in the fifth instar nymph of the tick *Ornithodoros moubata* (Germond *et al.* 1982). Metabolic studies using radiolabelled E injected at various intervals of the molting cycle demonstrated that nymphs of this last species were capable of 1) hydroxylating E to 20E, the major hormone during ES peak, and to 20,26-dihydroxyecdysone, and 2) metabolizing these free hormones to polar and apolar products (Bouvier *et al.* 1982). The more apolar of these apolar metabolites, also formed after ingestion of 20E, were found to be esters of 20E where one of four different fatty acids (palmitic, stearic, oleic, and lineolic) were bound to the C-22 position (Diehl *et al.* 1985).

Ecdysteroids have also been found in vitellogenic females of several tick species and, similarly to insects, these maternal ecdysteroids may be incorporated into the developing oocytes. Depending upon the tick species, freshly laid eggs may contain 1) free E and 20E (i.e., *A. hebraeum* [Connat *et al.* 1985] and *Amblyomma variegatum* [Connat and Dotson 1988]), 2) apolar

conjugates of ecdysteroids (*Boophilus micraplus* [Wigglesworth *et al.* 1985] and *O. moubata* [Connat *et al.* 1984]) or 3) a combination of free and conjugated ecdysteroids (*Rhipicephalus appendiculatus* and *Hyalomma dromedarii* [Connat and Dotson 1988]). These apolar conjugates have the same retention times on reversed phase HPLC as the fatty acid esters of 20E described in *O. moubata* nymphs (Diehl *et al.* 1985) and have been identified in *B. microplus* as fatty acid esters of E (Crosby *et al.* 1986).

The purpose of these ES in the freshly laid eggs remains unclear. *O. moubata* embryos did not appear to reuse the radiolabelled apolar conjugates incorporated into the eggs after vitellogenic females had been injected with tritiated E or 20E (Connat *et al.* 1988). In addition, the embryos produced more endogenous conjugates at the appearance of each ecdysteroid peak (Dotson *et al.* 1991). In *B. microplus*, these conjugates were converted to ecdysteroid-26-oic acids and fatty acyl esters of these acids (Crosby *et al.* 1987 and Dotson unpublished results). These acids and acid conjugates were presumed to be inactivation metabolites.

In this study, we attempted to determine the role of incorporated free E and 20E in *A. hebraeum* eggs. We searched for eventual fluctuations of ES levels and possible correlations with cuticle development. We also studied the modifications of these incorporated maternal ES throughout embryonic development.

MATERIALS AND METHODS

Fully engorged *A. hebraeum* females were provided by CRA Ciba-Geigy, St. Aubin (FR) Switzerland. The preovipositional period lasted approximately 11 days, and the females oviposited for 20 to 30 days. The females were allowed to lay eggs in plastic vials and every day or two days, the females were removed, leaving the eggs in the vial. This was done to minimize handling the eggs, which may disrupt development. When collecting eggs for extraction, dried and nondeveloping eggs were removed. The females and eggs were kept in 97% RH (above a saturated K_2SO_4 solution) at 28°C and in total darkness. More information about the biology and physiology of vitellogenesis of this species may be found in Norval (1977) and Connat *et al.* (1985).

Cytology

Embryos collected every two days during development were fixed in 6.25% glutaraldehyde in a 0.1 M Sörensen phosphate buffer (pH 7.4) containing 2% sucrose (Sabatini *et al.* 1963) and held at 4°C for several days. The eggs had to be pierced with a fine tungsten needle to allow penetration of the fixative. The tissue was then washed 3 times for 10 min and once overnight with a 0.2 M Sörensen buffer (pH7.4) containing 5% sucrose. The embryos were postfixed for 2 hrs at 4°C with OsO_4 in Palade (1962) buffer (pH7.4) containing 5% sucrose and rinsed 3 times for 10 min with the Palade buffer containing 5% sucrose. The tissue was dehydrated in acetone and embedded in Spurr resin.

The blocks were cut on a Sorval ultramicrotome. Semi-thin sections (~0.75 µm), cut with glass knives, were placed on albumin coated slides and stained with toluidine blue. Thin sections (~0.08µm), cut with a Diatom diamond knife, were collected on carbon-coated, parlodion-coated grids. They were contrasted in a 50% ethanol solution saturated with uranyl acetate for 15 min and then in Reynolds lead citrate solution (Reynolds 1963) for 30 min. The ultrastructure was observed with a Philips 201 transmission electron microscope (TEM).

Chemicals

Ecdysone (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one) and 20-hydroxy-ecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one) were purchased from Simes (Milan, Italy). Labelled ecdysone ([23,24^{3}H]-ecdysone ([^{3}H]-E)) (sp. act. 53.6 Ci/mmol) was obtained from New England Nuclear Corporation (Boston, MA, U.S.A.) and was purified on high performance liquid chromatography (HPLC) when necessary. Labelled 20-hydroxy-ecdysone was produced by incubating [^{3}H]-E with Malpighian tubules from 5th instar larvae of *Locusta migrataria* and purifying the extract of culture medium on HPLC.

Extractions for Radioimmunoassay (RIA)

In preliminary experiments, for every two days of development, 10-40 mg of eggs were weighed and homogenized in 3 ml 80% methanol. The homogenate was sonicated for 2 min,

centrifuged for 10 min at 10,000 *g* and the supernatant saved. The pellet was reextracted twice with 1 ml 100% methanol. The combined supernatants were dried under reduced pressure on a Rotary Film Evaporator (Büchi, Switzerland), and the extracts were resuspended in 1.5 ml 100% methanol. An aliquot equivalent to 2 mg of eggs was dried and resuspended in 0.1 M citrate buffer (pH 6.2) for RIA measurements.

In later experiments, 20-50 mg of eggs were weighed and stored in 500 µl 100% methanol at -20°C. The eggs were ground in a 1.5 ml centrifuge tube with a teflon coated piston which was rinsed afterwards with 500 µl of pure methanol. The samples were vortexed, sonicated and centrifuged for 10 min at 10,000 *g*. After centrifugation, an aliquot equivalent to 2 or 5 mg of eggs was dried and resuspended in 0.1 M citrate buffer (pH 6.2).

Radioimmunoassay

Ecdysteroid concentrations in the crude methanol extracts were determined by the radioimmunoassay method of De Reggi *et al.* (1975) under the conditions described in Connat *et al.* (1985).

Injections

A Hamilton 10 µl syringe was used to inject 500,000 or 1,000,000 cpm of [^{3}H]-E or [^{3}H]-20E dissolved in 5 µl of TC199 into vitellogenic *A. hebraeum* females one day after the beginning of oviposition.

Extractions for high performance liquid chromatography

After females had been injected with either of the labelled hormones, the oviposited eggs were collected daily. Part of the daily egg batch was extracted to determine the radioactive profile at the beginning of development. The remainder of the eggs were allowed to develop to various ages before being extracted. Radiolabelled eggs (10-40 mg) were homogenized in 500 µl methanol in 1.5 ml centrifuge tube with a teflon coated piston. The piston was then rinsed with 500 µl methanol.

High performance liquid chromatography (HPLC)

HPLC analyses were done with the same apparatus and conditions described in previous papers (Connat *et al.* 1987, Dotson *et al.* 1993).

Aliquots of extracts containing approximately 12,000 cpm were dried under a N_2 flow and were resuspended in a volume of pure methanol by vortexing and sonicating. Two volumes of Tris buffer were added, and the sample was vortexed again. This protocol was needed to recover the apolar products which are probably adsorbed on the plastic tube walls during the evaporation step. Finally, to determine if the labelled hormones comigrated with the UV peaks of standards, cold ecdysone and 20-hydroxyecdysone standards were added to the sample.

Normal phase HPLC using a silica column (Merck Lichrospher 5 µm, 25 cm x 4 mm) was employed with chloroform/isopropanol/water 125/25/1.25 (v/v/v) as the solvent. The flow rate was 2 ml/min and 30 second fractions (1 ml) were collected.

Enzymatic hydrolysis

Aliquots of methanolic extracts equivalent to 5 mg of eggs were dried in 3 ml glass bottles under a N_2 flow and resuspended in 950 µl borate buffer (O.1 M, pH 8.3) by vortexing and sonicating for 3 min. Pig liver esterase (E.C. 3.1.1.1, Boerhinger, Mannheim, Germany, 50 µl, 50 IU) was added. After an overnight incubation at 37°C, the hydrolyzed sample was diluted with 3 ml of 100% methanol and centrifuged to precipitate the denatured enzyme. The supernatant was dried with a rotary film evaporator and was then collected in 1.5 ml pure methanol. The sample was then dried under reduced pressure and was assayed using RIA.

Radioactive samples were first separated into 3 fractions (2 ml 30% methanol, 5 ml 60% methanol, and 5 ml 100% methanol) using a SEP PAK@ (Waters, Milford, MA, USA) according to method of Lafont *et al.* (1982). The third fraction was dried under reduced pressure in a rotary film evaporator or N_2 flow and subjected to esterase hydrolysis under the above conditions.

For hydrolysis with *Helix* enzymes, the radioactive sample was dried under a N_2 flow, resuspended in 100 µl 0.5 M acetate buffer pH 5.5, vortexed and sonicated for 3 min. Two to three µl of *Helix* juice (IBF) were added and then vortexed. The sample was incubated at room temperature for 1 hr and injected in HPLC in a 30% methanol solution along with cold standards.

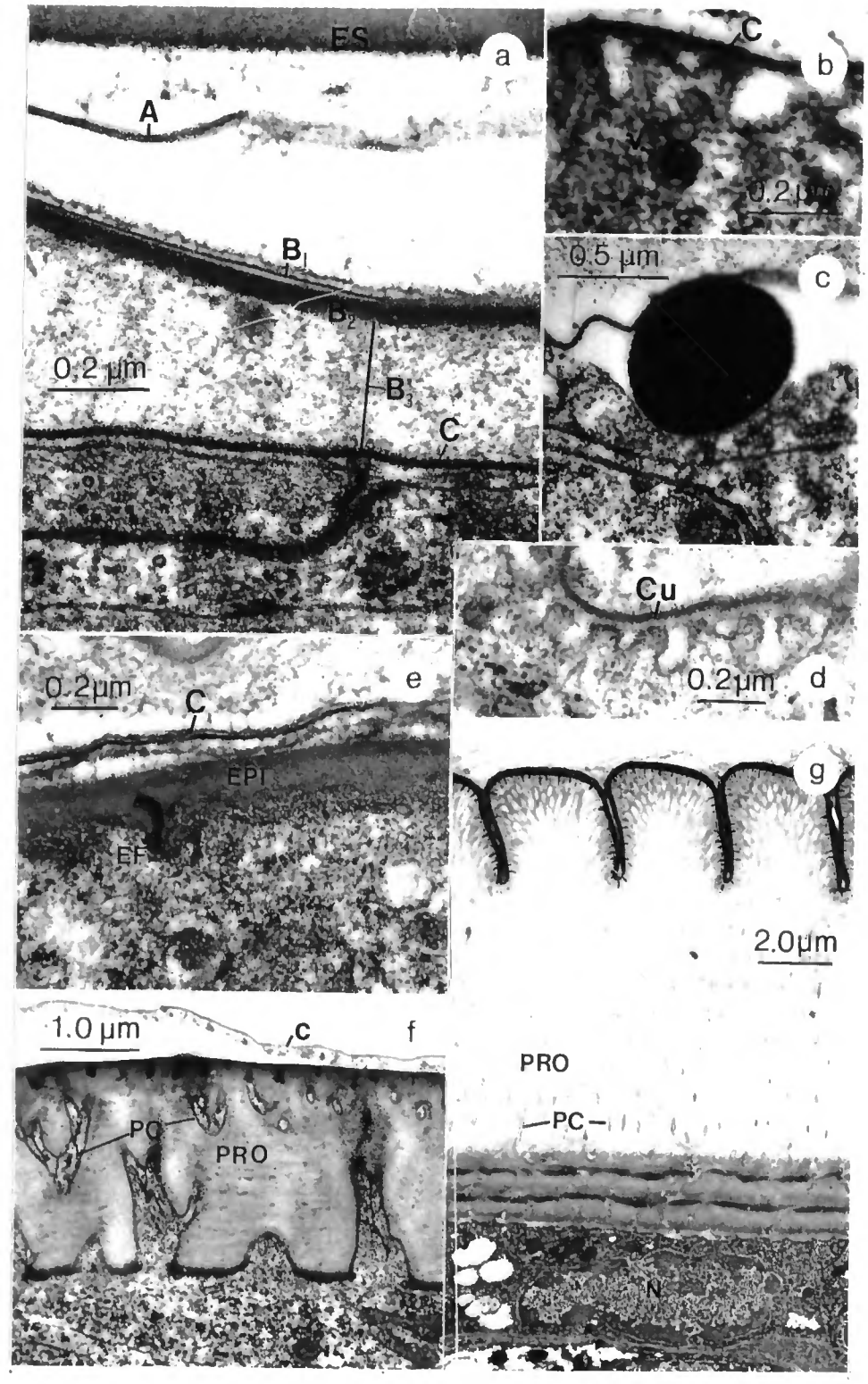

Figure 1: a) Embryo, 28 days after oviposition. "Cuticles" A, B and C are present. ES-eggshell, b) Embryo, 30 days after oviposition. Electron dense plaques are on microvilli just beneath layer C. v - coated vesicle, c) Embryo, 28 days after oviposition. An electron dense ball is located just beneath layer C, d) Embryo, 40 days after ovipostion. Cuticulin (Cu) is being deposited at the tips of microvilli. v - vesicle, e) Embryo, 46 days after oviposition. Epicuticle (EPI) is being deposited. C - embryonic cuticle C, EF - epicuticular filament, f) Embryo, 53-54 days after oviposition. Nonextensible cuticle of the scutum. C- embryonic cuticle C, PC - pore canal, PRO - procuticle, g) Larva. The cuticle is opisthosomal region is composed of epicuticle and amorphous and lamellate procuticle (PRO). PC -pore canals, N - nucleus

Liquid Scintillation Counting

We used an automatic liquid scintillation counter Kontron MR 300 DPM and Riatron scintillation cocktail (KONTRON, Zürich, Switzerland). In the case of HPLC fractions, results are expressed in noncorrected cpm since in our conditions, only a small difference of quenching existed between the different HPLC fractions.

