Induction of an inactivation pathway for ecdysteroids in larvae of the cotton leafworm, *Spodoptera littoralis*

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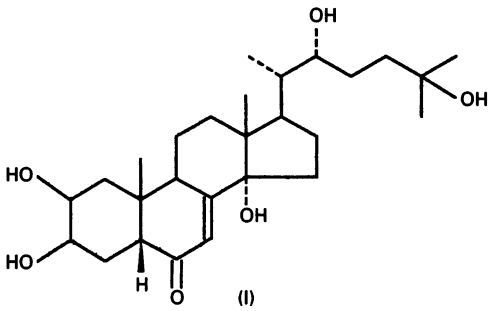
Treatment of the last-instar larvae of the cotton leafworm (*Spodoptera littoralis*) with ecdysteroids (moulting hormones) results in the induction of an ecdysteroid-inactivation pathway. Administration of ecdysone, 20-hydroxyecdysone or an ecdysteroid agonist, RH 5849, leads to induction of an ecdysteroid 26-hydroxylase activity. This induction occurred in both early sixth-instar larvae and in older larvae which had been head-ligated to prevent the normal developmental increase in ecdysone 20-mono-oxygenase activity. The induction of 26-hydroxylase activity requires both RNA and protein synthesis, as demonstrated by experiments involving actinomycin D and cycloheximide. The 26-aldehyde derivative of ecdysone and ecdyson-26-oic acid were also formed from ecdysone in the RH 5849-induced systems. Formation of the aldehyde and the corresponding 26-oic acid (ecdysonoic acid) from 26-hydroxyecdysone was directly demonstrated in a cell-free system, thus establishing the following inactivation pathway:

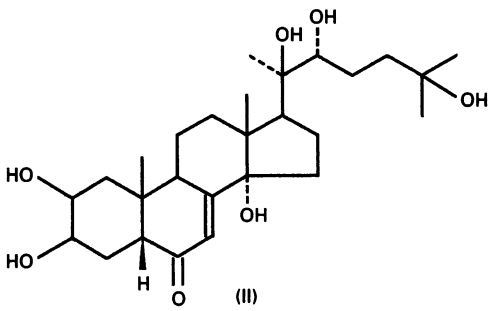
Ecdysteroid → 26-hydroxyecdysteroid → ecdysteroid 26-aldehyde → ecdysteroid 26-oic acid

INTRODUCTION

Moulting in insects is controlled by the insect moulting hormones (ecdysteroids). In most species, the major active hormone is 20-hydroxyecdysone (II), which is generally accompanied by its precusor, ecdysone (I; Koolman, 1989). The ecdysone 20-mono-oxygenase-catalysed C-20 hydroxylation of ecdysone (I), yielding 20-hydroxyecdysone (II), results in hormone activation (Smith, 1985; Weirich, 1989).



(I)



(II)

At specific stages in development, the ecdysteroid titre exhibits distinct peaks. Decreases in titre result from enhanced ecdysteroid inactivation, possibly with elevated excretion (Koolman and Karlson, 1985). A number of transformations may contribute to the inactivation of ecdysteroids, including the formation of ecdysteroid 26-oic acids, various conjugates (phosphates, acetates, glycosides, fatty acyl esters), and 3-epi-(3α-hydroxy) derivatives (Rees and Isaac, 1984; Lafont and Connat, 1989). Although the nature and abundance of the ecdysteroid-inactivation products depend upon species and developmental stage, the 26-oic acid derivatives are apparently ubiquitous. These acids are hormonally inactive and represent end products of inactivation. It has been shown *in vivo* that the 26-oic acids are formed from the parent ecdysteroid via the corresponding 26-hydroxy derivative (Isaac et al., 1983; Lafont et al., 1983). 26-Hydroxyecdysteroids were first isolated from the tobacco hornworm, *Manduca sexta* (Thompson et al., 1967; Kaplanis et al., 1973) and have subsequently been shown to be formed from ecdysteroids in several species, both *in vivo* and by various tissues *in vitro* (Koolman and Karlson, 1985).

