Pergamon
0305-0491(94)00140-5

Comp. Biochem. Physiol. Vol. 110B, No. 1, pp. 155–166, 1995

0305-0491/95 $9.50 + 0.00

Ecdysteroid titre and metabolism and cuticle deposition during embryogenesis of the ixodid tick *Amblyomma hebraeum* (Koch)

Ellen M. Dotson,*† Jean-Louis Connat† and Peter A. Diehl

Institut de Zoologie, Université de Neuchâtel, Chantemerle 22, 2007 Neuchâtel, Switzerland

Three embryonic cuticles are formed before larval cuticle deposition during embryonic development of *Amblyomma hebraeum*. The quantity of radioimmunoassay-positive material varied between 50 and 200 pg ecdysone equivalents per mg, but no significant peaks were detected. Maternally incorporated [^{3}H]-20-hydroxyecdysone and [^{3}H]-ecdysone contained in freshly laid eggs appear to be conjugated to C-22 fatty acid esters and 3α epimers of those esters and, thus, appear doubly inactivated. In addition, ecdysone is converted to an unknown product called 2′. The role of these maternally derived ecdysteroids is unknown.

Key words: *Amblyomma hebraeum*; Apolar conjugates; Cuticle development; Ecdysteroids; Ecdysteroid metabolism; 3α Ecdysteroids; Embryonic cuticles; Epimer; Tick.

Comp. Biochem. Physiol. 110B, 155–166, 1995.

Introduction

Knowledge of the endocrinology of ticks has greatly increased in the last 15 years (see Diehl *et al.*, 1986; Connat, 1987). Although the 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) was 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

Correspondence to: E. M. Dotson, Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, GA 30460-8056, U.S.A.

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

†Present address: Université de Bourgogne, Laboratoire de Zoologie, F-21000 Dijon, France.

Received 30 December 1993; revised 18 June 1994; accepted 28 June 1994.

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 microplus*, 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. *Ornithodoros 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; 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

Animals

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 2 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 2 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 were pierced with a fine tungsten needle to allow penetration of the fixative. The tissue was then washed three times for 10 min and once overnight with a 0.2 M Sörensen buffer (pH 7.4) containing 5% sucrose. The embryos were postfixed for 2 hr at 4°C with OsO_4 in Palade (1962) buffer (pH 7.4) containing 5% sucrose and rinsed three 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 ($\sim 0.75\ \mu m$), cut with glass knives, were placed on albumin-coated slides and stained with toluidine blue. Thin sections ($\sim 0.08\ \mu m$), 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-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, U.S.A.) and purified on high performance liquid

chromatography (HPLC) when necessary. Labelled 20-hydroxyecdysone was produced by incubating [^{3}H]-E with Malpighian tubules from fifth instar larvae of *Locusta migratoria* and purifying the extract of culture medium on HPLC.

Extractions for radioimmunoassay (RIA)

In preliminary experiments, for every 2 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 re-extracted 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. The piston was rinsed afterwards with 500 μl of pure methanol which was then added to the homogenate. The samples were vortexed, sonicated and centrifuged for 10 min at 10,000 ***g***. After centrifugation, an aliquot of the supernatant 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 1 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 was 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. The homogenate and rinse were combined, then vortexed, sonicated and centrifuged for 10 min at 10,000 ***g***.

High performance liquid chromatography (HPLC)

Reverse and normal phase HPLC analyses were done with the same apparatus and conditions described in previous papers (Connat *et al.*, 1987; Dotson *et al.*, 1991). Aliquots of extracts containing approximately 12,000 cpm were dried under a N_2 flow, and resuspended in a volume of pure methanol by vortexing and sonicating. Two volumes of Tris buffer (pH 7.5, 20 mM) were added, and the sample was vortexed again. This protocol was needed to recover the apolar products which are probably absorbed on the plastic tube walls during the evaporation step. Finally, to determine whether the labelled hormones comigrated with the UV peaks of standards, cold ecdysone and 20-hydroxyecdysone standards were added to the sample.

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 (0.1 M, pH 8.3) by vortexing and sonicating for 3 min. Pig liver esterase (EC 3.1.1.1, Boerhinger Mannheim, Germany, 50 μl, 50 IU) was added. After an overnight incubation at 37°C, the hydrolysed 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 then collected in 1.5 ml pure methanol. The sample was then dried under reduced pressure and was assayed for RIA.

