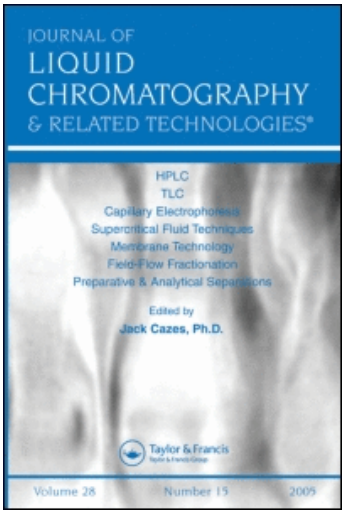

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METABOLISM OF ECDYSTEROIDS DURING THE EMBRYOGENESIS OF *MANDUCA SEXTA*

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ABSTRACT

The combination of ion suppression, reverse phase high pressure liquid chromatography (IS-RPHPLC) and radioimmunoassay (RIA) employing two relatively non-specific, complimentary antisera was used to identify and quantify ecdysteroids of divergent polarities during embryogenesis of the tobacco hornworm, Manduca sexta. Newly layed eggs (0-1 hr) contained high levels (>30 μg/g) of a maternally derived, polar conjugate of 26-hydroxyecdysone (26E) but less than 0.6 and 0.2 μg/g of the polar conjugates of ecdysone (E) and 20-hydroxyecdysone (20E), respectively. The only free ecdysteroid detected was 26E (0.4 μg/g). Between 4 and 12 hr after oviposition, marked deconjugation activity occurred as titers of free 26E increased to 14 μg/g while levels of conjugated 26E fell to 19 μg/g. By this time there were only negligible levels of free E (less than 0.1 μg/g). After 12 hr, the deconjugation of the maternal polar 26E-26-phosphate conjugate slowed and levels of free 26E fell as it was metabolized. At 24 hr, low titers of free 20,26-dihydroxyecdysone (20,26E, 0.4 μg/g) and a β-D-glucose conjugate of 26E (0.8 μg/g) were detected, presumably formed by embryonic 20-monooxygenase and uridine diphospho-α-D-glucose (UDPG) glucosyltransferase activities, respectively. About the time of the deposition of the first larval cuticle (66-72 hr), these 26E metabolites reached

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high concentrations, (5 μg/g free 20,26E and 7 μg/g 26E-glucose conjugate) while free 26E fell to 2 μg/g. In newly hatched larvae, the 26E-glucose conjugate was the major detectable ecdysteroid. At no time during embryogenesis did the concentrations of free E and 20E exceed 0.5 μg/g and 0.3 μg/g, respectively.

INTRODUCTION

It is generally accepted that 20-hydroxyecdysone (20E) is the principal hormone that coordinates molting and metamorphosis during insect postembryonic development. 20E is the product of the hydroxylation of ecdysone (E) by a P-450 20-monooxygenase following the synthesis and secretion of E by the prothoracic glands (1,2). The presence of ecdysteroids in eggs suggest that these steroids may also be involved in the control of embryonic molting. In several insect species, e.g. Oncopeltus fasciatus (4), Blaberus craniifer (5), Leucophaea maderae (6), Nauphoeta cinerea (7), Clitumnus extradendatus (8), Locusta migratoria (9), Schistocerca gregaria (10) and Bombyx mori (11), a correlation was clearly evident between molting events in the embryo and the peaks of molting hormone bioactivity or ecdysteroid immunoreactivity [ranging from at least 1 μg/g (2 μM) to more than 4 μg/g of E and 20E].

In most insects studied, the ecdysteroids in newly oviposited eggs, and during most of embryonic development, are usually conjugates of E, 20E and other ecdysteroids that are more polar than the corresponding free ecdysteroids, and are in much higher concentration. Thusfar, phosphates and/or acyl esters of the 2,3 (both the α and β epimers), 22, and 26 carbon hydroxyl groups of

ecdysteroids have been demonstrated unequivocally in eggs and embryos (12-15).

To investigate the putative role of ecdysteroids during the embryogenesis of Manduca sexta it was first necessary to determine their qualitative nature and then follow the fluctuation of individual ecdysteroids during embryogenesis. Once these data have been obtained, one can probe the function and origin of ecdysteroids during embryogenesis. At present, it appears that all of the embryonic ecdysteroids can be traced back to the maternal ecdysteroids, mainly conjugates, found in eggs prior to the initiation of embryogenesis (14). The present investigation has benefited from the excellent chemical analyses provided by the U.S.D.A. group at Belstville, MD (Kaplanis, Robbins, Thompson, Svoboda, etc.) who have demonstrated the presence of E, 20E, 26E, 20,26E and their 3-α-epimers in Manduca eggs and embryos of various ages (16-19). More recently, they determined that 26E-26-phosphate was the major ecdysteroid conjugate of newly layed eggs (21 μg/g) (15,20). A sensitive, analytical technique involving ion suppression, reverse phase high pressure liquid chromatography coupled to two complimentary radioimmunoassays (IS-RPHPLC/RIA) that recently enabled us to measure free and conjugated ecdysteroids during the pupal-adult development of Manduca sexta (21), was used to investigate the origin and titers of E, 20E and other ecdysteroids that may act to coordinate morphogenetic events during embryogenesis.