RESULTS

Production of embryonic and larval cuticles

Observations of semi-thin and thin sections of various ages of *A. hebraeum* embryos demonstrate that three "embryonic cuticles" are formed before the deposition of the larval cuticle. These cuticles are labelled layers A, B and C following the terminology used in embryos of the tick *O. moubata* (Vogel 1975, Dotson *et al.* 1991). The first of these cuticles, layer A, forms on the 3rd to 4th day of development. This thin, electron dense layer is 10-12 nm thick and occasionally has a trilaminar appearance (Fig. 1a). The second more complex cuticle forms around the 8th day of development when the germinal disk appears. This cuticle consists of a thin outer layer (B_1, approx. 3 nm), separated from a thicker electron dense layer (B_2, 40 nm) by a thin electron translucent space, and an inner fibrous layer of varying thickness (B_3, 250nm-600nm) (Fig. 1a). Formation of layer C begins around the 28-30th day of development (Fig. 1a & b). This layer is composed of a thin electron dense layer which is deposited by electron dense plaques at the end of microvilli (Fig. 1b) and a fibrous inner layer is sometimes present (not shown). Electron dense balls appear to attach to the inner surface of the electron dense layer of C (Fig. 1c). They do not appear to be vesicles which add electron dense material to layer C; in fact, they are still present as distinct balls after larval cuticle formation is complete. Resorption or exuviation of these embryonic cuticles does not occur and timing of apolyses is difficult to observe because of the violent retraction of the egg shell when it is pierced during the fixation process.

Larval cuticle production begins around day 40 of embryonic development with the deposition of the first epicuticular layer, cuticulin, at the end of microvilli (Fig. 1d). Formation of the dense layer occurs after day 40 and, by the 46th day of development, the epicuticle is approximately 0.1 µm thick (Fig. 1e). Procuticle deposition follows. By the 54th day the scutal cuticle has increased in width to 2.0 µm (Fig. 1f) and when the larva hatches out, this type of cuticle will be approximately 5.0 µm thick and will not possess lamellae. No procuticle is deposited after hatching. The opisthosomal cuticle of the larvae is composed of a thin epicuticle and an inner and outer procuticle. In the opisthosomal region, epicuticular folds begin forming around the 40th day of development and are gradually filled with outer procuticle. These folds are generally 2-3 µm wide but can be as wide as 8.5 µm, and are from 0.6 to 3 µm deep. The outer procuticle below the folds is composed of 9-10 lamallae (ea. 0.4-0.8 µm) with pore canals dispersed throughout. The inner procuticle is deposited after hatching and is composed of 3-6 lamellae which are slightly thicker than those of the outer procuticle (Fig. 1g).

Ecdysteroid titer during embryonic development

When crude methanolic extracts of the various ages of embryos from 5 different series of *A. hebraeum* eggs were analyzed using RIA, no distinct peak of ecdysteroid immunoreactive material was detected (Fig. 2a). The amount of immunoreactive material appeared to remain between 50-200 pg equivalents of E per mg. However, a few samples around the 12th day of development (following the production of embryonic layer B and the germinal disk) and around the 44-46th day (following the beginning of larval epicuticle production) contained as much as 350 pg/mg and one sample contained 1100 pg/mg.

Extracts from *A. hebraeum* embryos 0, 10, 20, 30, 40 and 48 days old were analyzed for ecdysteroid immunoreactive material before and after hydrolysis with porcine liver esterase. In the extracts used, the RIA-positive material before hydrolysis decreased from 180 pg E/mg at oviposition to 110 pg 20 days later and remained about the same thereafter (Fig. 2b). Interestingly, esterase hydrolysis did not increase the RIA-positive material in the Day 0 and Day 10 samples but hydrolysis increased the ecdysteroid content in the Day 20 and older samples to approximately that of the Day 0 and 10 samples.

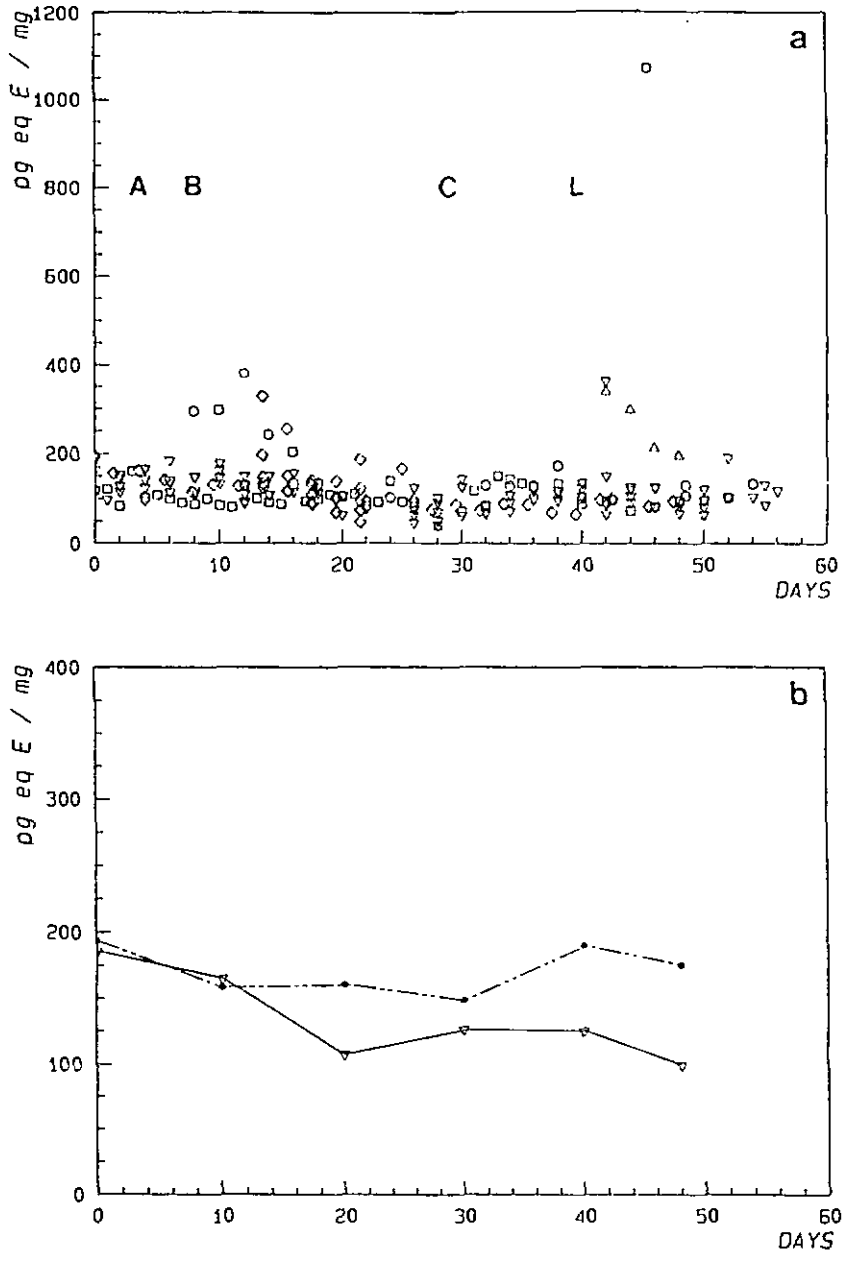

Figure 2: a) Ecdysteroid immunoreactive material in methanolic extracts of *A. hebraeum* eggs for every day or two days of development, the duration of which varied from 48-60 days. Each symbol represents a different series of egg extracts. A, B, and C indicates the time of deposition of the three embryonic "cuticles." L marks the beginning of larval epicuticle, b) A comparison of immunoreactive material present in egg extracts before (—▽—) and after (- -●- -) hydrolysis with porcine liver esterase.

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

When [^{3}H]-20E was injected into *A. hebraeum* females at the beginning of oviposition, approximately 76% of the radiolabelling was transferred to the eggs, 95% of which was found in its free form (Fig. 3a). The remaining 5% corresponded to a compound more polar than 20E, which had the same retention time as product 2 described in females of this species (Connat *et al.* 1987). In a preliminary experiment, we monitored the fate of the 20E and 2 every 15 days of development. At day 15 none of the 20E had been metabolized, whereas at day 30, all of it had been converted to apolar products AP1 and AP2. AP2 comigrated on reversed phase HPLC with esters of 20E (Fig. 3b) which have already been described in *O. moubata* nymphs (Diehl *et al.* 1985). The AP1 were more polar than AP2 and comigrated with the unidentified esterase labile

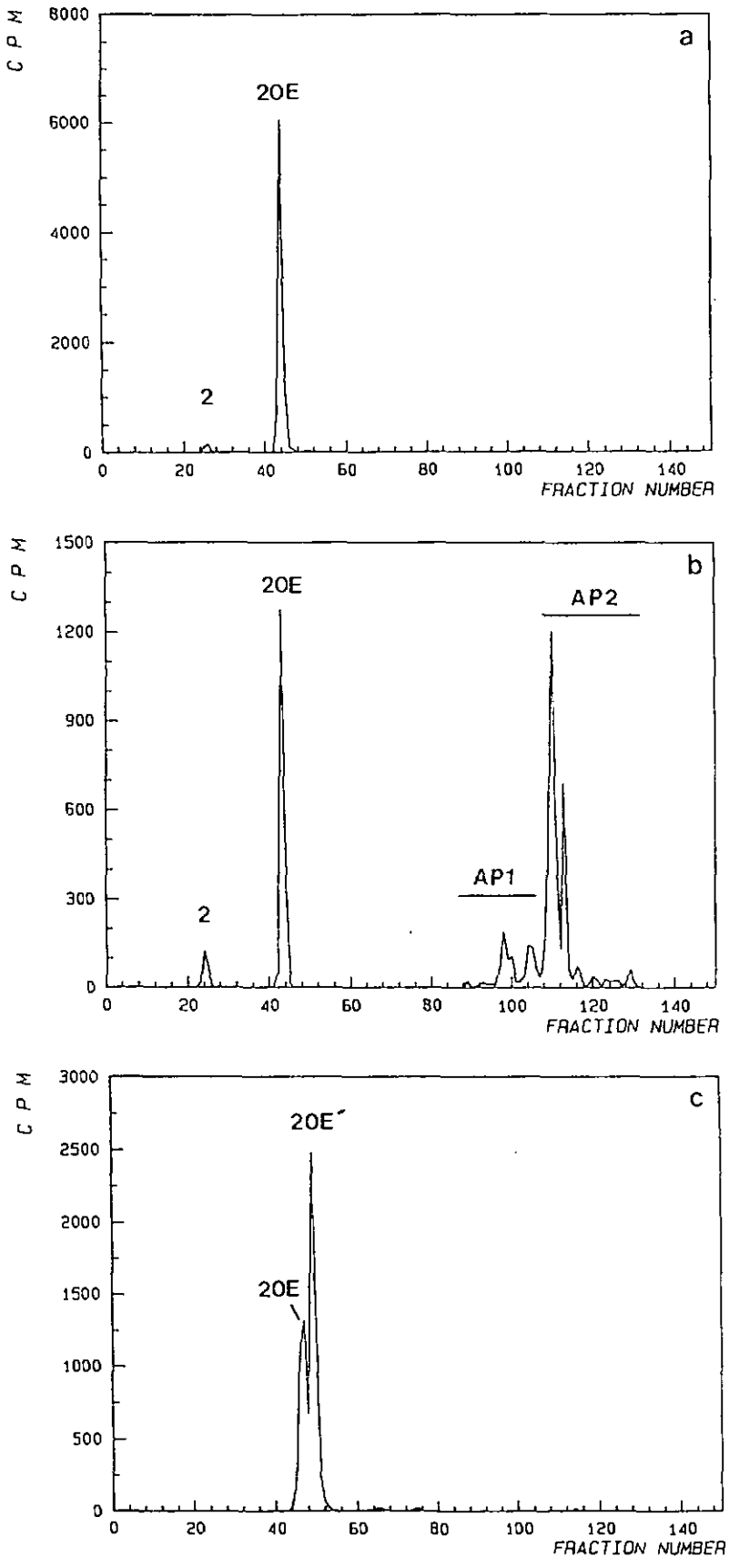

Figure 3: Reversed phase HPLC radiochromatograms of eggs from *A. hebraeum* females injected with [^{3}H]-20-hydroxyecdysone during vitellogenesis. 20E marks the retention of authentic 20-hydroxyecdysone. 2 is an unidentified polar product and AP1 and AP2 are apolar products with similar retention times as fatty acid ester conjugates. 20E' marks the retention of the 3αepimer of 20-hydroxyecdysone. a) freshly laid eggs, b) embryos, 20 days after oviposition, c) esterase hydrolysis of apolar metabolites from embryos, 20 days after oviposition.