Radioactive samples were first separated into three fractions (2 ml 30% methanol, 5 ml 60% methanol, and 5 ml 100%

methanol) using a reverse phase C-18 SEP PAK@ (Waters, Milford, MA, U.S.A.) 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 microlitres of *Helix* juice (IBF) was 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.

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 non-corrected 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 in 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, 250–600 nm) (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 material that 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 that 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 eggshell 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 or 10 lamellae (each 0.4–0.8 μm) with pore canals dispersed throughout. The inner procuticle is deposited after hatching and is composed of 3–6 lamellae that are slightly thicker than those of the outer procuticle (Fig. 1g).

Ecdysteroid titre during embryonic development

When over 200 crude methanolic extracts of the various ages of embryos from five different series of *A. hebraeum* eggs were analysed using RIA, no distinct peak of ecdysteroid immunoreactive material was detected. The amount of immunoreactive material appeared to remain between 50 and 200 pg equivalents of E per mg.

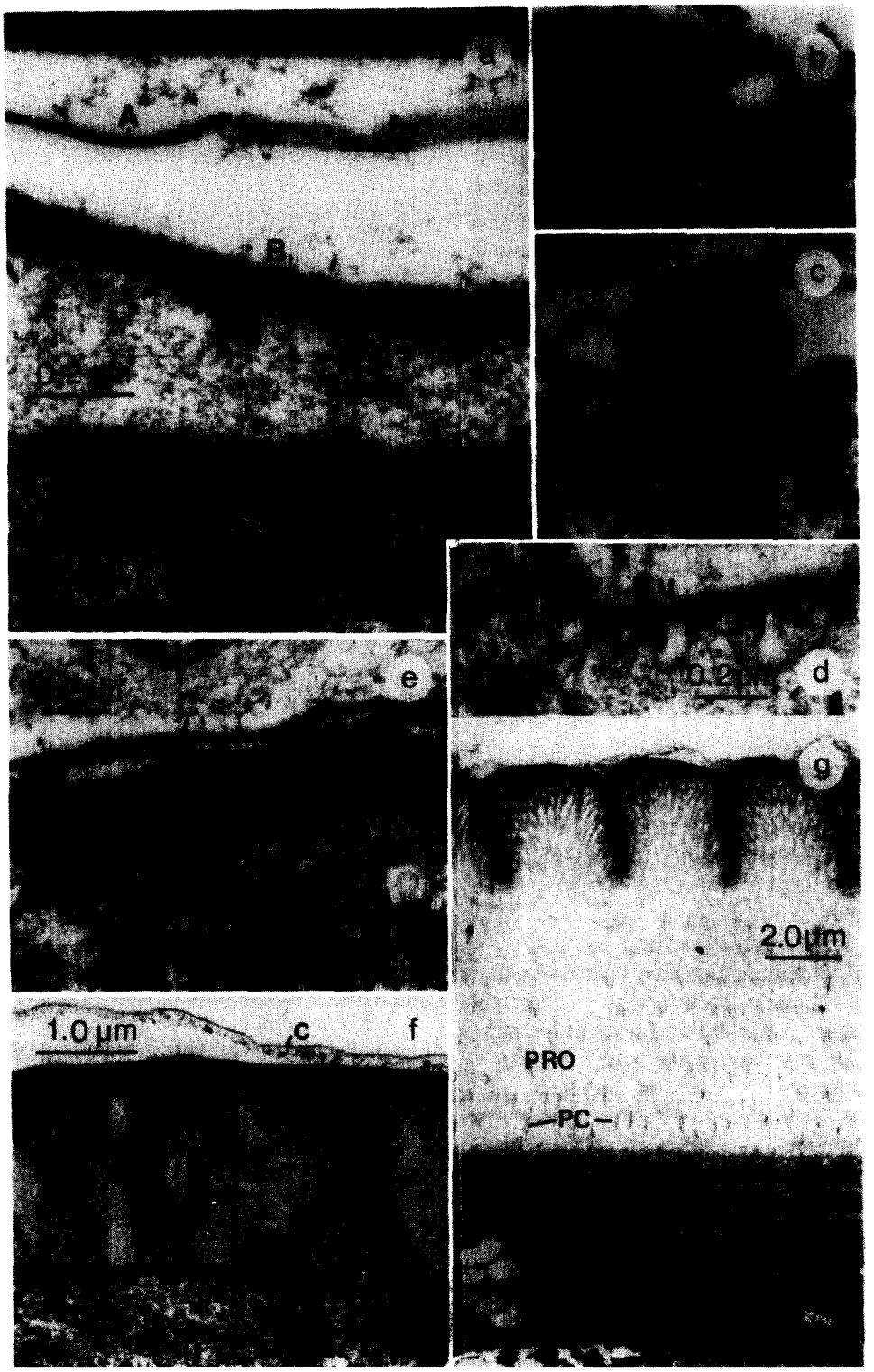