MATERIALS AND METHODS

Materials

Solvents, chemicals and ecdysteroid standards were obtained as previously noted (21). A mixed (age, sex) population of adult Manduca sexta moths were held at 25°C, 60% relative humidity under a "long day" (16L:8D) photoperiod. Eggs were oviposited during the scotophase on surrogate leaves suspended in the cage and coated with tobacco leaf extract. The adults were fed diluted honey in open cups. Each morning before "lights-out" the cage was cleared of eggs. Eggs collected during the first two hr of darkness were discarded in case some had been retained from the previous light phase. Subsequently, hourly collections of newly layed eggs were made throughout the dark cycle and were kept in the dark at 25°C, 60% humidity.

Eggs (0.25 g, approx. 250 eggs) of various ages, especially during the first 12 hr and at about the time of deposition of the first larval cuticle (66–72 hr) were quick frozen and kept under acetonitrile (ACN) at -70°C until processed. The term embryo used here encompasses the embryonated egg, i.e. the embryos were not dissected free of the chorion and yolk. Young, first instar larvae were similarly frozen and stored in ACN as soon as they hatched. The timetable of Manduca embryonic development will be published in the near future (A. Dorn et al.).

Sample Preparation

Samples were thawed and homogenized in ACN (10 volumes x 2) using an all-glass tissue homogenizer or a rotating blade blender

(Sorvall), and the pellets re-extracted with 50% ACN (10 volumes x 2). The extracts were pooled and neutral lipids extracted with hexane (equal volume x 2) and the hexane layer then discarded. For the identification of the late embryonic 26E conjugate, the residue from newly hatched larvae (80 g) following homogenization, extraction, and hexane partition was further partitioned in chloroform/ethanol/H_2O (1:1:1) with each phase being back extracted with fresh counter-phase through two cycles. The aqueous-ethanol phases containing polar ecdysteroid conjugates were pooled.

Each residue, following evaporation (reduced pressure, <35°C) of the pooled aqueous ACN or ethanol phases, was dissolved in buffer (0.02 M TRIS/perchlorate, pH 7.5) and applied to one or more primed C_{18} Sep Paks (Waters). After washing the mini-columns with buffer (5 ml), ecdysteroids (both free and conjugated) were eluted into methanol (7 ml). Following evaporation of the methanol, the residue was taken up in buffer (1 ml) and the solution centrifuged (2000 x g, 5 min), prior to IS-RPHPLC. Recovery of ecdysteroids was monitored by RIA of aliquots of the various extracts resulting from the sample preparation procedures.

Radioimmunoassay

Radioimmunoassays were performed as described previously (21,22). [^{3}H]-Ecdysone (62.7 pg, 60 Ci/mmol, New England Nuclear) and the correct dilution of anti-ecdysone antibodies, i.e. that dilution which bound 30% of total radiolabel in 100 mM borate buffer (0.2 ml, pH 8.5) containing 0.05% bovine serum albumin

(Sigma, fraction V), was added to aliquots (usually 10-20 µl) of IS-RPHPLC fractions, to aliquots of aqueous solutions of evaporated fractions following normal phase HPLC (NPHPLC) or high-performance, thin-layer chromatography (HPTLC), to evaporated portions of extracts following extraction and clean-up procedures, and to aliquots of hydrolytic enzyme reaction mixtures. Incubation was overnight at 4°C; termination employed protein A from Staphylococcus aureus; pellets were counted in Scintiverse I (Fisher) with an LKB Rackbeta spectrometer; and concentrations were expressed initially as ng ecdysone equivalents per tube following data reduction using a logit-log RIA program. These concentrations were later adjusted for cross-reactivity. Rabbit antisera were obtained following immunization with: 1) ecdysone-22-succinyl thyroglobulin amide (E-22-ST) from Dr. D.H.S. Horn (23) (C.S.I.R.O., H-22); or 2) 20-hydroxyecdysone-2-succinyl thyroglobulin amide (20E-2-ST) from Dr. J.D. O'Connor (24) (UCLA, H-2, DHS1-15). Ecdysone standards were quantified by u.v. absorbance (242 nm, ε = 12,400) following HPLC or HPTLC purification and the data used to determine ecdysteroid cross-reactivities with the RIAs. The ability of the antibodies to detect biological conjugates purified by HPLC was assessed by comparing the RIA response prior to, and following total deconjugation with enzymes (see below). Cross-reaction is expressed as the ratio of the concentrations required for 50% inhibition of [^{3}H]-ecdysone binding by the metabolite and ecdysone: I_{50} metabolite/I_{50} ecdysone.