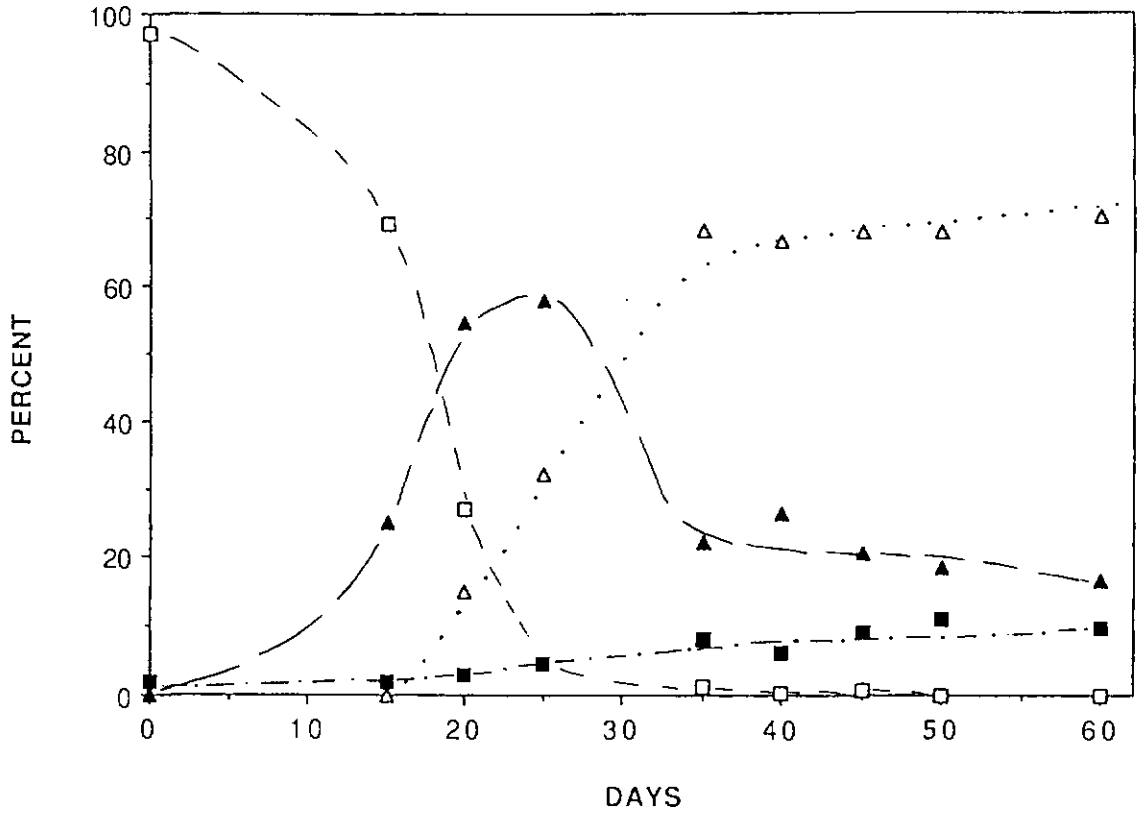

Figure 4: Metabolism of metabolites incorporated into the eggs after injection of [^{3}H]-20E. The results are expressed as present of total radioactivity in the sample. 20-hydroxyecdysone (- -□- -), polar product 2 (-·-■-·-), apolar products 2 (—▲—), apolar products1(· · △ · ·)

products described in *O. moubata* larvae, nymphs and females (Connat *et al.* 1988) and in *A. hebraeum* females (Connat *et al.* 1987). We then monitored the radiolabel every 5-10 days beginning with day 15 to determine when conjugation begins. Figure 4 summarizes the fate of the radioactive compounds in the embryos in this experiment. The 20E was completely metabolized between 15 and 35 days to AP1 and AP2. After 25 days of development, the AP2 decreased to represent about 20-25% of the radiolabel around day 35 and remained at that level until hatching. AP1 first appeared in the embryos after 20 days of development (Fig. 3b & 4) and gradually increased, as the AP2 decreased, to represent approximately 70-75% of the radiolabel in embryos 35 days old and older. The percentage of radiolabel corresponding to product 2 remained low in the first 40 days (3-6%) but increased to about 10% in the latter part of embryonic development.

Esterase hydrolysis of the AP1 and AP2 in the day 20 sample released two peaks of material: 35% of the released material comigrates with 20E and 63% comigrates with the 3α epimer of 20E (20E') (Fig. 3c). The ratio of 20E' to 20E remained the same in the 30 and 45 day embryos and the larvae.

Metabolism of [^{3}H]-ecdysone in the embryos.

When [^{3}H]-E was injected into engorged *A. hebraeum* females at the beginning of oviposition, a mixture of E, 20E, polar product 2' (and in some cases a small amount of polar product 2 which is probably the 20-hydroxy homologue of polar product 2') and apolar products AP1 and AP2 were found in the eggs (Table 1a). Percentages of these products varied with each day of oviposition. As oviposition progressed, the proportion of radiolabel corresponding to E decreased as the other products increased in the freshly laid eggs. The AP2 were found only in eggs which were produced after 4-6 days of oviposition. The fate of the radiolabelled compounds was monitored throughout embryonic development with reference to the percentages found in the corresponding freshly laid eggs (Table 1). By day 15, the E had been reduced to slightly more than half and the 2' had doubled. The amount of 20E had only decreased by 4%. The radioactive E was completely metabolized by day 30 (Fig. 6). The amount of radiolabel corresponding to 2' increased while the E was being metabolized. Because product 2' had a retention time of 5 min instead of 15 min when H_2O was used in the place of Tris buffer as the

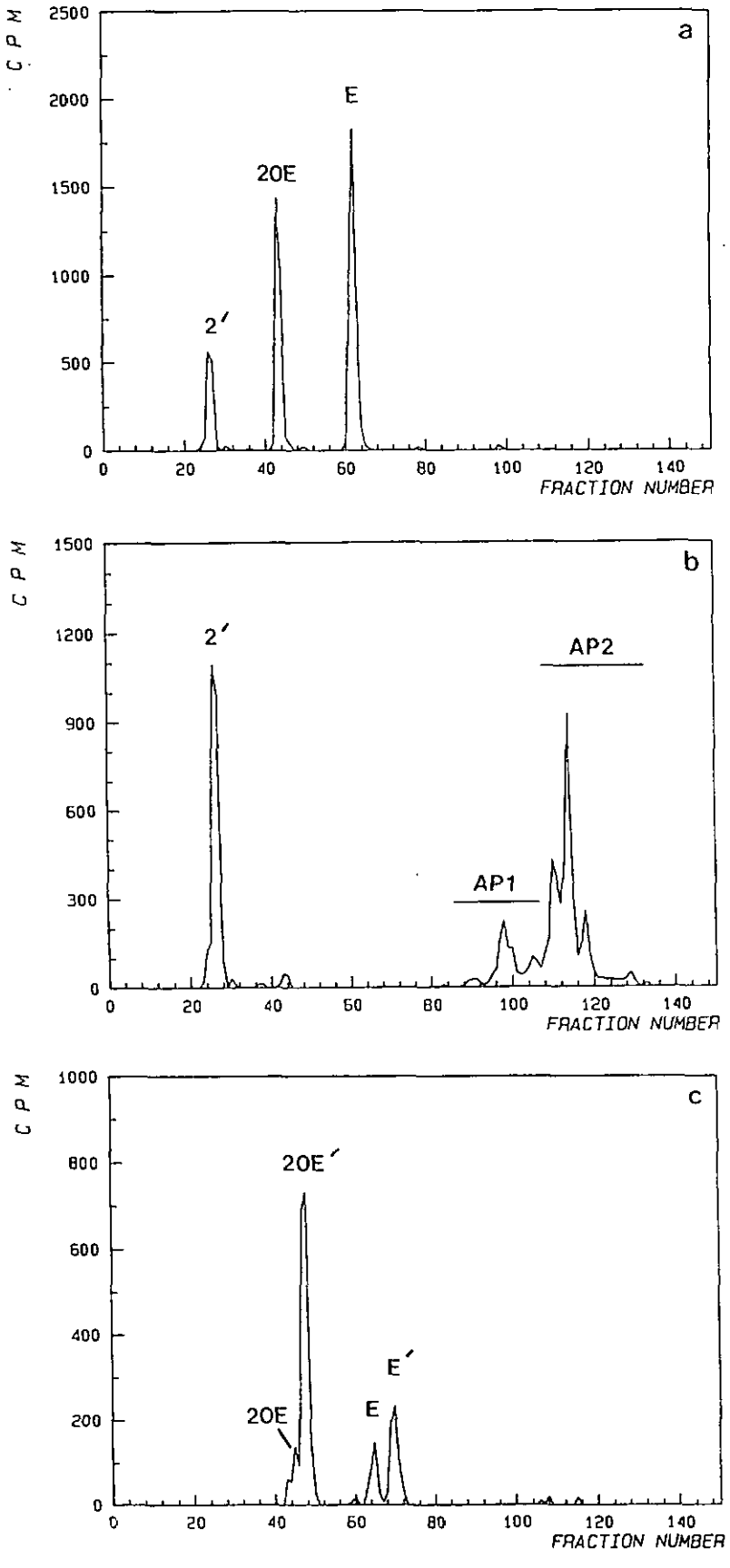

Figure 5: Reversed phase HPLC radiochromatograms of eggs from *A. hebraeum* females injected with [^{3}H]-ecdysone during vitellogenesis. E and 20E mark the retention of ecdysone and 20-hydroxyecdysone standards, respectively. 2' is an unidentified polar product and AP1 and AP2 are apolar products with similar retention times as fatty acid ester conjugates. E' and 20E' mark the retention time of the 3α epimers of E and 20E respectively. a) freshly laid eggs oviposited 2 days after injection, b) embryos, 25 days after oviposition, c) esterase hydrolysis of apolar metabolites from embryos 25 days after oviposition.