It has not been possible to demonstrate 26-hydroxylation in experiments using homogenates or subcellular fractions of certain tissues under the same conditions as those used for ecdysone 20-hydroxylation. This is surprising, since the same intact tissues effect the reaction (see Lafont and Koolman, 1984). Similarly, the cell-free transformation of 26-hydroxyecdysteroids to the 26-oic acid has not been reported (Koolman and Karlson, 1985). Furthermore, there are reports that ecdysone 20-mono-oxygenase activity in both microsomal (Feyereisen and Durst, 1980) and mitochondrial (Srivatsan et al., 1987; Keogh et al., 1989) systems can be induced by administration of ecdysteroid *in vivo*.

We now report that administration of ecdysone, 20-hydroxyecdysone or RH 5849, a non-steroidal ecdysteroid agonist (Wing, 1988; Wing et al., 1988), to sixth-instar larvae of the cotton leafworm, *Spodoptera littoralis*, leads to induction of ecdysteroid 26-hydroxylase activity, but not of the 20-hydroxylase activity. This was demonstrated in both normal larvae and after head ligation, which prevents the normal developmental increase in ecdysone 20-mono-oxygenase activity (Keogh et al., 1989). Furthermore, the 26-aldehyde derivative of ecdysone and ecdysonoic acid were also formed in the induced systems. The

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intermediacy of the aldehyde in the conversion of 26-hydroxyecdysone into the corresponding 26-oic acid has also been demonstrated.

EXPERIMENTAL

Animals

Spodoptera littoralis were reared on an artificial diet under a photoperoid of 18 h light: 6 h dark at 28 °C and 60% relative humidity (Hoggard and Rees, 1988). Last (sixth)-instar larvae were used in this study, the instar lasting for 5 days. An almost synchronous population of animals was obtained at the end of the fifth instar and the larvae resynchronized at the beginning of the sixth instar; second-gate animals were mainly used (Zimowska et al., 1989). [A few larvae moult during a particular dark period ('first-gate' larvae), whereas the majority moult during the subsequent dark period ("second gate" larvae).]

Chemicals

[23,24-$^3H_2$]Ecdysone (82.8 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). D-Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, actinomycin D and cycloheximide were from Sigma (Poole, Dorset, U.K.). NADPH was obtained from Boehringer Mannheim (Lewes, East Sussex, U.K.). Ecdysteroid standards were kindly given by Dr. G. B. Russell, D.S.I.R., Palmerston North, New Zealand (20-hydroxyecdysone) and Dr. M. Feldlaufer, Insect Neurobiology and Hormone Laboratory, U.S.D.A., Beltsville, MD, U.S.A. (26-hydroxyecdysone). RH 5849 was a gift from Dr. K. Wing, Rohm and Haas Co., Spring House, PA, U.S.A..

Animal ligations and injections

Animals to be ligated and injected were anaesthetized on ice. Head ligations were carried out 46 h into the sixth instar between the head and prothoracic segments using waxed dental floss. All injections were made via abdominal segments using a 10 μl Hamilton syringe, and the injection sites were sealed with low-melting-point wax. Sham-injected animals served as controls. Ecdysone (8.0 μg/injection/larva), 20-hydroxyecdysone (8.0 μg/injection/larva) or RH 5849 (5.0 μg/injection/larva) were administered to the animals as double injections at 46 h and 65 h, respectively, into the instar. The inducing compounds were also administered into early sixth-instar animals at the times noted in the Results section as triple injections for ecdysone and 20-hydroxyecdysone (4.0 μg/injection/larva) and double injections for RH 5849 (2.0 μg/injection/larva). Cycloheximide and actinomycin D were administered to the animals as noted in the Results section. Injection volumes were all less than 5 μl each time.

Enzyme-activity assay

The fat body from the last-instar larvae was dissected (at the appropriate time of development) and homogenized using a Potter–Elvehjem homogenizer in ice-cold isotonic Hepes buffer (0.037 M, containing 0.3 M sucrose, 0.1 M KF), pH 7.5. Mitochondria were isolated by differential centrifugation and washed with the same buffer and then resuspended in a hypotonic Hepes buffer (0.037 M, containing 0.05 M sucrose and 0.1 KF) as described previously (Hoggard and Rees, 1988). The resulting broken mitochondrial suspension was used for ecdysone 26-hydroxylase assay. Briefly, the reaction mixture contained [23,24-$^3H_2$]ecdysone (final specific radioactivity 0.42 Ci/mmol), cofactors [NADPH (0.2 mM), glucose 6-phosphate (2.0 mM), glucose-6-phosphate dehydrogenase (0.2 unit)] and broken mitochondrial fraction (50 μl) in a total volume of 300 μl hypotonic Hepes buffer, pH 7.5. The reaction, which was carried out at 37 °C, was started by addition of the cofactors (30 μl) and was terminated by addition of methanol (300 μl). The mixture was then centrifuged for 10 min at 8800 *g* at 4 °C, and an aliquot of the supernatant was used for h.p.l.c. analysis.