Chromatography

Ecdysteroids were separated prior to RIA using a liquid chromatography system [Waters, 6000 A pumps, 720/730 integrator/system controller, Schoeffel UV detector (242 nm) and an LKB fraction collector]. For IS-RPHPLC, a C_{18} μBondapak column (Waters, 3.9 nm x 15 cm, 5 μm spherical packing) was employed in conjunction with a pre-column filter containing C_{18} packing material (Waters, 35-75 μm), and was eluted in the gradient mode with an ACN/aqueous buffer (20 mM TRIS/perchlorate, pH 7.5) (25). Gradient conditions for system 1 were: initial conditions 1 ml/min, 100% aqueous HPLC buffer A (95% buffer/5% ACN) changing over 60 min to 15% organic HPLC buffer B (5% buffer/95% ACN). Conditions remained isocratic at 15% B/85% A for 5 min, followed by a 10 min column cleansing shift to 100% B for 15 min prior to a return to initial conditions over 10 min. Fraction size varied between 0.3 and 1.0 ml depending on the resolution required. To help identify ecdysteroids, evaporated fractions following IS-RPHPLC were subjected to NPHPLC employing a μCN column (Waters, 3.9 mm x 30 cm, 10 μm) used in the normal phase mode. System 2: 3 ml/min, isocratic isooctane/isopropanol/methanol (90:5:5), 1 min fractions.

Free and conjugated ecdysteroids were also separated on HPTLC silica gel 60 plates (Merck, u.v. 254, 10x10 cm) utilizing a solvent system of chloroform/methanol (8:2 v/v, system A). Polar ecdysteroid conjugates were further differentiated on HPTLC plates with a solvent system of ethyl acetate/ethanol/H_2O (2:8:1, system

B). Immunoreactive, u.v.-absorbing compounds were eluted from the plates with chloroform/methanol (1:1).

Hydrolysis of Ecdysteroid Conjugates

Following IS-RPHPLC or HPTLC of eggs, embryos or larvae and RIA analysis of aliquots of eluted fractions, residues following lyophilization were dissolved in buffered enzyme solution (50 mM acetate, pH 5.1, 1 ml) containing 0.2 mg partially purified powder from Helix pomatia (Sigma type H-1 β-glucuronidase/arylsulfatase) and 0.2 mg lyophilized powder from potatoes (Sigma type II acid phosphatase) and incubated overnight at 37°C. Aliquots of the hydrolyzed fractions were again assayed by RIA employing the 2 antisera. Selected immunoreactive peaks were pooled, pre-purified on a C_{18} Sep-Pak and re-subjected to IS-RPHPLC and subsequently, to NPHPLC or HPTLC in order to characterize the deconjugated ecdysteroids present.

Spectroscopy

Positive-ion fast atom bombardment (FAB+) spectra were recorded on a ZAB 4F VG Micromass, U.K., using a primary zenon atom beam with energy of 7.8 KeV. Samples were applied to the probe dissolved in glycerol.

Proton magnetic resonance spectra (PMR) were recorded on an IBM-200. Samples were dissolved in > 99% D_2O and the H_2O impurity was used as an approximate internal reference.

RESULTS

The Manduca egg is a closed system except for respiratory exchange and weighs about 1 mg when oviposited. This weight does

not vary significantly until hatching. More than 80% of the eggs kept at 24°C hatched by 117 hours (±4 hours) after oviposition. Since polar and non-polar ecdysteroids, both free and conjugated, were expected to be present, only a hexane partition was performed initially in order to remove neutral lipids that might damage the column. Greater than 95% of the immunoreactive (RIA-positive) ecdysteroids in the initial extract was recovered in the methanol eluate from the C_{18} Sep Pak. Repeated extractions of egg and larval residues produced no additional RIA-positive material.

Radioimmunoassay Sensitivity

The cross-reactivities of the H-22 and H-2 antisera and their cooperative utility in quantifying known Manduca ecdysteroids and metabolites have been described previously (21). Basically, the H-22 antibodies will bind ecdysteroids that have undergone covalent modification of the side-chain, close to the point of thyroglobulin attachment (C-22) in the immunogen (26) (Fig. 1). They are able to detect metabolites from phase-1 reactions (oxidations) occurring at C-20 and C-26, in addition to esters and acetals of inorganic and organic acids, carbohydrates, and other molecules conjugated at the C-22 and C-26 hydroxyls (phase-2 reactions).