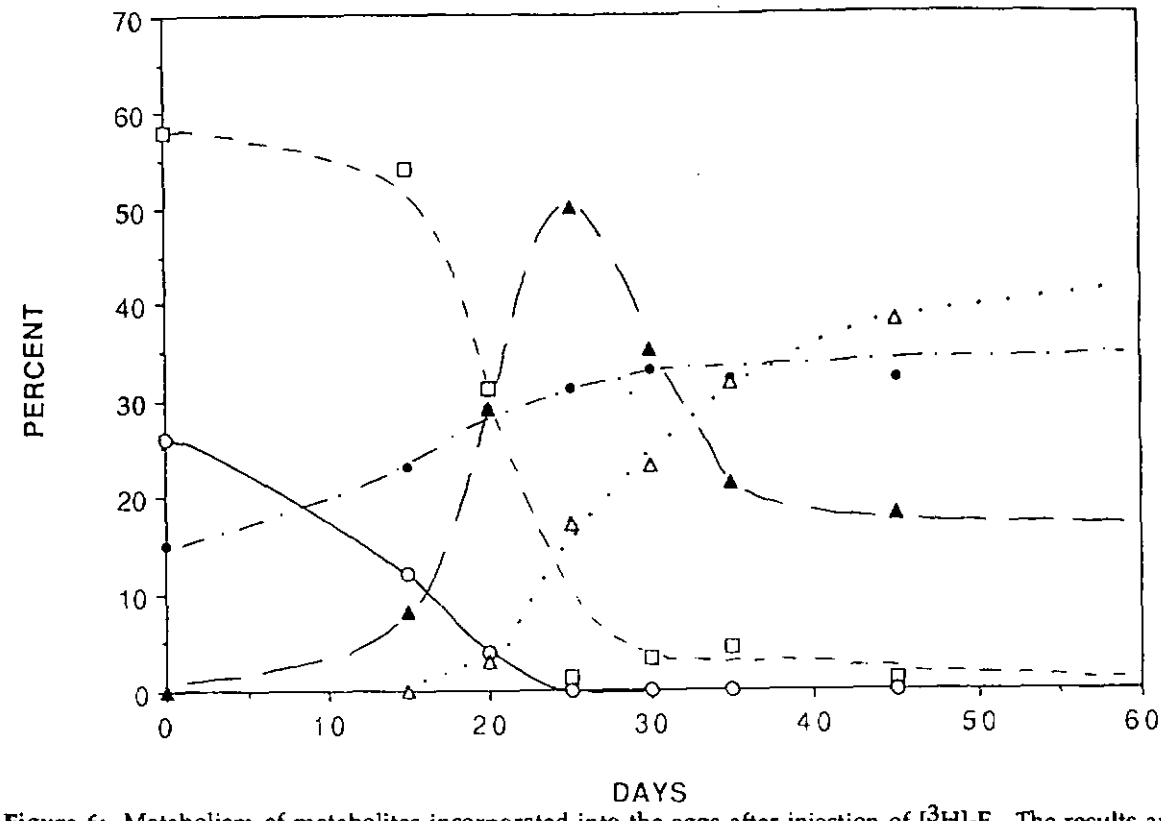

Figure 6: Metabolism of metabolites incorporated into the eggs after injection of [^{3}H]-E. The results are expressed as present of total radioactivity in the sample. Ecdysone (—○—), polar product 2' (- —●- -). For other metabolites see Figure 4.

elutant on HPLC (data not shown), it appeared to be ionic in nature. In addition, it was resistant to *Helix* and esterase hydrolysis.

Production of AP2 followed a similar pattern to that observed with the metabolism of [^{3}H]-20E (Fig. 6). The formation of these products was first observed in the 15 day cultures (8% of the radiolabel). AP2 increased to 50% of the radiolabel in the 25 day culture (Fig. 5b) and then decreased to represent only 18% of the radiolabel at the end of development (Fig. 6). AP1 were first observed in the 20 day culture (Table 1b). These products increased as the AP2 were decreasing and reached 38% of the metabolites in the 45 day embryos.

Esterase hydrolysis of the AP1 and AP2 released four peaks of material which corresponded to the incorporated E and 20E and to their 3α epimers, E' and 20E' (Fig. 5c). In the 25 day old embryos, E and 20E made up 11 and 9%, respectively, of the AP whereas E' and 20E' represented 20% and 58%, respectively. When calculated as a percentage of the total radioactivity of the sample, the 20-hydroxy compounds (20E and 20E') represented 45% and the E and E' together represented 21%. In comparison with the homologous freshly laid eggs (Table 1a), the E compounds appeared to have decreased from 48% to 21% of the radioactivity. Not only was the E epimerized and conjugated, it was also hydroxylated to form 20E compounds which increased from 34 to 45%. In addition, E was converted to polar product 2' which increased from 15.6 to 31% (Fig. 7).

DISCUSSION

Cuticle development

The three embryonic (A, B and C) cuticles produced by *A. hebraeum* embryos are very similar in thickness and structure to those produced in embryos of *O. moubata* (Vogel 1975, Dotson *et al.* 1991) and of *B. microplus* (Crosby *et al.* 1987 and Dotson unpublished results). The fact that these cuticles are found in tick species from both of the major tick families suggests that these cuticles may form in all tick embryos.

Whether all of these three embryonic cuticles are true cuticles is questionable. The thin electron dense layer A may not correspond to a cuticle. However, of the three produced, the second embryonic cuticle, B, is the most similar to cuticles found in immatures and adults. Its outer two layers and the electron dense layer of C may be remnants of an epicuticle and the inner layer of B and of C, a very diffuse procuticle.

Table 1. Ecdysone and its metabolites eggs and embryos from eggs oviposited days 2-4 after injection of [^{3}H]-E into vitellogenic *A. hebraeum* females, expresssed as percentages of the total radiolabel recovered in extracts.

Table 1a. Freshly laid eggs.

Time of oviposition (Days) after injection of [^{3}H]-E	PP2'	20E	E	AP1	AP2
2	15.5	34	48	–	–
3	15	58	26	–	–
4	23	53	15	6	1

Table 1b. Developing embryos[1]

Age of embryo (days)	Oviposition day after injection	PP2'	20E	E	AP1	AP2
15	3	23	54	12	–	8
20	3	29	31	4	3	29
25	2	31	1.3	–	17	50
30	3	33	3	–	23	35
35	4	39	4.3	–	32	21
45	4	38	1.1	–	38	18

[1] To determine the amount a compound has decreased or increased in the developing embryos, the percentages in this table must be compared with the percentages in the freshly laid eggs oviposited the same day after injection from Table 1a.

PP2'-polar product 2', 20E-20-hydroxyecdysone, E-ecdysone, AP1 and AP2-apolar products.

Formation of embryonic cuticles has been well documented in crustaceans and insects (see Dotson *et al.* 1991). Less is known about these embryonic membranes in arachnids. The "vitelline membrane" of a Lychosid spider resembles cuticle A (Kondo 1969). In the spider *Chiracanthium virescens*, the "embryonic" cuticles which form after the embryo breaks the egg membrane could correspond to those of the ticks (Canard 1987).

Our results show that the larval cuticle of *A. hebraeum* is composed of an epicuticle, an outer procuticle deposited before hatching and an inner procuticle that is deposited after hatching. The outer procuticle appears to correspond to the exocuticle that is deposited before ecdysis of other tick stages (Nathanson 1967, 1970; Beadles *et al.* 1973; Beadle 1974; Diehl *et al.* 1982; Stauffer and Connat 1990) and the inner procuticle corresponds to endocuticle deposited after ecdysis. No endocuticle was found beneath sclerotized exocuticle of the scutum of the larvae of *A. hebraeum*. This is the case in females and larvae of *B. microplus* and *B. decoloratus* (Beadle 1974, and Filshie unpublished results in Hackman 1982). In the opisthosomal region of *A. hebraeum*, nonsclerotized exocuticle and endocuticle are present (Fig. 1g) and both layers appear lamellate. This differs from the much studied *B. microplus* female (Hackman and and Filshie 1982) and the larvae of *B. decoloratus* and *B. microplus* (Beadle 1974) and *O. moubata* (Vogel 1975 and Dotson *et al.* 1991) where no lamellae are distinguishable in the exocuticle. Lamellae have been found in the exocuticle of *Ixodes ricinus* females (Lees 1952 cited in Hackman and Filshie 1982).

Ecdysteroid titers

In embryos (Dotson *et al.* 1991) and nymphs (Germond *et al.* 1982) of *O. moubata* and in nymphs of the ixodid ticks *A. hebraeum* (Diehl *et al.* 1982) and *A. variegatum* (Stauffer and Connat 1990) high ecdysteroid titers were temporally correlated with cuticle deposition. It is possible that they also are needed for stimulation of, at the least, the larval cuticle during embryonic development of *A. hebraeum*. In the embryos examined, larval epicuticle production begins around the 40th day. However, because of poor synchronization of development and the protocol using approximately 200 eggs or more, the combination of the high titers in embryos at the time of epicuticle production with low titers of embryos at a slightly different stage of development may have obscured any peak. However, the fact that a few samples assayed around the 44th day of development had titers as great as 350 pg suggests that such a peak exists.

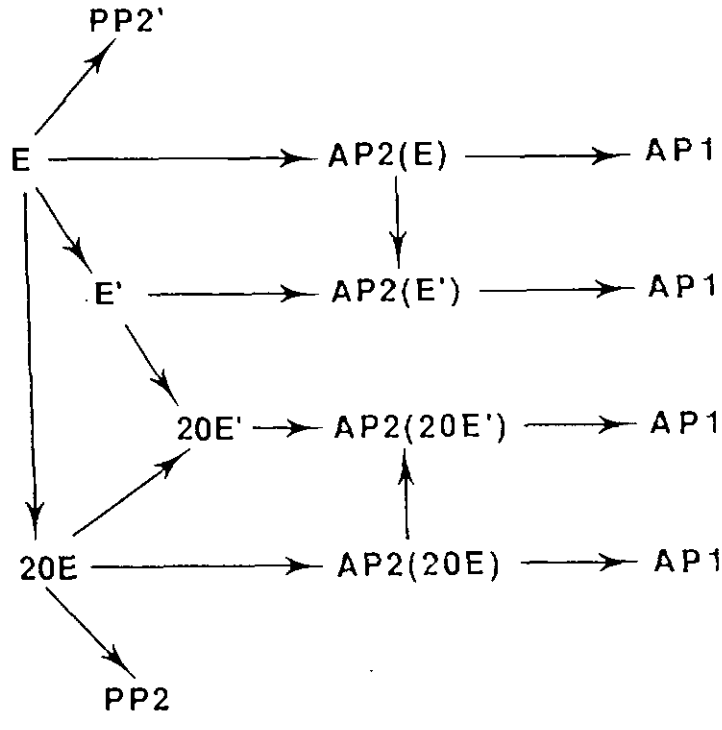

Figure 7: Possible metabolic pathways of [^{3}H]-ecdysone in *A. hebraeum* embryos.

Esterase hydrolysis of extracts from embryos of different stages never released more RIA positive material than those of the first day of development. This is unlike *O. moubata* embryos and larvae where at the appearance of each ecdysteroid peak, an increase in the RIA-positive material releasable by esterase hydrolysis was noted (Dotson *et al.* 1991). In *A. hebraeum* embryos endogenous ecdysteroids are probably conjugated to esters of ecdysteroids between 10 and 20 days of development. The metabolism of tritiated E and 20E in the embryos to form AP1 and AP2 corroborates this idea.

The role of free endogenous ecdysteroids

Previous studies with radioimmunoassay revealed that high titers of 20E and some E are incorporated into the eggs of *A. hebraeum* (Connat *et al.* 1985). The role of these maternal hormones in embryogenesis is unknown. Several experiments with insect embryos have shown that maternal ecdysteroids are necessary for early development. In the posterior pole of the *Locusta* oocyte where the nucleus is located, the ecdysone titers increase twice, at the first and second meiotic reinitiation (Lanot *et al.* 1989b). This role of ecdysone has also been demonstrated in *Periplaneta americana* and *Gryllus bimaculatus* (Lanot *et al.* 1989a). Furthermore, studies on *Manduca sexta* demonstrated that cultured germ bands separated from the yolk will not undergo elongation or segmentation; however, the addition of ecdysteroid laden yolk, or free ecdysteroids (E, 20E or Makisterone A) to the cultures stimulates these processes (Dübendorfer 1989). In two mutant strains of *Drosophila*, a decrease in the production of ovarian ecdysteroids occurs when the females are kept at a certain restrictive temperature and the eggs produced fail to develop (see Dübendorfer 1989). The oocytes of *Locusta* females reared on fenpropimorph-treated wheat have a reduced ecdysteroid titer (by as much as 80%) and will not undergo meiotic reinitiation *in vivo*. *In vitro* ecdysone applied to these oocytes dissected from the females will stimulate this process in 50% of the oocytes (Lanot *et al.* 1989b). It would be interesting to see if the development of *A. hebraeum* embryos could be affected by decreasing the amount of ecdysteroids incorporated into the eggs.

In the tick *O. moubata*, the apolar conjugates incorporated into the eggs do not appear to be used by the embryo (Connat *et al.* 1988). The free ecdysteroids incorporated into *A. hebraeum* eggs appear to be completely inactivated by the 25-30th day of development. They may be involved in events during oocyte maturation or early embryogenesis. Diehl *et al.* (1985) suggested that the ecdysteroids may play a role in eggshell production by the oocyte, a process

that resembles cuticle production (Aeschlimann and Hecker 1969). They may be involved in the production of A and B which are produced before the free hormones are metabolized. Unfortunately, the poor synchronization of development and the large number of embryos used for each experiment do not allow us to conclude whether or not [^{3}H]-E or [^{3}H]-20E are released from AP to stimulate larval cuticle production or other developmental processses. It is also possible that they act as feeding deterrent to predators in a way similar to the allochemicals found in the eggs of certain plant feeding insects (Connat *et al.* 1987).