H.p.l.c. analysis

H.p.l.c. analysis was carried out using two systems. System 1 was a reversed-phase Nova-Pak C_{18} Cartridge (10 cm × 8 mm, particle size 4 μm; Waters Associates) eluted at 1 ml/min with a linear gradient (60 min) of acetonitrile in Tris/$HClO_4$buffer (20 mM, pH 7.5) changing from 10 to 40% (v/v). The radioactivity was monitored by an on-line radioactivity monitor (Radiomatic A-200 Flo-one/beta; Canberra Packard, Pangbourne, Berks., U.K.). System 2 was an APS-Hypersil column (25 cm × 4.6 mm, particle size 5 μm; Shandon Southern Products, Runcorn, Cheshire, U.K.) eluted isocratically with 6% (v/v) methanol in 1,2-dichloroethane at 2 ml/min. Fractions collected at 1 min intervals were evaporated to dryness and assayed by liquid-scintillation counting. Ecdysteroid standards were detected by u.v. absorbance at 254 nm.

Chemical transformations

Reduction of ecdysone 26-aldehyde to 26-hydroxyecdysone was performed using sodium borohydride. The aldehyde was dissolved in 1 ml of dry redistilled ethanol/dry redistilled tetrahydrofuran (1:1, v/v), sodium borohydride (1 mg) was carefully added and the mixture allowed to react for 10 min at room temperature. The reaction was terminated by addition of 15 μl of acetic acid and then evaporated to dryness under a stream of N_2.

Ecdysteroid acids dissolved in methanol (1 ml) were methylated by addition of excess diazomethane in diethyl ether until the yellow colour persisted. The mixture was allowed to react for 20 min at room temperature and the reaction terminated by evaporation of the diazomethane together with solvent under a stream of N_2.

Protein assay

Protein was determined by the method of Lowry et al. (1951), using BSA as standard.

RESULTS

Induction of ecdysone 26-hydroxylase

As reported earlier, the activity of fat-body ecdysone 20-monooxygenase increases approx. 7-fold during the last larval instar of *Spodoptera littoralis* (Chen et al., 1994). In contrast, ecdysone 26-hydroxylase activity was undetectable *in vitro*, under the standard assay conditions, throughout the instar. This latter enzyme activity, however, can be induced by administration of either ecdysone, 20-hydroxyecdysone or an ecdysteroid agonist, RH 5849. In initial experiments, *S. littoralis* larvae were head-ligated 46 h after the fifth ecdysis to prevent the normal developmental increase in ecdysone 20-mono-oxygenase activity (Keogh et al., 1989). As shown in Figure 1, the head ligation prevented the developmental increase in ecdysone 20-mono-oxygenase activity. The administration of RH 5849 into the head-ligated larvae at 46 h and 65 h induced ecdysone 26-hydroxylase activity at 72 h

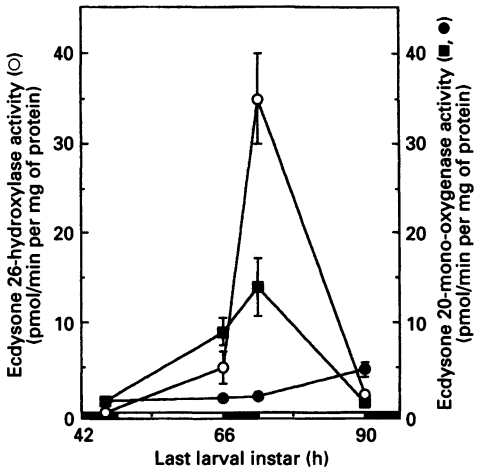


Figure 1 Effects of head ligation and administration of RH 5849 on ecdysone 20-mono-oxygenase and ecdysone 26-hydroxylase activities in last-instar larvae of *Spodoptera littoralis*