The H-2 antibodies can quantify phase-1 metabolites of the A-ring such as the 3-α-ecdysteroid epimers, the result of oxidation/reduction of the C-3 hydroxyl. Unfortunately, neither antiserum can measure some ecdysteroids resulting from metabolism of both the side-chain and A-ring such as the 3-α-epimers of 26E, 20,26E, and the epimeric ecdysonic acids (polar

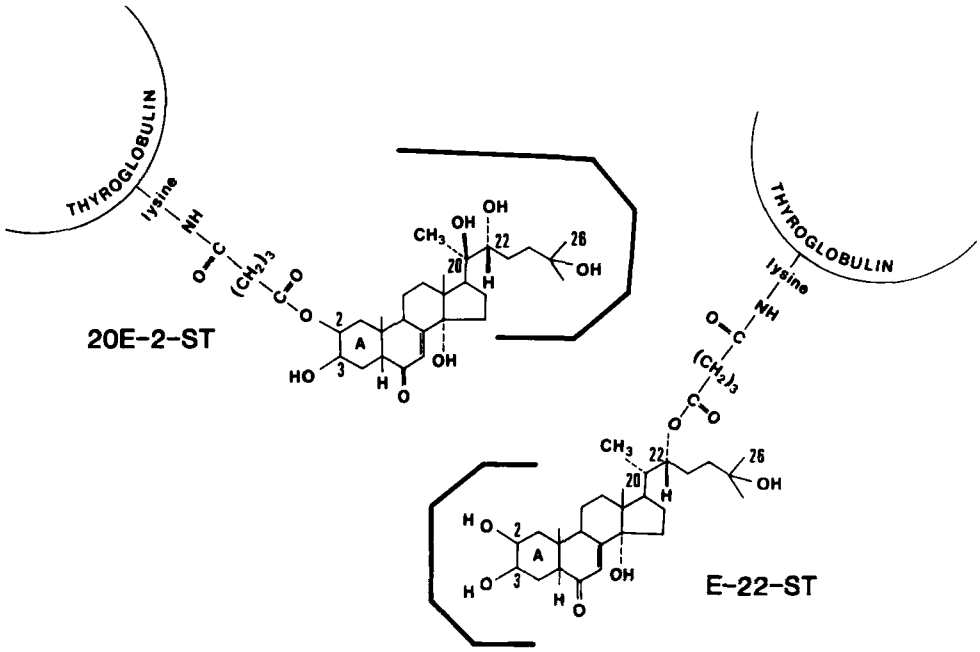

FIGURE 1. Immunogens for production of H-2 (20E-2-ST) and H-22 (E-22-ST) antisera. 20E = 20-hydroxyecdysone, ST = succinyl thyroglobulin, E = ecdysone.

ecdysteroids with the C-26 primary alcohol oxidized to the acid). The former two of these biologically inactive ecdysteroids have been found in developing hornworm embryos (18). The cross-reactivities for the major free ecdysteroids with the H-22 antisera are: E(1), 26E(1), 20E(4.5), 20,26E(5) and ecdysonic acid(1). With the H-2 antisera they are: E(1), 20E(3), 26E(15), 20,26E(60) and ecdysonic acid(25). The H-22 antibodies bind well to the side-chain ecdysteroid conjugates 26E-26-phosphate (1.6) and 26E-β-D-glucose (2.0); the H-2 antibodies do not recognize these metabolites well, but do bind similar conjugates of the A-ring hydroxyls of E and 20E (21).