Ecdysteroid metabolites

A. hebraeum embryos E and 20E appear to use similar pathways as the adult to metabolize ecdysteroids. The polar pathway leads to the still unknown polar products 2 and 2'. They appear to be esterase and *Helix* resistant, which indicates that they are probably not conjugates. Because polar product 2 is incorporated into the eggs when [^{3}H]-20E is injected into ovipositing females, and polar product 2' is incorporated when [^{3}H]-E is injected, polar product 2 may be the 20-hydroxy homologue of polar product 2'. Interestingly, the product 2 increases only slightly during embryonic development whereas product 2' increases to almost 40 % of the radiolabel in the eggs. The increase appears to be due to a conversion of [^{3}H]-E.

The apolar pathway leads to AP1 via the production of AP2. The AP2 found in *A. hebraeum* appear to be the same as those described in *O. moubata* nymphs (Diehl *et al.* 1985) and in *B. microplus* females (Crosby *et al.* 1986) namely, long chain fatty acid esters conjugated to the C-22 position.

The most interesting phenomenon noted in this study was the release of 3αepimers of E and 20E by esterase hydrolysis of the apolar products. However, no free epimers are observed during development. This suggests either that the epimers are formed after conjugation or that once they are formed they are quickly conjugated. The fact that the relative proportions of the α-epimers to the 3-β form of the ecdysteroids did not change after day 30 suggests that, after day 30, the conjugated ecdysteroids are sequestered to a place where epimerization cannot take place or that the epimerization does not take place after conjugation. Although epimerization probably does not begin until around day 15, homogenates of all stages of *A. hebraeum* embryos (even frozen eggs) are able to convert E to E' via the formation of 3-dehydroecdysone (Dotson, Connat and Lafont, unpublished results).

Epimers of E and 20E were found in conjugated form in *A. hebraeum* females at the end of oviposition (Connat *et al.* 1987) and are released in the nonconjugated form into the media when *A. hebraeum* ovaries are cultured (Connat *et al.* 1986). These compounds have also been found in a few Lepidopteran and Orthopteran species (see reviews by Koolman and Lafont 1984, Koolman and Karlson 1985) and most likely, they represent inactivation metabolites (Lafont and Connat 1989). The E and 20E incorporated into the freshly laid eggs of *A. hebraeum* appear to be doubly inactivated during embryonic development.

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VI

In vitro Metabolism of [^{3}H]-Ecdysone in Embryos of the Ixodid Tick *Amblyomma hebraeum*: the Presence of 3-Dehydroecdysone and 3-Epiecdysone

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ABSTRACT

We investigated the metabolism of [^{3}H]-ecdysone *in vitro* by using cultures of homogenized or broken open *Amblyomma hebraeum* embryos of various stages of development. All stages studied converted ecdysone to 3-epiecdysone, presumably via the formation of 3-dehydroecdysone. This latter metabolite was identified with CI/D mass spectrometry. Other unidentified products were also produced. In addition, a product comigrating with 20-hydroyxecdysone on RP-HPLC was detected in cultures of broken open embryos on the 40th day of development. The epimerization corroborates the *in vivo* formation of epimers in embyros of this tick species (Dotson *et al.* submitted).

* The data presented in this study is a part of this author's doctoral thesis at the Université de Neuchâtel.

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INTRODUCTION

The first described 3-epi-ecdysteroid (3-epi-ES) was 3-epi-20-hydroxyecdysone (20E') from the meconium of *Manduca sexta* pupae (Thompson *et al.* 1974). Since that time several 3-epi-ES have been described in a few Lepidopteran and Orthopteran species (see reviews by Lafont and Koolman 1984, Koolman and Karlson 1985, Lafont and Connat 1989). 3-Epi-ES have been found as free ES in the eggs of *Galleria mellonella* (Hsiao and Hsiao 1979), *M. sexta* (Lafont and Koolman 1984) and *Schistocerca gregaria* (Isaac *et al.* 1981) and as phosphate esters in *Locusta migratoria* (Tsoupras *et al.* 1982). *In vitro* studies of various insect tissues have shown that the formation of 3-epi-ES takes place through the production of 3-dehydroecdysteroids (see Lafont and Koolman 1984, Koolman and Karlson 1985, Lafont and Connat 1989); however, the only 3-epi-ES isolated *in vivo* is 3-dehydroecdysone (3DE) which was isolated after long term [^{14}C]-cholesterol incubations in *Pieris* (Blais and Lafont 1984). The enzymes for this(ese) reaction(s) appear to be present in the midgut cytosol of several insect species and the properties of these enzymes have been studied extensively (see reviews by Weirich 1989, Weirich *et al.* 1989, 1991). Epimerization at C-3 is an irreversible reaction in several insect species and is thought to be an inactivation process (Lafont and Connat 1989).

In vitro studies of the metabolism of [^{3}H]-ecdysone ([^{3}H]-E) in isolated tissues of the tick *Amblyomma hebraeum* revealed that, in addition to hydroxylating ecdysone (E) to 20-hydroxyecdysone (20E) and conjugating these compounds to form AP, the ovary and carcass were capable of converting the E to 3-epiecdysone (E') via the conversion to 3DE (Connat *et al.* 1986). Large quantities of 3DE and E' were isolated from the medium of ovary cultures and these compounds were identified by CI/D mass spectrometry. *In vivo*, E' and 20E' were found as apolar ester conjugates at the end of oviposition in whole body extracts of *A. hebraeum* females that had been injected with tritiated ecdysteroids before or at the beginning of oviposition (Connat *et al.* 1987). In addition, they have also been isolated in conjugated form in developing *A. hebraeum* embryos (Dotson *et al.* submitted).

We undertook this study of *in vitro* metabolism of [^{3}H]-E throughout embryonic development of *A. hebraeum* to determine 1) whether these life stages are able to convert E to E', to hydroxylate E to 20E and to form apolar conjugates and 2) whether qualitative or quantitative modification of the metabolites produced occurs throughout these developmental stages.

MATERIALS AND METHODS

Animals

Fully engorged *A. hebraeum* females which had fed on young bovines were provided one or two days postdrop by CRA Ciba-Geigy, St. Aubin (FR) Switzerland. Ten to 15 days later the females began to oviposit (ovipositional period lasts from 20 to 30 days). The females were allowed to lay eggs in plastic vials and every day or every two days the females were removed, leaving the eggs to minimize disruption of development. When collecting eggs for cultures, abnormal or dried eggs were carefully removed. The females and eggs were kept above a saturated K_2SO_4 solution (RH 97%) at 28°C and in total darkness.

Chemicals

Ecdysone (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one) and 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one) were purchased from Simes (Milan, Italy). Labelled ecdysone ([23,24^{3}H]-ecdysone ([^{3}H]-E)) (sp. act. 53.6 Ci/mmol) was obtained from New England Nuclear Corporation (Boston MA, USA) and when necessary was purified with high performance liquid chromatography (HPLC).

Cultures

80 Milligrams of embryos from each stage cultured (0, 10, 15, 20, 30, 40) or larvae were ground in 400 µl of TC199 adjusted to 390 mOsmol with NaCl and containing 500 U penicillin and 500 µg streptomycin per ml. 100 Microliters of the homogenate was placed in each of the 4 receptacles of a sterile Nunclon culture dish. The homogenizer was rinsed with 400 µl TC199, which was then distributed among the 4 cultures and 100,000 cpm [^{3}H]-E in 50 µl of TC199 was added to each. In one of the cultures, an additional 50 µl of TC199 containing 18 µg cold ecdysone/ml was added to give a final concentration of 3 µg/ml which is the approximate ES titer in freshly laid eggs. The surcharged culture was incubated for 48 hrs and the other three for 6, 24 and 48 hrs. Thus 20 mg of embryos were cultured in 300 µl of TC199 at 27°C at different incubation times but all represented the same pool of embryos.

We also cultured "whole" embryos of which the egg shell had been broken open. 20 Milligrams of each stage investigated were cultured for 48 hrs at 27°C in 300 µl TC199 containing 100,000 cpm [^{3}H]-E, either with or without 3 µg cold E per ml of medium.

Extractions

Each of the cultures was extracted twice in 1.0 ml methanol, dried under a N_2 flow, resuspended in 200 µl methanol and vortexed. Then, 400 µl of Tris buffer (pH 7.5, 20 mM) were added and the samples were vortexed again. Finally, cold E and 20E standards were added to the sample before injection on reversed phase HPLC.

High performance liquid chromatography (HPLC)

HPLC analyses were done with a Perkin Elmer Series 3 Chromatograph or a Kontron system with two 420 pumps piloted by an IBM-PC computer with a variable wave-length spectrophotometer set at 242 nm. The samples were separated on a Merck Lichrosorb RP-18 (7 µm phase) column (25 cm x 4 mm) with a precolumn (4 cm x 4 mm) packed with Merck RP-8 40 µm phase. A solvent gradient with methanol and Tris buffer pH7.5 (20mM) was generally used: 30% methanol to 45% (10 min), isocratic 45% (15 min), 45% to 100% (20 min), purge 100% methanol (20 min).

Normal phase chromatography was done using a Perkin-Elmer Silica A column with chloroform/isopropanol/H_20 (100:25:1.25) as the eluant. The flow rate was 1 ml/min and 30 second fractions were collected for the first 20 minutes and one minute fractions were collected thereafter.

Liquid Scintillation Counting

We used an automatic liquid scintillation counter Kontron MR 300 DPM and Riatron scintillation cocktail. Results are expressed in noncorrected cpm since only small differences of quenching existed among the different HPLC fractions.

Preparation of samples for mass spectrometry

To produce large quantities of metabolites, 5 grams of frozen newly laid eggs of *A. hebraeum* were ground in 100 ml TC199 medium (adjusted to 390 mOsmol with NaCl) containing 500 U penicillin and 500 µg streptomycin per ml, 500 µg cold E and 2 x 10^6 cpm [^{3}H]-E. The homogenate was incubated in a water bath held at 30°C for 48 hrs. To oxygenate the culture, air was bubbled through the medium. After incubation, the homogenate was placed in centrifuge tubes. The incubation beaker was rinsed with 20 ml methanol and this was added to the homogenate. The homogenate was centrifuged for 10 min at 10,000*g*. The supernatant was passed on three SEP PAK C-18 cartridges (Waters). Each SEP PAK was rinsed with 10 ml H_2O to remove the salts and the metabolites were removed with 10 ml 100% methanol. The metabolites were further purified with a Zorbax ODS preparative column (25 cm, i.d. 9.4 mm) in a gradient of acetonitrile in 1% trifluoroacetic acid (TFA) with a flow rate of 4 ml/min. Fractions corresponding to UV peaks were collected separately. These fractions were further purified on a semi-preparative Zorbax-SIL column (25 cm, i.d. 9.4 mm) with methylene chloride/isopropanol/H_2O (125:20:1.50). The flow rate was 2 ml/min.

Mass spectrometry

A Riber 10-10B spectrometer (Nermag, France) was used to analyze the samples after direct probe introduction. Spectra were recorded using a chemical ionization/desorption (CI/D) procedure with ammonia as the reagent gas (Lafont *et al.* 1981).

RESULTS

Metabolism of [^{3}H]-ecdysone in egg homogenates

All homogenates of *A. hebraeum* embryos, including those with a surcharge of E and frozen eggs, were able to metabolize the [^{3}H]-E (Table 1, Fig. 1). Most of the metabolites were less polar than ecdysone. The major metabolite produced was a product which comigrated on reversed and normal phase HPLC with the 3α epimer of E (designated E'). Other less polar metabolites consisted of 1) a product which comigrated with 3-dehydroecdysone (3DE), 2) a group of products less polar than 3DE which sometimes formed 2-3 peaks and are labelled Y_{1-3} (Table 1, Fig. 1) and 3) a single peak of material found in fractions 106-109, designated by the letter Z. In some cases, some radiolabelled products less polar than Z were also found. Z and these apolar products may be apolar esters. However, Z has retention times similar to 22,25-dideoxyecdysone or 2,22,26-trideoxyecdysone. The products more polar than ecdysone are designated by the letters A, B, C, D and F (E corresponds to ecdysone itself.). The exact nature of these products is unknown. However, B comigrates with 20E or 20E'. The percentage of radioactivity in the combined polar products may be as high as 10% but is generally lower than 5%.

The metabolic rate was slowest in the freshly laid eggs (half-life of the hormone 21.7 hrs) with 35% of the E unmetabolized in the 48 hr culture. The metabolic rate increased in the 10 and 15 day cultures (half-lives of 15 hrs and 2.5 hrs) and by day 20, approximately 95% of the E was metabolized within the first 6 hrs. Interestingly, in all of the cultures surcharged with cold E, even that of the freshly laid eggs, more than 95% of the [^{3}H]-E was metabolized.

In the large culture containing 5 grams of frozen *A. hebraeum* eggs, E' production was very low, and the CD/I spectrum of this compound was not obtained. The intermediate metabolite, 3DE, however, was the major metabolite produced. The CD/I spectrum of this compound showed ion m/e 480 ($M + H^+ + NH_3$), 463 ($M + H^+$), 445 ($M + H^+ - H_2O$) and 427 ($M + H^+ - 2\ H_2O$) which is typical for 3DE.