Larvae were head-ligated 46 h after the fifth ecdysis and administered with RH 5849 (5.0 μg/injection) at 46 and 65 h into the instar and enzyme activities were determined at the times indicated. Enzyme activities in fat-body mitochondria of larvae without treatment were also determined for control. ●, head-ligation only; ○, head-ligation + RH 5849 adminstration; ■, untreated control. The ecdysone 26-hydroxylase activity in fat-body mitochondria was undetectable (< 0.5 pmol/min per mg of protein) by the standard assay in sham-injected larvae (2.5 μl of methanol/injection per larva). Light and dark periods are indicated by open and black boxes respectively on the time scale. Values represent the mean (with range) for two independent experiments, except in the case of untreated control and at 72 h, where values are the means for at least three experiments.

Table 1 Induction of ecdysone 26-hydroxylase activities in last-instar larvae of *Spodoptera littoralis*

In the late stage, larvae were administered with the inducing compounds (ecdysone: 8 μg/injection; 20-hydroxyecdysone: 8 μg/injection; RH 5849: 5 μg/injection) at 46 and 65 h into the instar and 26-hydroxylase activity in fat-body mitochondria was determined at 72 h. In early stage, larvae were administered with the inducing compounds (ecdysone, 4 μg/injection; 20-hydroxyecdysone, 4 μg/injection; RH 5849, 2 μg/injection) 18, 24 and 42 h after the fifth ecdysis, and 26-hydroxylase activities in fat-body mitochondria were determined at 48 h, except for RH 5849 induction, in which the enzyme activity was assayed at 42 h. Values are means (with range) for two independent experiments, except in the case of RH 5849 induction in the late stage, where values are means (with S.E.M.) for three experiments. Sham-injected animals (2.5 μl of methanol/injection) served as controls.

Stage within sixth instar	Inducing compound	Ecdysone 26-hydroxylase activity (pmol/min per mg of protein)
Late (ligated)	Methanol	Not detectable (< 0.5)
Late (ligated)	Ecdysone	3.9 ± 0.8
Late (ligated)	20-Hydroxyecdysone	10.6 ± 3.0
Late (ligated)	RH 5849	32.0 ± 8.7
Late	RH 5849	29.4 ± 8.3
Early	Methanol	Not detectable (< 0.5)
Early	Ecdysone	3.5 ± 0.7
Early	20-Hydroxyecdysone	5.1 ± 0.6
Early	RH 5849	6.2 ± 0.7

(35 pmol/min per mg of protein); thereafter enzyme activity decreased sharply (Figure 1). In contrast, a single administration of RH 5849 at 46 h caused only a limited induction of ecdysone 26-hydroxylase activity (results not shown). On the basis of these observations, in subsequent experiments, inducing compounds were administered at 46 h and 65 h into the instar and enzyme activity was determined at 72 h. The ecdysone 26-hydroxylase activity in fat-body mitochondria was undetectable by the standard assay in sham-injected control larvae, whereas ecdysone, 20-hydroxyecdysone and RH 5849 all significantly enhanced the activity (Table 1). 20-Hydroxyecdysone was more effective than ecdysone, whereas RH 5849 showed a particularly strong inductive effect on enzyme activity (32 pmol/min per mg of protein) (Table 1). RH 5849 also induced ecdysone 26-hydroxylase activity to the same level in fat-body mitochondria of larvae which had not been head-ligated (Table 1). The induction of 26-hydroxylase with these compounds was also observed at an early stage of the last-instar larvae in which head ligation had not been performed, since the ecdysone 20-mono-oxygenase activity was naturally low (Table 1; also see Chen et al., 1994).

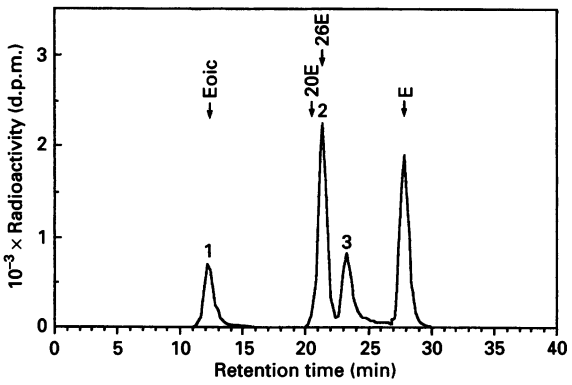


Figure 2 Reversed-phase h.p.l.c. radiochromatogram of the ecdysone metabolites formed by fat-body mitochondria from RH 5849-induced larvae

Fat-body mitochondria from RH 5849-induced *S. littoralis* larvae were incubated with [^{3}H]ecdysone under standard assay conditions and the reaction products were analysed by reversed-phase h.p.l.c. (system 1). The positions of elution of authentic ecdysone (E), 20-hydroxyecdysone (20E), 26-hydroxyecdysone (26E) and ecdysonoic acid (Eoic) are shown.