Fig. 1. (a) *Amblyomma hebraeum* embryo, 28 days after oviposition. "Cuticles" A, B and C are present, B_1, B_2 and B_3 are the thin outer layer, thicker electron-dense layer and inner fibrous layer, respectively, of cuticle B. ES: Eggshell. (b) Embryo, 30 days after oviposition. Electron-dense plaques 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 oviposition, Cuticulin (Cu) is being deposited at the tips of microvilli. (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 of the opisthosomal region is composed of epicuticle and amorphous and lamellate procuticle (PRO). PC: pore canals; N: nucleus.

However, a few samples around the 12th day of development and around the 44–46th day contained as much as 350 pg/mg and one sample contained 1100 pg/mg, but these were not correlated with a developmental event.

Extracts from *A. hebraeum* embryos 0, 10, 20, 30, 40 and 48 days old were analysed 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 equivalents of E per mg at oviposition to 110 pg 20 days later and remained about the same thereafter (Fig. 2). 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 day 10 samples.

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

When [^{3}H]-20E is injected into *A. hebraeum* females at the beginning of oviposition, approximately 76% of the radiolabelling is transferred to the eggs, 95% of which is found in its free form (Fig. 3a). The remaining 5% corresponded to a compound more polar than 20E, which has 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

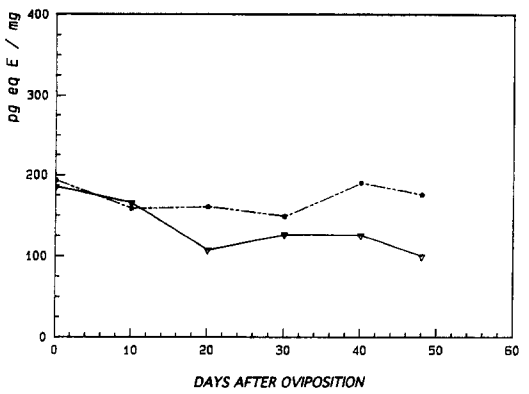

Fig. 2. A comparison of immunoreactive material present in crude methanolic extracts of various ages of *A. hebraeum* eggs before (—▽—) and after (----●----) hydrolysis with porcine liver esterase. The results are expressed in pg equivalents ecdysone/mg of eggs.