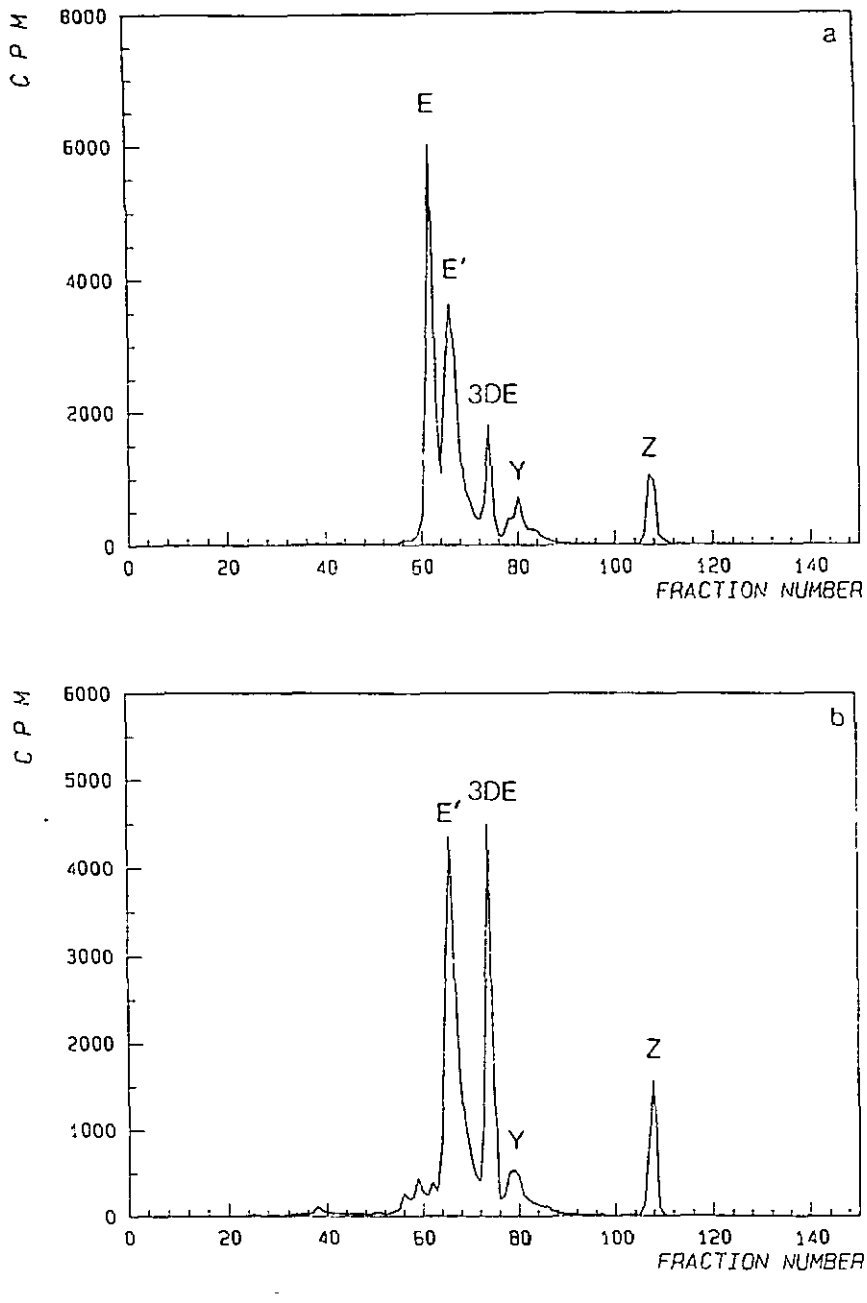

Figure 1: RP-18 HPLC radiochromatograms of extracts of homogenate cultures containing 20 mg of freshly laid *A. hebraeum* eggs incubated in TC199 containing [^{3}H]-ecdysone. **E** - retention times of cold ecdysone, **E'**-3-epiecdysone, **3DE**-3-dehydroecdysone, **Y** and **Z** - unidentified apolar products, (a) 48 h culture of day 0 embryos, (b) 48 h culture of day 0 embryos with a surcharge of 3 ng/ml of cold E

Metabolism of [^{3}H]-ecdysone in broken open embryos

Because homogenization of the tissue destroyed tissues and cells, we decided to break open the eggs using forceps in a method similar to that used with eggs of *O. moubata* (Dotson *et al.* in press). The two stages (eggs 0 and 40 days old) cultured for 48 hrs, with and without 3 ng/ml surcharge of cold ecdysone, were able to metabolize the [^{3}H]-E (Table 1). The metabolites appeared to be the same as those produced in homogenate cultures. However, in the freshly laid eggs of this experiment, the Y compounds appeared to be the major metabolites, comprising 15% of the radiolabel. E' and 3DE corresponded to 7.4% and 7.7%, respectively. As with the homogenate cultures, those having an added concentration of cold E metabolized the [^{3}H]-E more quickly.

Table 1. Percentages of recovered radiolabel corresponding to [^{3}H]-E or metabolites produced in cultures of *A. hebraeum* egg homogenates or broken open eggs.

Age (Days)	Hrs	A	B	C	D	F	E	E'	3DE	Y_1	Y_2	Y_3	Z_1	Z_2	Total
0	6	--	--	--	0.5	5.0	80.7	3.4	0.8	6.8	1.4	--	0.2		98.8
	24	1.0	--	1.2	2.8	1.6	47.8	19.6	6.4	7.2	6.7	--	2.5	--	96.8
	48	--	--	--	--	--	35.8	34.1	9.3	7.4	2.5	2.3	6.6	--	98.0
	[1]E48		--	--	--	4.7	2.8	44.8	25.1	7.7			8.6	--	93.7
	[2](f)E48	0.9	--	--	--	4.6	1.8	33.2	28.8	15.6	5.3	--	5.6	--	95.8
10	6	--	--	--	0.4	1.0	68.1	23.8	1.3	3.2	--	-	0.5	--	98.3
	24	0.7	--	0.6	0.7	2.2	40.1	25.8	5.6	10.7	1.5	--	4.7	1.0	93.6
	48	--	--	--	--	0.9	9.5	55.1	10.7	13.6		--	4.0	0.9	94.7
	[1]E48	--	--	--	--	2.0	3.6	43.5	18.5	15.2	4.7	--	5.0	--	92.5
15	6	0.4	--	--	1.5	0.7	29.2	48.5	2.6	4.6	2.7	--	4.4	2.7	97.3
	24	1.0	0.5	0.9	2.8	2.7	10.9	42.6	6.9	12.7	4.0	6.1	4.4	3.6	99.1
	48	--	--	--	1.4	2.0	1.5	40.7	9.7	17.2	11.1	--	4.5	3.9	92.0
	[1]E48	--	--	--	--	1.9	2.4	54.8	17.6	7.4	3.1	--	5.2	1.2	93.6
20	6	1.9	1.7	1.0	3.8	5.7	5.8	39.8	5.5	17.4	8.8	0.8	2.3	2.7	97.2
	24	2.5	3.0	1.4	4.6	4.5	3.5	43.9	8.1	15.1	5.7	0.9	1.6		94.7
	48	--	--	3.6	1.2	2.0	2.7	43.5	8.2	15.2	7.3	2.9	3.9	3.4	93.9
	[1]E48	--	--	--	--	1.7	2.8	47.9	15.8	16.2		--	2.9	--	87.3
30	6	0.8	--	1.3	1.4	1.3	--	82.4	--	6.0	4.0	--	--	--	97.2
	24	1.4	1.7	1.4	3.3	2.5	--	62.0	4.7	10.6	4.0	--	1.7	--	93.3
	48	--	1.1	1.6	--	1.7	1.5	74.6	6.6	3.4	4.9	--	--	--	95.4
	[1]E48	--	--	--	--	2.9	1.7	69.7	10.4	4.9	4.2	--	1.3	--	95.1
40	6	2.2	0.4	--	6.1	5.6	2.2	60.7		10.8	4.1	--	1.5	0.9	94.5
	24	4.5	--	4.5	6.4	5.1	5.8	18.7	7.6	11.3	4.1	--	3.4	--	71.4
	48		3.9		1.9	2.7	2.5	27.9	4.9	>10	6.0	5.4	16.6	--	81.7
	[1]E48	--	--	--	--	5.2	3.1	52.8	7.8	16.8		--	0.8	-	86.5
L	6	1.7	--	1.0	6.5	3.8	4.8	53.4	--	>10	6.7	0.9	3.1	3.7	95.6
	24	--	--	--	2.6	8.8	3.5	38.6	5.0	21.5	2.9	1.6	2.5	1.5	88.5
	48	--	--	--	--	4.0	3.6	66.1	3.4	10.1	1.0	--	2.2	2.2	92.3
	[1]E48	--	--	--	5.5	5.7	7.8	37.7	4.8	17.9	2.4	--	4.2	--	86.0
O-B[3]	48	2.2	--	1.2	4.7	3.1	41.2	7.4	7.6	15.6	6.5	--	6.4	--	95.9
	[1]E48	5.1	--	3.6	5.6	5.6	7.0	8.3	15.8	17.4	11.0	--	11.1	--	90.5
40-B[3]	48	--	8.6	3.7	3.9	5.6	15.8	16.4	7.3	13.4	6.4		1.5		88.2
	[1]E48														
Days	Hrs	A	B	C	D	F	E	E'	3DE	Y_1	Y_2	Y_3	Z_1	Z_2	Total

1- A surchage of cold ecdysone (3 mg/ml) was added to culture

2- Frozen eggs were homogenized for this culture

3- Broken open embryos were cultured instead of homogenates

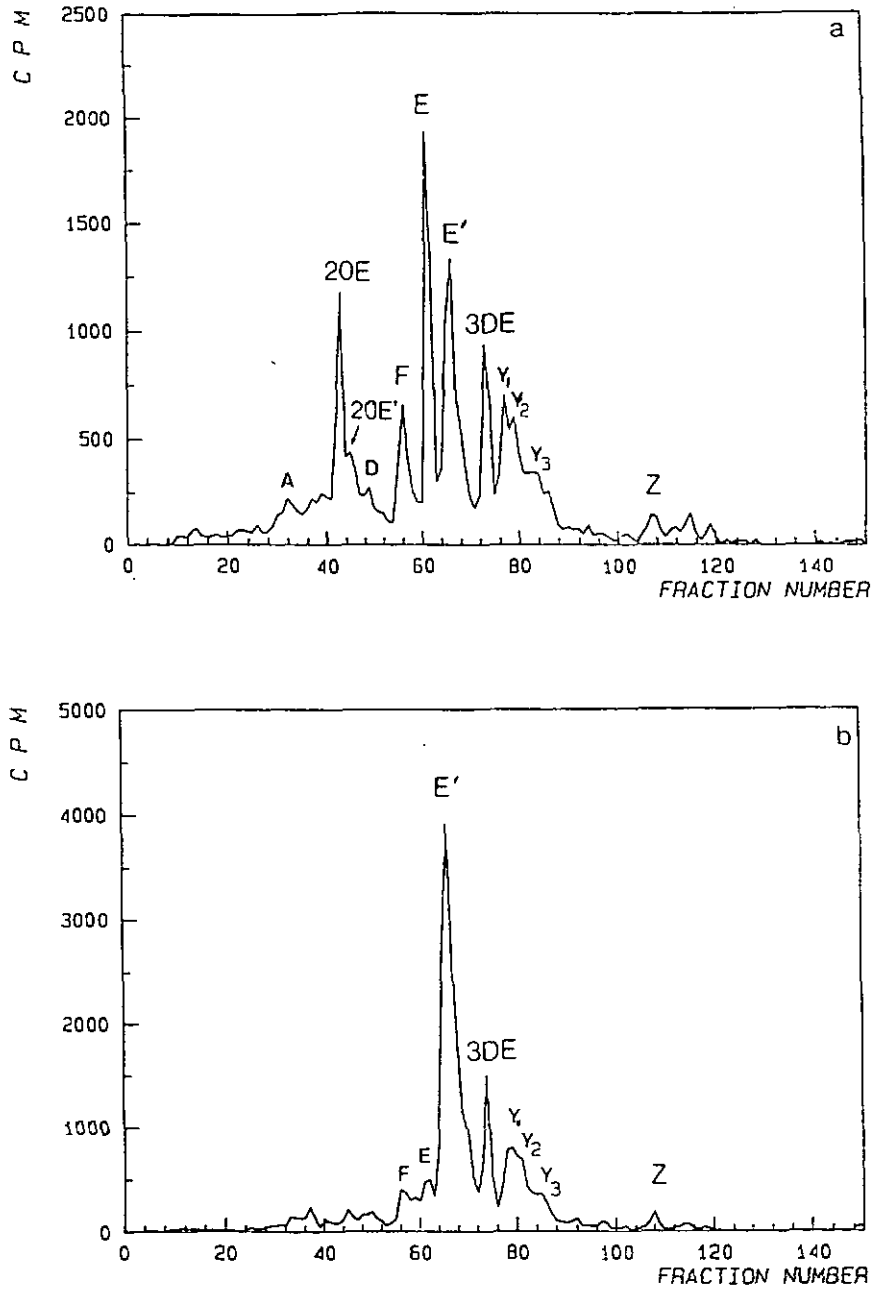

Figure 2: RP-18 HPLC radiochromatograms of extracts of cultures of 20 mg of broken open 40 day *A. hebraeum* embryos incubated in TC199 containing [^{3}H]-ecdysone. E and 20E - retention times of cold ecdysone and 20-hydroxyecdysone, respectively, E'-3-epiecdysone, 3DE - 3-dehydroecdysone, Y and Z - unidentified apolar products, A, D & F - unidentified polar products, (a) 48 h culture of day 40 broken open embryos, (b) 48 h culture of day 40 broken open embryos with a surcharge of 3 ng / ml of cold E

In the 40 day culture without E, only 15.8% of the label comigrated with E. The most interesting product in this culture is one that comigrates with 20E (8.6%) and 20E' (3.7%) (Fig. 2a). Because of its retention time, D may correspond to 3D20E (3.94 %) and F may be a 20E form of Y which may be an isomer of 3D20E. In the surcharged culture no 20E appeared to be produced. Most of the E was metabolized to E' (Fig. 2b).