Identification of metabolites in the induced systems

Fat body mitochondria from induced larvae were incubated with [^{3}H]ecdysone under standard assay conditions and the reaction products in the methanolic solution, after removal of protein, were analysed by h.p.l.c. (system 1). Figure 2 shows a typical reversed-phase h.p.l.c. radiochromatogram of the products obtained after incubation of RH 5849-induced *S. littoralis* fat-body mitochondria with [^{3}H]ecdysone substrate. A major radioactive peak co-chromatographed with authentic 26-hydroxyecdysone (peak 2, retention time 21.5 min) just after 20-hydroxyecdysone. In addition to this major peak, two other metabolites were apparent, peak 1 (retention time 12.3 min) co-chromatographing with ecdysonoic acid and peak 3 (retention time 23.2 min).

The identity of the putative 26-hydroxyecdysone was further verified by h.p.l.c. analysis on an adsorption APS-Hypersil column (system 2). The major reaction product was purified by reversed-phase h.p.l.c. and subjected to h.p.l.c. analysis on system 2. As shown in Figure 3, the majority of the radioactivity co-chromatographed with authentic 26-hydroxyecdysone (retention time 26.0 min), whereas a small peak corresponded to authentic 20-hydroxyecdysone. The latter is the product of the ecdysone 20-mono-oxygenase-catalysed reaction, which is detectable in fat-body mitochondria from ligated animals throughout the

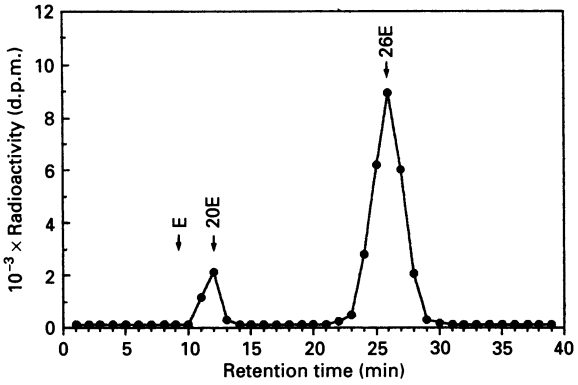


Figure 3 Identification of 26-hydroxyecdysone by adsorption h.p.l.c.

Peak 2 of Figure 2 was purified by reversed-phase h.p.l.c. and analysed by adsorption h.p.l.c. on an APS-Hypersil column (system 2). The positions of elution of authentic ecdysone (E), 20-hydroxyecdysone (20E) and 26-hydroxyecdysone (26E) are shown.

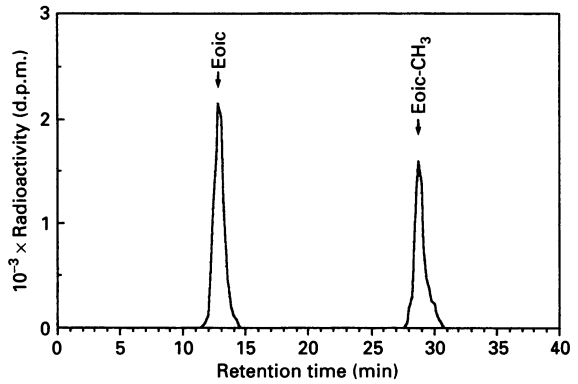


Figure 4 Identification of 26-ecdysonoic acid by methylation followed by reversed-phase h.p.l.c.

Peak 1 of Figure 2 was purified, subjected to the methylation reaction and the resulting products analysed by reversed-phase h.p.l.c. (for conditions, see the legend to Figure 2). The positions of elution of authentic ecdysonoic acid (Eoic) and the corresponding methyl ester (Eoic-CH_3) are shown.

developmental time under investigation [46–90 h; see Figure 1; see also Chen et al.(1994)].