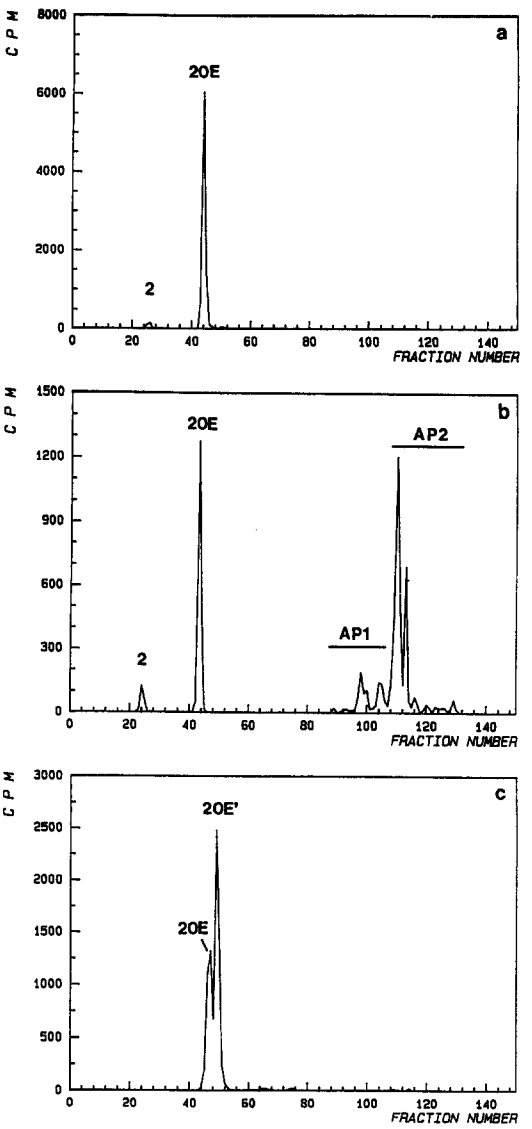

Fig. 3. Reversed phase HPLC radiochromatograms of eggs from *A. hebraeum* females injected with [^{3}H]-20-hydroxyecdysone during vitellogenesis. 20E marks the retention of authentic 20-hydroxyecdysone. 2 is an unidentified polar product and AP1 and AP2 are apolar products with similar retention times as fatty acid ester conjugates. 20E′ marks the retention of the 3α epimer 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.

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) that have already been described in *O. moubata* nymphs (Diehl *et al.*, 1985). The AP1 are

more polar than AP2 and co-migrated with the unidentified esterase labile 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 (Figs 3b and 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 remains low in the first 40 days (3–6%) but increases 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.

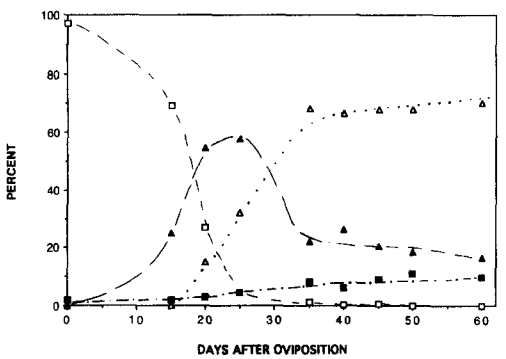

Fig. 4. Metabolism of maternally incorporated radioactive compounds found in the eggs after injection of [^{3}H]-20E into ovipositing *A. hebraeum* females. The results are expressed as percentage of total radioactivity in the sample. 20-hydroxyecdysone (— —□— —), polar product 2 (— · —■— · —), apolar products 2 (— —▲— —), apolar products 1 (····△····).

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

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

PP2′-Polar product 2′; 20E: 20-hydroxyecdysone; E: ecdysone; AP1 and AP2: apolar products.

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

When [^{3}H]-E was injected into engorged *A. hebraeum* females at the beginning of oviposition, 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 1). Percentages of radiolabel corresponding to 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 that 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. 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. 5). 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 (pH 7.0) was used in the place of Tris buffer (pH 7.5, 20 mM) as the 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. 5). 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. 6b) and then decreased to represent only 18% of the radiolabel at the

end of development (Fig. 5). AP1 were first observed in the 20 day culture. 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. 6c). In the 25-day-old embryos, E and 20E make 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 1), 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%. 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; Dotson, unpublished results). The fact that

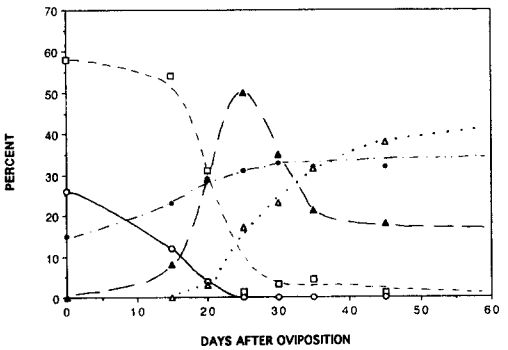

Fig. 5. Metabolism of maternally incorporated radioactive compounds found in the eggs after injection of [^{3}H]-E into ovipositing *A. hebraeum* females. The results are expressed as percentage of total radioactivity in the sample. Ecdysone (—○—), polar product 2′ (—·—●—·—). For other metabolites see Fig. 4.