DISCUSSION

All stages of developing *A. hebraeum* embryos appear to possess the enzymes necessary for transforming ecdysone E to E'. The presence of 3DE in the cultures suggests that

the formation of E' takes place via the oxidation of E to form 3DE which is then reduced to form E'. This is similar to the process of epimer formation in insects (Weirich 1989).

The rate at which E is metabolized appears to increase as the embryos aged from 0 to 15 days. This suggests that the embryos were producing more of these enzymes as they developed. In older embryos it was not possible to determine if an increase occurred because most of the E was metabolized within 6 hrs. Interestingly, it appeared that by increasing the amount of E in the culture with the addition of cold E, the rate of the reaction increased. One explanation for this may be that the enzyme is allosteric and ecdysone may have a positive homotropic effect on the enzyme (Dixon *et al.* 1979). However, detailed kinetic studies need to be done before statements can be made about the nature of this(ese) enzyme(s).

Unlike the *in vitro* studies of *O. moubata* embryos (Dotson *et al.* 1993), we were not able to make correlations between the qualitative or quantitative metabolism of E and the developmental stages. The most abundant metabolite formed in almost all stages was E'. Homogenizing the tissue may have destroyed some enzyme systems. The 40 day old broken open embryos produced some 20E, but the homogenized 40 day old embryos in the same conditions did not appear to hydroxylate E to form 20E. However, homogenates of *O. moubata* embryos were able to hydroxylate E to form 20E (Dotson unpublished). In addition, another enzyme system that appears to be missing or inhibited in *A. hebraeum* embryo cultures is that which conjugates ecdysteroids to form apolar esters. Formation of [^{3}H]-ecdysteroid esters *in vivo* began around 15 days of development (Dotson *et al.* submitted). However, no or very small amounts of apolar products with the same retention times as the apolar ester conjugates were observed in the *A. hebraeum* cultures, and it is not known if they are esters of E. The production of apolar esters is very prominent in cultures of *O. moubata* embryos and has been observed in homogenates of *O. moubata* and *B. microplus* embryos. The reactions forming 3DE and E' may proceed at much faster rates than the conjugation reaction(s) to form apolar products. This would explain why more E' was formed. However, if enzymes for conjugation are present, it would have seemed likely that the E' would have been conjugated. It is possible that a cosubstrate necessary for conjugation reaction is missing in the culture, or it may be that the enzymes for conjugation do not recognize 3α epimers. If the latter is the case, then the epimers observed *in vivo* were formed after conjugation took place. However, in *Calliphora*, the presence a C-22 hydroxy group was necessary for epimerization to take place (Koolman 1985).

The apolar peak Z has the same retention times as 22,25-dideoxyecdysone or 3,22,25-tridcoxyecdysone. It does not seem likely that the embryos have removed three hydroxy groups to form this ecdysteroid which is putative insect precursor of ecdysone (Rees 1985). More studies need to be done before conclusions can be made about the identity of these unknown products and the properties of the enzyme systems present in these embryos.

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VII

Preliminary studies on [^{3}H]-Ecdysone Metabolism in *Boophilus microplus* Embryos

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ABSTRACT

When ovipositing *Boophilus microplus* females are injected with [^{3}H]-ecdysone ([^{3}H]-E), as much as 97% of the radiolabel is recovered in the eggs. The majority of the label is found in the eggs oviposited during the first four days after injection, with the highest amount incorporated on the second day. Of the radiolabel found in the female at the end of oviposition, the majority is found in the midgut.

The [^{3}H]-E is incorporated into the eggs in the form of apolar ecdysteroid conjugates (AP2) which are fatty acid ecdysteroid esters. Esterase hydrolysis of the AP2 releases only ecdysone. Between days 0 and 10 of embryonic development, much of the AP2 are transformed into polar products (PP), which may be ecdysteroid-26-oic acids, and into apolar conjugates AP1, which are more polar than AP2 and are conjugates of PP. The PP and AP1 are the major metabolites present throughout the remainder of development. Esterase hydrolysis of extracts of day 10 and older embryos releases mostly PP.

*The data presented in this paper is a part of this author's doctoral thesis at the Université de Neuchâtel.

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INTRODUCTION

The presence of maternally derived ecdysteroids (ES) in the newly laid eggs is a common occurrence in insects (Hoffmann and Lagueux 1985) and ES or ES immunoreactive material has been found in eggs of several tick species (Connat *et al.* 1985, Connat and Dotson 1988, Dotson *et al.* 1991). After injection of tritiated ES into vitellogenic ticks, the radiolabel has been recovered from the eggs as free ecdysone (E) and 20-hydroxyecdysone (20E) in two *Amblyomma* species (Connat *et al.* 1987, Connat and Dotson 1988), as apolar conjugates (AP) in *Ornithodoros moubata* (Connat *et al.* 1984, Connat and Dotson 1988) and *Boophilus microplus* (Wigglesworth *et al.* 1985) and as a mixture of free and conjugated ecdysteriods in *Rhipicephalus appendiculatus* and *Hyalomma dromedarii* (Connat and Dotson 1988). Apolar conjugates have been identified as fatty acid esters of 20E in the fifth instar nymphs of *O. moubata* (Diehl *et al.* 1985) and as esters of E in newly laid eggs of *B. microplus* (Crosby *et al.* 1986).

The role of maternal ES in embryonic development is unknown. Connat and colleagues (1984) and Wigglesworth and coworkers (1985) hypothesized that the conjugates were inactive storage forms which were to be hydrolyzed when needed during embryonic development. In fact, homogenates of developing *B. microplus* embryos hydrolyzed tritiated apolar conjugates (Wigglesworth *et al.* 1985). However, *O. moubata* embryos do not appear to use the apolar conjugates. The tritiated AP found in the eggs after vitellogenic females were injected with [^{3}H]-ecdysone ([^{3}H]-E) remained unchanged during embryonic development and were gradually tranformed into slightly more polar "esters" of E and 20E during larval development. Esterase treatment of these AP throughout embryonic and larval development of *O. moubata* released the same relative proportions of E and 20E as were incorporated into the embryo (Connat *et al.* 1988). Endogenous increases in esterase-labile ecdysteroid conjugates were noted at the appearance of each ES peak in embryos and larvae of this species (Dotson *et al.* 1991). These data suggested that *O. moubata* embryos and larvae did not use these AP as a source for ES.

The purpose of this study is to analyze the fate of radiolabelled AP incorporated into the eggs of *B. microplus* and to compare the results with those found in another ixodid tick, *Amblyomma hebraeum*, and the argasid tick *O. moubata*.

MATERIALS AND METHODS

Ticks

Engorged *B. microplus* females one or two days postdrop from bovine hosts were generously supplied by CIBA-GEIGY (CRA, St. Aubin, Switzerland). The females were allowed to lay eggs in plastic vials. Each day the females were removed and the eggs were left in the vial, in order to minimize handling the eggs which may disrupt development. When collecting eggs for extraction, dried and nondeveloping eggs were removed. The females and eggs were kept in 97% RH (above a saturated K_2SO_4 solution) at 28°C and in total darkness.

Chemicals

Ecdysone (2β,3β,14α,22R,25-pentahydroxy-5β-cholest-7-en-6-one) and 20-hydroxy-ecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one) were purchased from Simes (Italy). Labelled ecdysone ([23,24^{3}H]-ecdysone ([^{3}H]-E)) (sp. act. 53.6 Ci/mmol) was obtained from New England Nuclear Corporation and was purified on high performance liquid chromatography (HPLC) when necessary.

Injections

Vitellogenic *B. microplus* females, one day after the beginning of oviposition, were injected with 100,000 cpm [^{3}H]-E (53 Ci/mmol) dissolved in 5 µl of TC199 Seromed medium (Biochrom, Berlin, Germany). The tritiated hormone was injected through the dorsal cuticle of the tick (perhaps into midgut) with a Hamilton 10 µl syringe.

Extractions for HPLC

Radiolabelled eggs were homogenized in 500 µl methanol in a 1.5 ml centrifuge tube using a teflon coated pestle. The pestle was then rinsed with 500 µl methanol. The sample was vortexed and centrifuged at 10,000*g* for 10 min. The supernatant was saved. The pellet was re-extracted with 500 µl methanol, vortexed and centrifuged. The supernatant was combined with previous supernatant.

Aliquots of extracts containing approximately 12,000 cpm were dried under a N_2 flow, and then the samples were resuspended in a volume of pure methanol by vortexing and sonicating. Two volumes of Tris buffer were added to the methanol, and the sample was vortexed again. This protocol was needed to recover the apolar products which were probably adsorbed on the plastic tube walls during the evaporation step. Finally, cold ecdysone (E) and 20-hydroxyecdysone (20E) standards were added to the sample. This was done to determine if the labelled hormones comigrated with the standards.

Enzymatic hydrolysis

Aliquots of methanolic extracts of the radiolabelled eggs were dried in 3 ml glass bottles under a N_2 flow and 950 µl of O.1 M borate buffer (pH 8.3) was added. The bottle was vortexed, sonicated for 3 min to insure solution of the dried extract and 50 µl (50 IU) pig liver esterase (E.C. 3.1.1.1, Boerhinger) was added. After an overnight incubation at 37°C, the hydrolyzed sample was extracted with methanol.

Cultures

Freshly laid eggs (60 mg) were ground in 300 µl of TC199 Seromed medium adjusted to 390 mOsmol with NaCl and containing 500 U penicillin and 500 µg streptomycin per ml. Three 100 µl aliquots of this homogenate was placed into a sterile Nunclon culture dish. The homogenizer was rinsed with 450 µl TC199, which was then distributed among the 3 cultures and 100,000 cpm [^{3}H]-E in 50 µl of TC199 was added to each. The three cultures, each containing 20 mg of same embryo pool in 300 µl of TC199, were incubated 6, 24, or 48 hrs at 27°C.

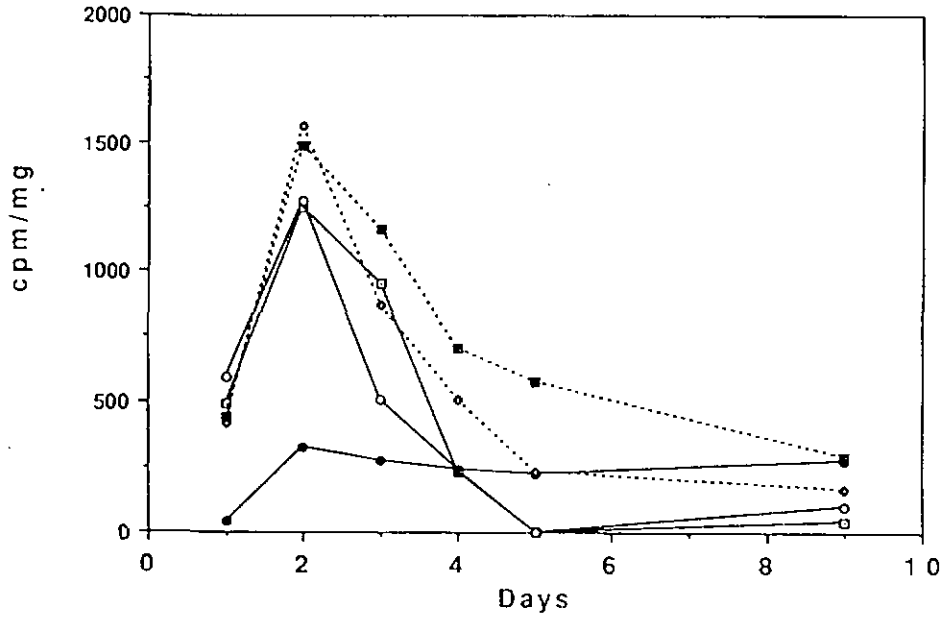

Figure 1: The amount of radiolabel found in the eggs on each day after injection of 100,000 cpm of [^{3}H]-ecdysone into five *Boophilus microplus* females. The results are expressed as cpm/mg.