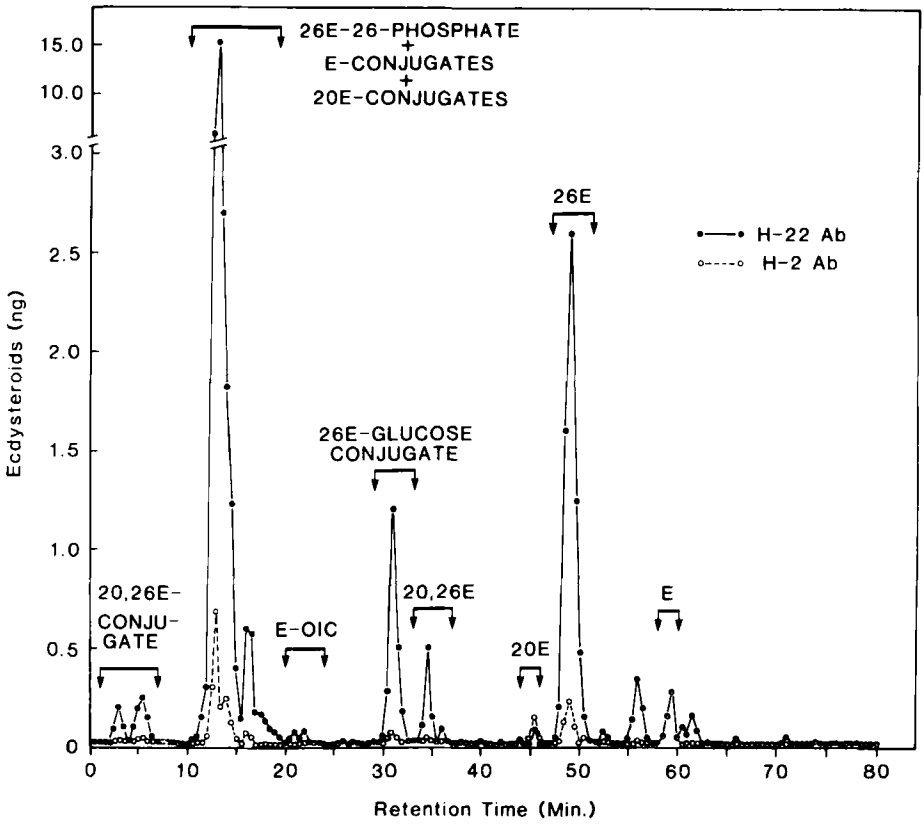

FIGURE 2. IS-RPHPLC/RIA analysis of ecdysteroids from 48 hr embryos. E = ecdysone, 20E = 20-hydroxyecdysone, 26E = 26-hydroxyecdysone, 20,26E = 20,26-dihydroxyecdysone, E-oic = ecdysonic acid, Ab = antibody.

HPLC

The recovery of RIA-positive material from the IS-RPHPLC elute was high in newly layed eggs (95%) but declined gradually as embryogenesis continued until only 75% was recovered from 96 hr embryos. The IS-RPHPLC/RIA analysis (expressed in ecdysone equivalents) of an extract of 48 hr embryos is shown in Fig. 2.

Designations indicate the identity of the eluted ecdysteroids, some following enzymatic deconjugation, as confirmed by additional reverse-phase and normal-phase co-chromatography with standards. Polar conjugates of ecdysteroids eluting before 25 min, and especially 26E-26-phosphate (13 min) are clearly separated from a less polar 26E conjugate at 31 min and the free ecdysteroids 20,26E (34.5 min), 20E (45.5 min), 26E (49 min) and E (59.5 min). The variable abilities of the two antisera to detect ecdysteroids are in accordance with their respective cross-reactivities, i.e. E is measured equally by both the H-2 and H-22 antisera while 20E is "seen" better by H-2 (x 4.5/3), and 26E by H-22 (x 15/1).

The advantages of the technique are more apparent in Fig. 3, showing the RIA analysis of IS-RPHPLC fractions of a gut extract from day 14 female Manduca developing adults (21). Three ecdysone conjugates of diverse polarities (23 min, 42 min, and 54.5 min) are detected readily by the H-22 antibodies, indicating the involvement of the C-22 hydroxyl with conjugating moieties. A C-2 ecdysone conjugate eluting at 56.5 min is detected differentially by the H-2 antibodies as are the 3-α-epiecdysteroids 20E' (50 min) and E' (62.5 min), and 2-deoxyecdysone (68 min).

Ecdysteroid Conjugate Concentrations in Developing Embryos

The individual ecdysteroid concentrations during embryogenesis, after correction for cross-reactivity and normalization per gram of eggs are presented in Fig. 4.