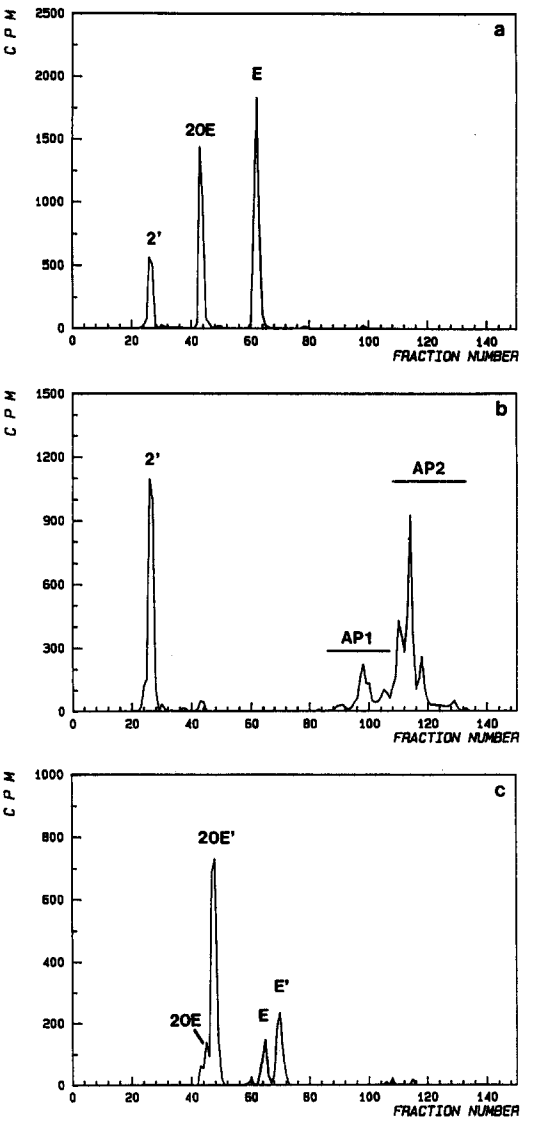

Fig. 6. Reversed phase HPLC radiochromatograms of eggs from *A. hebraeum* females injected with [^{3}H]-ecdysone during vitellogenesis. E and 20E mark the retention of ecdysone and 20-hydroxyecdysone standards, respectively. 2′ is an unidentified polar product and AP1 and AP2 are apolar products with 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.

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

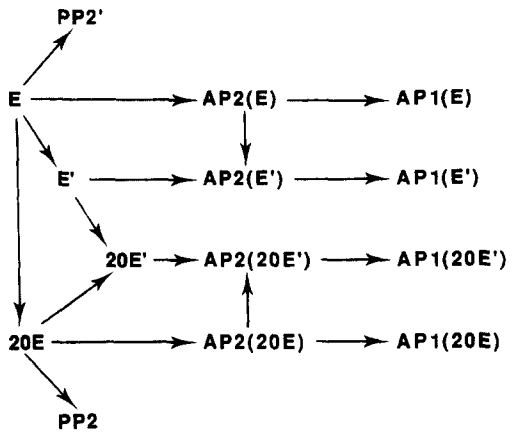

Fig. 7. Possible metabolic pathways of [^{3}H]-ecdysone in *A. hebraeum* embryos.

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.

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 desposited 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 *A. hebraeum* larvae. This is the case in females and larvae of *B. microplus* and *B. decoloratus* (Beadle, 1974; 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 Filshie, 1982) and the larvae of *B. decoloratus* and *B. microplus* (Beadle, 1974) and *O. moubata* (Vogel, 1975; 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 titres

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 titres 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 titres in embryos at time of epicuticle production with low titres of embryos at a slightly different developmental stage may have obscured any peak.

Esterase hydrolysis of extracts from embryos of different stages never released more RIA-positive material than those of the 1st 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 ecdysteroid esters 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 titres 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 titres 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 titre (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 whether decreasing the amount of ecdysteroids incorporated into the eggs would affect the development of *A. hebraeum* embryos.

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.* (1986) 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. It is also possible that they act as a 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

Amblyomma hebraeum embryos appear to use similar pathways as the adults 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 (Fig. 7). 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 3α epimers 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 Lafont and Koolman, 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.

Acknowledgements—We thank Ms Martha Joiner for critical reading of this manuscript. We gratefully acknowledge the Swiss National Foundation for its financial support (no. 3.662.84). During this study, Ellen Dotson benefitted from a 2 year scholarship from the Swiss Confederation.

References

Aeschlimann A. and Hecker H. (1969) Vitellogénèse et formation cuticulaire chez l'oeuf d'*Ornithodorus moubata* Murray (Ixodoidea: Argasidae). Étude au microscope électronique. *Acarologia* **11,** 180–192.

Beadle D. J. (1974) Fine structure of the integument of the ticks, *Boophilus decoloratus* Koch and *B. microplus* (Canastrini) (Acarina: Ixodidae). *Int. J. Insect Morphol. Embryol.* **3,** 1–12.