Extractions of Cultures

The cultures were extracted twice in 1 ml methanol, dried under a N_2 flow and resuspended in 200 µl methanol and vortexed. Then, 400 µl of Tris buffer (pH 7.5, 20 mM) were added and the samples were vortexed again. Finally, cold E and 20E standards were added to the sample before injection on reversed phase HPLC.

High performance liquid chromatography (HPLC)

HPLC analyses were done with a Perkin Elmer Series 3 Chromatograph or a Kontron system with two 420 pumps piloted by an IBM-PC computer with a variable wave-length spectrophotometer set at 242 nm. The samples were separated on a Merck Lichrosorb RP-18 (7 µm phase) column (25 cm x 4 mm) with a precolumn (4 cm x 4 mm) packed with Merck RP-8 40 µm phase. A solvent gradient with methanol and Tris buffer (pH 7.5, 20 mM) was generally used: 30% methanol to 45% (10 min), isocratic 45% (15 min), 45 to 100% (20 min), purge 100% methanol (20 min).

Liquid Scintillation Counting

We used an automatic liquid scintillation counter (Kontron MR 300 DPM) and Riatron scintillation cocktail. In the case of HPLC fractions, results are expressed in noncorrected cpm since, in our conditions, only a small difference of quenching existed between the different HPLC fractions.

RESULTS AND DISCUSSION

Boophilus microplus females were injected with [^{3}H]-E on the day they began to oviposit and radiolabel was found in the eggs oviposited the following day. The highest amount of radiolabel (mean $\pm$ SD = 1179 $\pm$ 497 cpm/mg) was incorporated into the eggs oviposited the second day after injection (Fig. 1). The percentage of the recovered radiolabel found in the eggs ranged from 76 to 97%. Of the radiolabel remaining in the female, only 6% remained in the ovary. The majority (68%) was found in the midgut. The dissection medium contained about 12% but most of this was probably contamination from the midgut contents. The remainder of the label was recovered in the Malpighian tubules (6%) and carcass (8%). This distribution of the radialabel at the end of oviposition is not different from that found in *A. hebraeum* embryos after injection of [^{3}H]-20E where the majority of the radiolabel is incorporated into the eggs and, of that left in the female, most is found in the midgut (Connat *et al.* 1987).

The majority of the radiolabel incorporated into the freshly laid eggs of *B. microplus* had retention times similar to the AP2 found in *O. moubata* embryos (Connat *et al.* 1984) and

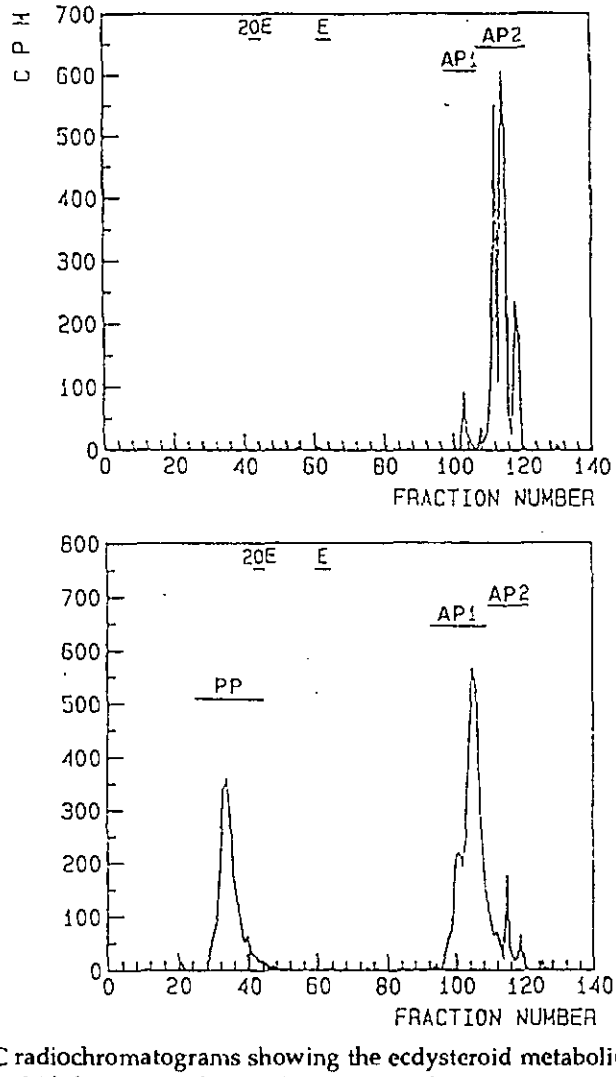

Figure 2: RP-18 HPLC radiochromatograms showing the ecdysteroid metabolites found in a) the freshly laid eggs and b) the larvae of *Boophilus microplus* females injected with 100,000 cpm of [^{3}H]-ecdysone. E and 20E - retention times of cold ecdysone and 20-hydroxyecdysone standards, respectively, PP-unidentified polar products (ecdysteroid-26-oic acids) AP1-apolar products, which appear to be conjugates of PP and AP2-apolar products with similar retention times as the esters of E and 20E found in *Ornithorodoros moubata*.

is probably composed of the fatty acid esters of E (Fig. 2a) which have been described in this tick (Wigglesworth *et al.* 1985). In some cases, very low amounts of radioactivity (3%) co-migrated with E (Table 1). Esterase hydrolysis, although incomplete, of the AP2 in freshly laid eggs released only E. By day 10, much of the AP2 had been transformed to products more polar than 20E (called polar products-PP) and to apolar products which had retention times similar to the AP1 described by Connat *et al.* (1984) (Fig. 2b). The PP may be ecdysteroid-26-oic acids (Crosby *et al.* 1987). Esterase hydrolysis of the sample increased the free E to only 5% but released a large amount of PP. Thus, it appears the AP1 of *B. microplus* are esters of PP and are not the more polar esters of E found in *O. moubata* (Connat *et al.* 1984, Connat *et al.* 1988) and in *A. hebraeum* (Connat *et al.* 1987, Dotson *et al.* submitted).

By the time the larvae hatch out (22-23 days after oviposition), the PP have increased to almost 40% of the radiolabel and the AP1 have decreased (Table 1, Fig. 2b). A slight increase in free E and AP2 was also noted. Again, esterase hydrolysis greatly increased the PP in the sample and the E made up 7.6% of the radiolabel.

It is difficult to state whether the embryro has reused any of the maternal conjugates as a source of ecdysteroids to stimulate formation of any of the 4 cuticles produced during embryonic development. The slight increase of free E and conjugated E at the end of development may suggest that perhaps some of the PP or AP1 was converted to E and this was changed back to PP or AP1 or conjugated to form AP2. Also, because we do not know the exact nature of the PP and AP1, we cannot tell whether any of the E was, at any time during development, hydroxylated to 20E which appears to be the principal hormone in the

Table 1. Distribution of radiolabel (expressed in percent) in crude methanol and esterase treated extracts of developing embryos from eggs oviposited after *Boophilus microplus* females were injected with 100,000 cpm of [^{3}H]-ecdysone.

	Methanolic Extracts				Esterase Treated Extracts			
Day	PP	E	AP1	AP2	PP	E	AP1	AP2
0	--	2.9	4.0	91.0	--	85.0	3.15	10.5
10	20	0.4	67	10.0	70	5	15.6	--
15	19.3	0.5	68.6	9.6	82	4.4'	8.3	--
21	27	--	66	7.0				
L	38.4	2.0	44.8	13.6	74	7.6	13.2	--

PP-unidentified polar products (ecdysteroid-26-oic acids), E-ecdysone, AP1-apolar products, which appear to be conjugates of PP, AP2-apolar products with similar retention times as the esters of E and 20E found in *O. moubata*.

ecdysteroid peak that is correlated with larval cuticle production in *O. moubata* (Dotson *et al.* 1991). However, formation of ecdysteroid-26-oic acids is an irreversible inactivation process in insects (Lafont and Connat 1989) and it does not seem likely that this embryo reverses the process. The slight increase of E observed may also be due to experimental error and more studies need to be done.

If the incorporation of radiolabelled products into the eggs of ticks after the females have been injected with a tritiated ecdysteroid and the metabolism of these radiolabelled products accurately reflect the endogenous situation, then at least three methods for dealing with maternal ecdysteroids have evolved in tick embryos: 1) the *O. moubata* system where the incorporated long chain fatty acid esters of E are not reused but are slowly converted to slightly more polar esters of E (Connat *et al.* 1988), 2) the *B. microplus* system where the incorporated long chain fatty acid esters of E appear to be transformed ecdysteroid-26-oic acids and esters of these acids, and 3) the *A. hebraeum* system where the incorporated free hormones are doubly inactivated after 15 days of development to conjugates of the free hormone and conjugates of the 3α epimers of the hormones (Dotson *et al.* submitted).

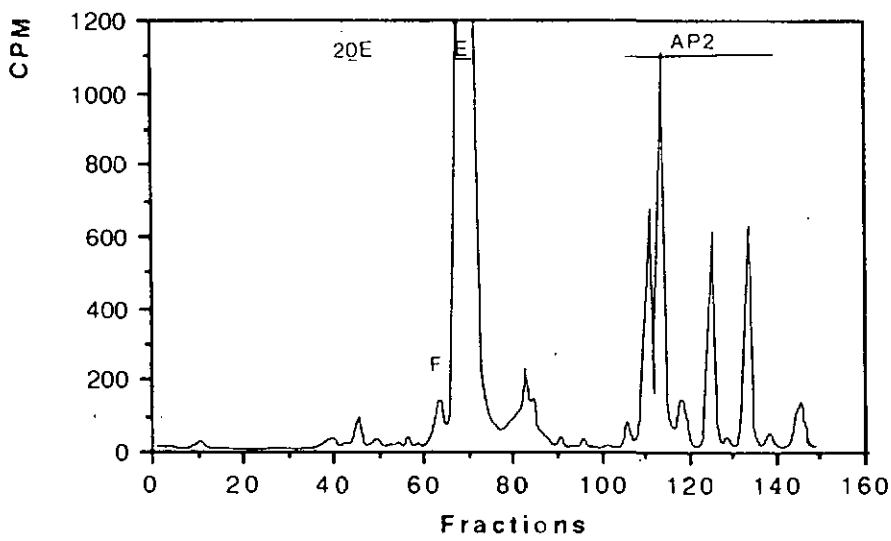

Figure 3: RP-HPLC radiochromatogram of 48 hr culture of *Boophilus microplus* egg homogenates. E and 20E - retention times of cold ecdysone and 20-hydroxyecdysone standards, respectively, AP2-apolar products with similar retention times as the esters of E and 20E found in *O. moubata*. F-unidentified polar product with retention times similar to an unidentified polar product found in *A. hebraeum* egg homogenate cultures.

Cultures of Egg Homogenates

Approximately 10% of the [^{3}H]-ecdysone was metabolized in the 6, 24 and 48 hrs cultures of the freshly laid eggs. The major metabolites (68-75% of metabolites) had retention times similar to AP2 (Fig. 3). AP2 have been found in cultures of broken open embryos and ovocytes (Dotson *et al.* 1993) and early embryo egg homogenates of *O. moubata* (Dotson unpublished). The enzymes for AP production appear to be incorporated into the ovocytes. However, because esterase hydrolysis was not carried out on these products in *B. microplus*, we can not be sure that they are esters of E.

Approximately 8% of the metabolites appeared to correspond to compound Y, unidentified metabolite found in *A. hebraeum* cultures (Dotson *et al.* submitted). However, they may represent contaminants because they were found after incubating [^{3}H]-E for 48 hrs in the culture medium without eggs or egg homogenates (Dotson unpublished). Approximately 10% of the metabolites appears to be more polar than E. The major polar peak (4-8% of metabolites) is just slightly polar than E, with retention times similar to the unidentified polar product F in cultures of *A. hebraeum* embryos (Dotson *et al.* submitted). The only other major polar product (represents 2% of metabolites in 48 hr culture) is a product which comigrates with 20E on RP-18 HPLC.

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