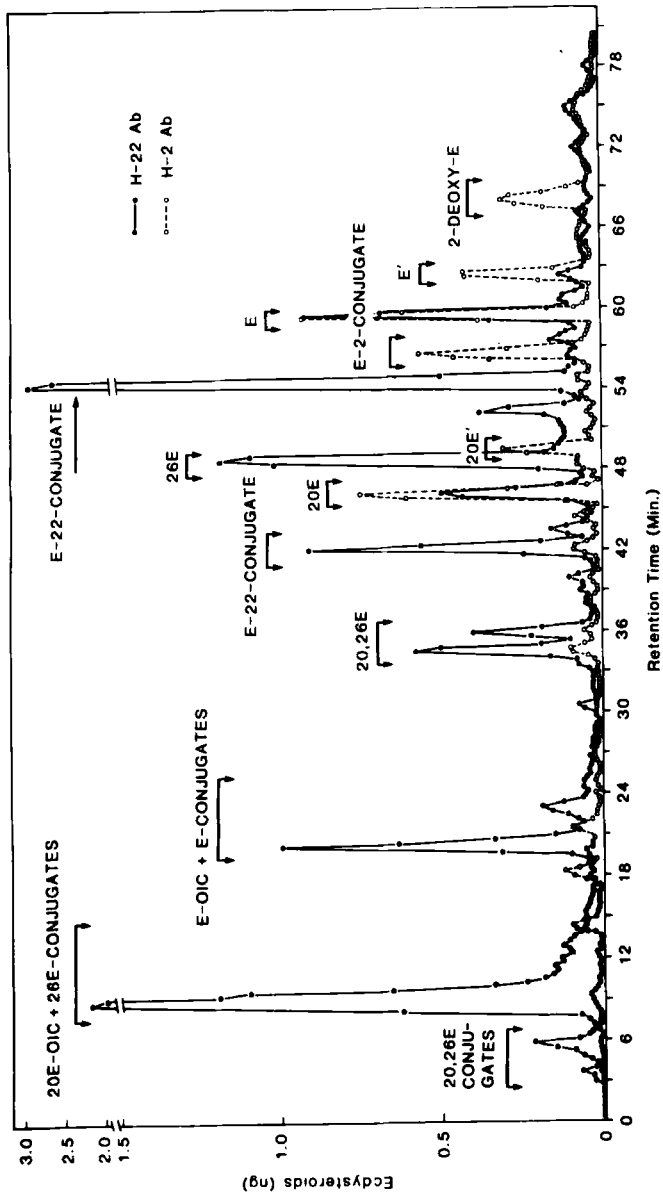

FIGURE 3. IS-RPHPLC/RIA analysis of ecdysteroids from the gut of day 14 female developing adults. Abbreviations as in Fig. 2. 20 E-oic = 20-hydroxyecdysonic acid, 2-deoxy-E = 2-deoxyecdysone, E' = 3-α-epimer of ecdysone, 20E' = 3-α-epimer of 20-hydroxyecdysone.

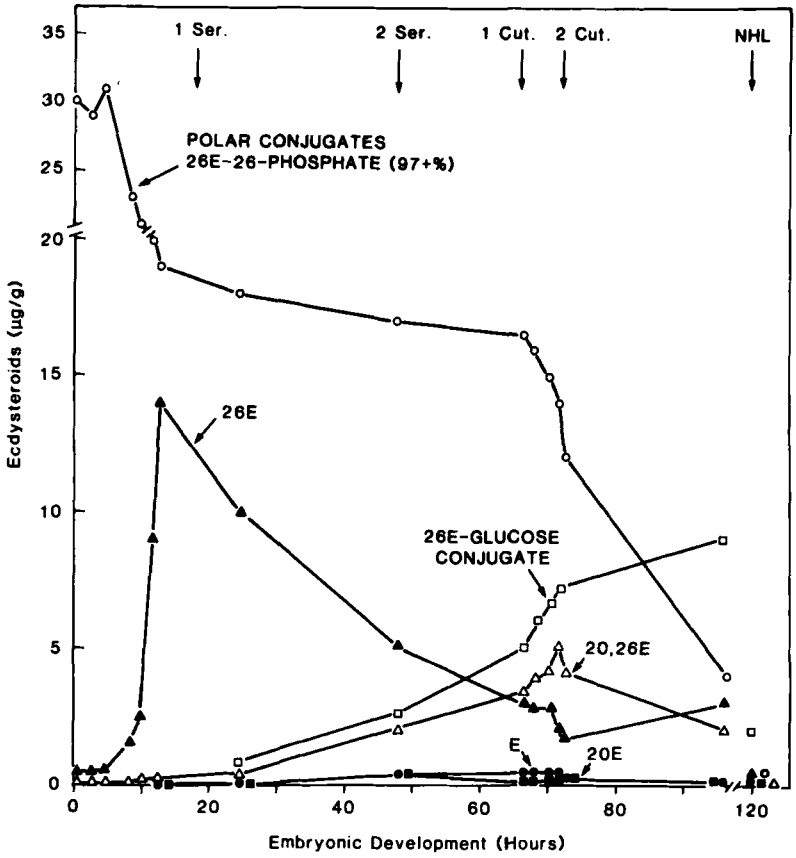

FIGURE 4. Ecdysteroid concentrations during embryogenesis. Abbreviations as in Figs. 2,3. Ser. = secretion of serosa, Cut. = secretion of cuticle, NHL = newly hatched larvae.