Beadles M. L., Tulloch G. S., Drummond R. O. and Miller F. H. (1973) Changes in the cuticle of the female lone star tick during engorgement. *Ann. Ent. Soc. Am.* **66,** 698–699.

Bouvier J., Diehl P. A. and Morici M. (1982) Ecdysone metabolism in the tick *Ornithodoros moubata* (Argasidae, Ixodoidea). *Rev. suisse Zool.* **89,** 967–976.

Canard A. (1987) Analyse nouvelle du développement postembryonnaire des araignées. *Rev. Arachnol.* **7,** 91–128.

Connat J.-L. (1987) Aspects endocrinologiques de la physiologie du développement et de la reproduction chez les tiques. Thése de Doctorate d'Etat, Université et Bourgogne, Dijon, France.

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

Connat J.-L. Diehl P. A. and Morici M. (1984) Metabolism of ecdysteroids during the vitellogenesis of the tick *Ornithodoros moubata* (Ixodoidea, Argasidae): accumulation of apolar metabolites in the eggs. *Gen. comp. Endocrinol.* **56,** 100–110.

Connat J.-L., Diehl P. A., Gfeller H. and Morici M. (1985) Ecdysteroids in females and eggs of the ixodid tick *Amblyomma hebraeum*. *Int. J. Invertebr. Reprod. Dev.* **8,** 103–116.

Connat J.-L., Lafont R. and Diehl P. A. (1986) Metabolism of [^{3}H]-ecdysone by isolated tissues of the female ixodid tick *Amblyomma hebraeum* (Ixodoidea; Ixodidae). *Mol. cell. Endocrinol.* **47,** 257–267.

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

Connat J.-L., Dotson E. M. and Diehl P. A. (1988) Apolar conjugates of ecdysteroids are not used as a storage form of molting hormone in the argasid tick *Ornithodoros moubata*. *Arch. Insect Biochem. Physiol.* **9,** 221–236.

Crosby T., Evershed R. P., Lewis D., Wigglesworth K. P. and Rees H. H. (1986) Identification of ecdysone 22-long-chain fatty acyl esters in newly laid eggs of the cattle tick *Boophilus microplus*. *Biochem. J.* **240,** 131–138.

Crosby, T., Lewis D. and Rees H. H. (1987) Ecdysteroids in adult and embryonic development of the cattle tick, *Boophilus microplus*. VIII Ecdysone Workshop, Marburg Germany, Poster 16, p. 41.

Delbecque J.-P., Diehl P. A. and O'Connor J. D. (1978) Presence of ecdysone and ecdysterone in the tick *Amblyomma hebraeum* Koch. *Experientia* **34,** 1379–1381.

De Reggi M. L., Hirn M. H. and Delaage M. A. (1975) Radioimmunoassay of ecdysone: an application to *Drosophila* larvae and pupae. *Biochem. Biophys. Res. Commun.* **66,** 1307–1315.

Diehl P. A., Germond J. E. and Morici M. (1982) Correlations between ecdysteroid titers and integument structure in nymphs of the tick *Amblyomma hebraeum* Koch (Acarina: Ixodidae). *Rev. suisse Zool.* **89,** 859–868.

Diehl P. A., Connat J.-L., Girault J. P. and Lafont R. (1985) A new class of apolar ecdysteroid conjugates: esters of 20-hydroxyecdysone with long-chain fatty acids in ticks. *Int. J. Invertebr. Reprod. Dev.* **8,** 1–13.

Diehl P. A., Connat J.-L. and Dotson E. M. (1986) Chemistry, function and metabolism of tick ecdysteroids. In *Morphology, Physiology, and Behavioral Biology of Ticks* (Edited by Sauer J. R. and Hair J. A.), pp. 165–193. Ellis Horwood, Chichester.

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

Dübendorfer A. (1989) Ecdysteroid action in embryonic systems. In *Ecdysone from Chemistry to Mode of Action* (Edited by Koolman J.), pp. 421–425. Georg Thieme, Stuttgart.

Germond J. E., Diehl P. A. and Morici M. (1982) Correlations between integument structure and ecdysteroid titres in fifth-stage nymphs of the tick, *Ornithodoros moubata* (Murray, 1877; *sensu* Walton, 1962). *Gen. comp. Endocrinol.* **46,** 255–266.

Hackman R. H. (1982) Structure and function in tick cuticle. *A. Rev. Entomol.* **27,** 75–95.

Hackman R. H. and Filshie B. K. (1982) The tick cuticle. In *Physiology of Ticks* (Edited by Obenchain F. D.), pp. 1–39. Pergamon Press, Oxford.

Kondo A. (1969) The fine structure of the early spider embryo. *Sci. Rep. Tokyo Kyoiku Daigku* **14B,** 1–35.

Koolman J. and Karlson P. (1985) Regulation of ecdysteroid titer: degradation. In *Comprehensive*

Insect Physiology, Biochemistry, and Pharmacology, Vol. 7, *Endocrinology I* (Edited by Kerkut G. and Gilbert L.), pp. 343–360. Pergamon Press, Oxford.

Lafont R. and Connat J.-L. (1989) Pathways of ecdysone metabolism. In *Ecdysone from Chemistry to Mode of Action* (Edited by Koolman J.), pp. 167–173. Georg Thieme, Stuttgart.

Lafont R. and Koolman J. (1984) Ecdysone metabolism. In *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones* (Edited by Hoffmann J. and Porchet M.), pp. 196–226. Springer, Berlin.

Lafont R., Pennetier L. L., Andrianjafintrimo M., Claret J., Modde J. R. and Blaise C. (1982) Sample processing for high-performance liquid chromatography of ecdysteroids. *J. Chromatogr.* **236,** 137–149.

Lanot R., Roussel J.-P. and Thiebold J. (1989a) Ecdysteroids and meiotic reinitiation in oocytes of *Periplaneta americana* (Dictyoptera) and *Gryllus bimaculatus* (Orthoptera). *Invertebr. Reprod. Dev.* **15,** 69–74.

Lanot R., Dorn A., Gunster B., Thiebold J., Lagueux M. and Hoffmann J. A. (1989b) Function of ecdysteroids in oocyte maturation and embryonic development of insects. In *Ecdysone from Chemistry to Mode of Action* (Edited by Koolman J.), pp. 262–270. Georg Thieme, Stuttgart.

Lees A. S. (1952) The role of cuticle growth in the feeding process of ticks. *Proc. Zool. Soc. London* **121,** 759–772.

Nathanson M. E. (1967) Comparative fine structure of sclerotized and unsclerotized integument of the rabbit tick, *Haemaphysalis leporispalustris* (Acari: Ixodides: Ixodidae). *Ann. Ent. Soc. Am.* **60,** 1125–1135.

Nathanson M. E. (1970) Changes in the fine structure of the integument of the rabbit tick, *Haemaphysalis leporispalustris* (Acari: Ixodides: Ixodidae), which occur during feeding. *Ann. Ent. Soc. Am.* **63,** 1768–1774.

Norval R. A. I. (1977) Studies on the ecology of the tick *Amblyomma hebraeum* Koch in Eastern Cape Province of South Africa. II. Survival and development. *J. Parasitol.* **63,** 740–747.

Palade G. (1952) A study of fixation for electron microscopy. *J. exp. Med.* **95,** 285.

Reynolds E. S. (1963) The use of lead citrate at high pH as an electron opaque-stain in electron microscopy. *J. Cell Biol.* **17,** 208–212.

Sabatini D. D., Bensch K. G. and Barnet R. J. (1963) Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* **17,** 9–58.

Solomon K. R., Mango C. K. A. and Obenchain F. D. (1982) Endocrine mechanisms in ticks: effects of insect hormones and their minics on development and reproduction. In *Physiology of Ticks* (Edited by Obenchain F. D. and Galun R.), pp. 399–438. Pergamon Press, Oxford.

Stauffer A. and Connat J.-L. (1990) Anteroposterior gradient during nymphal-adult moulting cycle of the tropical bont tick, *Amblyomma variegatum* (Acarina: Ixodidae). Correlation between ecdysteroid titers and integument structure. *Roux's Arch. dev. Biol.* **198,** 309–321.

Vogel B. E. (1975) Morphologische Untersuchungen über Integument and Hautung sowie Entwicklung des vierten Beinpaares bei *Ornithodoros moubata* (Murray) 1877 (Acarina, Ixodoidea, Argasidae). Ph.D. Dissertation, University of Basel, Basel, Switzerland.

Wigglesworth K. P., Lewis D. and Rees H. H. (1985) Ecdysteroid titre and metabolism to novel apolar derivatives in the adult female *Boophilus microplus* (Ixodidae). *Arch. Insect Biochem. Physiol.* **2,** 39–54.