Concentrations of conjugates are expressed as the amount of free ecdysteroid released following total hydrolysis. In newly layed eggs (0-1 hr) as well as in 2-3 and 4-5 hr embryos, polar conjugates predominate. They consist of large amounts of 26E-26-phosphate (29 µg/g) and much smaller amounts of polar E and 20E

conjugates (0.6 μg/g and 0.2 μg/g, respectively). Other unidentified ecdysteroid conjugates, which following hydrolysis elute at 52.5, 56, 60.5, 61.5, 66 and 71 min (see Fig. 2) are consistently detected in eggs of all ages. Whenever free E and 20E were detected (48-72 hr) the same relative proportions of these other free ecdysteroids were present. Throughout embryonic development, even as the concentration of total polar conjugates falls, the relative amounts of these unhydrolyzed conjugated ecdysteroids (but not of 26E-26-phosphate) remained the same. They may represent methyl or other alkyl analogues of 26E and E such as 20-deoxymakisterone A, a C-24 methyl analogue of E identified in Drosophila ring gland secretions (27) and tentatively identified as a product of the prothoracic glands of Manduca pupae (unpublished information).

From 48 hr on however, small but increasing amounts of polar 20,26E conjugates are formed, either by embryonic conjugation of free 20,26E or hydroxylation of 26E-26-phosphate. On the basis of its chromatographic behavior trace amounts of ecdysonic acid may also be present. These are minor metabolites compared to the prominent ecdysteroid identified as β-D-glucopyranoside of 26E (see below) (31 min) which first appears in 24 hr embryos (0.9 μg/g) and which later attains a concentration of 9 μg/g prior to hatching. It is the most prominent RIA-positive ecdysteroid in newly hatched larvae (2 μg/g).

Free Ecdysteroids in Developing Embryos

In 4-5 hr embryos, 26E (0.4 μg/g) is the only free ecdysteroid detectable. During the next 8 hr, a period of yolk

cell formation and embryonic cell division, differentiation and movement, levels of free 26E rise dramatically to 14 μg/g, presumably as a result of the hydrolysis of 26E-26-phosphate. At this time, and 12 hr later, only trace amounts (0.10 g/g) of E or 20E are present.

In addition to 26E-β-D-glucose, another metabolite of 26E is detectable at 24 hr, i.e. 20,26E (0.4 μg/g). Its concentration increases to 5 μg/g by 72 hr as that of free 26E falls to 2 μg/g. Between 48 and 72 hr, less than 0.5 μg/g E and 0.3 μg/g 20E are present, along with the above mentioned unidentified ecdysteroids eluting between 52 and 72 min (Fig. 2). The concentrations of all ecdysteroids except the 26E-glucose conjugate declined prior to hatching and were at low levels in newly hatched larvae.

Identification of β-D-Glucose Conjugate of 26E

The RIA-positive (H-22 antisera) ecdysteroid at 31 min (Fig. 2) was identified as a conjugate since 26E was released upon enzymatic hydrolysis. The conjugate was not ionizable as its retention time did not change as the pH or counter-ion of the RPHPLC buffer was varied (25). Using material initially isolated during the above analytical procedures, the TLC characteristics (R_f = 0.6, HPTLC system B) and solvent/solvent partition coefficient (0.2, chloroform/ethanol/H_2O, 1:1:1) were determined for this ecdysteroid conjugate. Following extraction, hexane and then chloroform/ethanol partitioning, IS-RPHPLC (4 injections) and HPTLC (4 plates, system B), 140 μg (determined by RIA and u.v. absorption) was isolated and FAB+ mass spectra and PMN spectra were obtained.

The mass spectra contained the following species: (ion), mass (m/e), relative ion intensity; (M+1), 643, 10%; ($M+1-OH^-$) 625, 35%; ($M+1-H_2O$), 625, 30%; ($M-C_6H_{11}O_6$), 463, 30%; ($M-C_6H_{11}O_6-H_2O$), 445, 35%; ($M-C_6H_{11}O_6-2H_2O$), 427, 20%; 331, 100%; 301, 35%; ($C_{16}H_{11}O_6$), 179, 40%. The PMN spectra (200 MHz) of the conjugate in D_2O showed methyl resonances at δ 0.74 (C18, 3H); 0.96 (C21, 3H, d, J=6 Hz); 1.01 (C19, 3H); 1.18 (C27, 3H); in addition to other protons at δ 4.54 (C1 glucose anomeric β-H, 1H, d, J=8 Hz) and 5.98 (C7 steroid vinyl H, 1H). A complex of absorptions between δ 3.0-4.1 represented protons attached to hydroxyl bearing carbons of glucose and 26E (C26, C22, C3 and C2).

DISCUSSION

Pharmacokinetic data on the metabolism of ecdysteroids in insects can be obtained rapidly from limited biological material by IS-RPHPLC/RIA analysis. Prior knowledge of the general metabolic pathways and possession of metabolite standards is certainly helpful, but unknown metabolites also can be identified and quantified with the technique, i.e. the β-D-glucopyranoside of 26E in developing Manduca sexta embryos.

Glucose conjugates have been observed in insects previously, primarily following the administration of xenobiotic phenols (28,29), including many insecticides and synergists (30). Glucosylation of 1-naphthol by the tobacco hornworm requires exogenous UDPG and a glucosyltransferase present in fat body and midgut cytosol (31). Similar in vitro studies with Manduca tissue suggested the glucosylation of ecdysteroids (32), but such

metabolites have not been found, or identified unequivocally, *in situ* (12,13,33).

The M+1 molecular ion (m/e 643) in the FAB+ mass spectra of the ecdysteroid conjugate from newly hatched larvae is consistent with a hexose conjugate of 26E. Subsequently, the molecule can either lose a molecule of water (625) or break up into hexose (179) and ecdysone fragments (463), which then sequentially lose water molecules in a characteristic fashion (34). Methyl resonances characteristic of 26E are present in the PMR spectra of the conjugate (15), as is an ecdysteroid C7 vinyl proton absorption (δ5.98) and an absorption characteristic of a glucose C1 anomeric proton in the beta configuration (δ 4.54) (35). Integration of these latter absorptions indicates a 1:1 molar ratio of ecdysteroid and glucose. Finally, the proton resonances of free β-D-glucose in the "finger print" region (δ 3.0-4.0) closely match those of the conjugate. It was not possible to determine the position of glucose conjugation of 26E from spectral data; this will require further chemical modification/mass spectral analysis, i.e. of the acetonide(s) of the conjugate. However, only glucosylation at the C22 or C26 hydroxyls is consistent with the preferential binding of the conjugates to the H-22 antibodies.

The metabolism of ecdysteroids in developing *Manduca* embryos probably involves three major enzymatic reactions. Between 4 and 12 hr, about 50% of the highly polar maternal ecdysteroid conjugates (primarily 26E-26-phosphate) are deconjugated,

resulting in very high titers of free 26E but little E early in development (Fig. 4). It would be interesting to determine whether this hydrolysis (phosphatase) activity is associated with the peripheral yolk cells that appear near the embryo at this time.

Beginning at 24 hr, and continuing almost to hatching, a second enzyme (20-monooxygenase) converts free 26E to 20,26E, which accumulates and reaches a maximum (5 μg/g) at 72 hr, about the time of deposition of the first larval cuticle. Unlike 26E, which is inactive in bioassays for molting hormone activity, 20,26E retains 10% of the activity of 20E (36). This sequence (26E to 20,26E) is analogous to the conversion of E to 20E by a mitochondrial monooxygenase during larval and pupal life (1). As in these latter developmental stages, 20,26E is further metabolized in embryos to very polar conjugates (presumably morphogenetically inactive). Thus, 20,26E may play a role in coordinating biochemical events late in embryonic development.

The third enzyme is probably a UDP-α-D-glucosyltransferase that acts to remove free 26E from the system. The conjugate, an acetal, is apparently stable to the deconjugating enzymes mentioned above and is found in high concentration in newly hatched larvae. The function of this metabolite in the embryo is unknown as is the putative role of 26E.

The levels of E and 20E attained during embryonic development contrast with those of the aforementioned ecdysteroids. Prior to 24 hr only traces of E are present; at 48 hr the concentrations of

free E and 20E are less than the titers of the maternal conjugates of E and 20E present in 0-1 hr eggs. A day later (72 hr), about the time of the deposition of the larval cuticle, E and 20E levels are about the same as the day before; they remain below 0.5 μg/g and decline further prior to hatching. The brevity of embryonic development in Manduca (117 hr) may have prevented the detection of short-lived titer fluctuations. Alternatively, developmental processes may be exquisitely sensitive to E and 20E. Otherwise, it does not seem likely that E or 20E function to coordinate embryonic cuticular events as they do during postembryonic development (2). Neither does it seem likely that embryonic prothoracic glands (or other tissues) synthesize significant amounts of E during development.

In Manduca, maternal "storage" conjugates are progressively hydrolyzed, mainly to free 26E, which is then metabolized to both a potentially active hormone (20,26E) and a stable (inactivated?) 26E-glucose conjugate. The titer of 20,26E is then modulated further by conversion to polar conjugates (presumably inactive). Ecdysteroid acids that are present in developing embryos of Schistocerca gregaria (37), were detected in Manduca embryos, but only in trace amounts and their titer and fate were not pursued further.

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