The identity of peak 1, co-chromatographing with ecdysonoic acid on reversed-phase h.p.l.c. (Figure 2), was corroborated by methylation. The reaction product co-chromatographed with methyl ecdysonoate produced by simultaneous methylation of authentic ecdysonoic acid (Figure 4). However, a substantial amount of unchanged ecdysonoic acid was also apparent. The methylation product of ecdysonoic acid was unstable and tended to demethylate during evaporation of the reaction mixture (results not shown).

Peak 3, which was eluted between 26-hydroxyecdysone and ecdysone on reversed-phase h.p.l.c. (see Figure 2) was conceivably the 26-aldehyde derivative of ecdysone and was thus subjected to sodium borohydride reduction. The resulting product was analysed by reversed-phase (system 1) and adsorption (system 2) h.p.l.c. Figures 5(a) and 5(b) show that the reduced compound co-chromatographed with 26-hydroxyecdysone in both h.p.l.c. systems, suggesting that peak 3 may be the 26-aldehyde de-

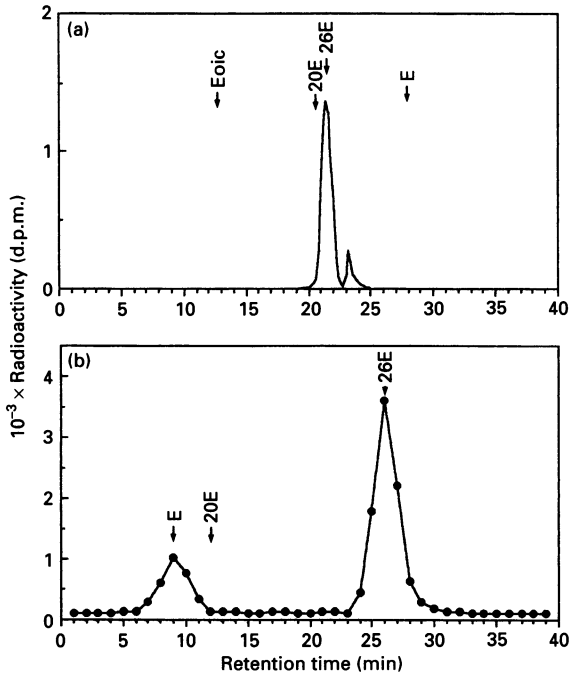


Figure 5 Identification of ecdysone 26-aldehyde by reduction with sodium borohydride treatment, followed by h.p.l.c. analysis

Peak 2 of Figure 2 was purified, subjected to reduction with sodium borohydride (see the text) and the product analysed by h.p.l.c. on system 1 (**a**) and system 2 (**b**). The positions of elution of authentic ecdysone (E), 20-hydroxyecdysone (20E), 26-hydroxyecdysone (26E) and ecdysonoic acid (Eoic) are shown.

rivative. A small amount of unchanged substrate was apparent on the reversed-phase system, and this eluted in the same position as ecdysone on the adsorption system.

Effect of actinomycin D and cycloheximide on induction of ecdysone 26-hydroxylase

To determine whether the induction of fat-body ecdysone 26-hydroxylase is dependent on transcription or protein synthesis, the effects of actinomycin D and cycloheximide were examined. Injection of actinomycin D (12.5 μg/injection per larva) together with RH 5849 (5.0 μg/injection per larva) at 46 and 65 h into the instar, with an additional administration of inhibitor at 54 h into insects ligated at 46 h, essentially abolished induction of fat-body mitochondrial ecdysone 26-hydroxylase activity by RH 5849 (Table 2). Cycloheximide injection (50 μg/injection per larva) at 22, 46 and 65 h into the instar, with head ligation of the insects at 46 h, caused an approx. 76% reduction in the RH 5849-induced fat-body mitochondrial ecdysone 26-hydroxylase activity (Table 2).

Transformations involved in the ecdysteroid inactivation pathway

In the RH 5849-induced system not only was 26-hydroxyecdysone formed under the standard assay conditions, but the putative 26-aldehyde derivative and ecdysonoic acid were detected as well (see Figure 2). This suggested that the 26-aldehyde may be an intermediate in the conversion of 26-hydroxyecdysone into ecdysone 26-oic acid and that the enzymes involved in these transformations might also be induced in the fat-body mitochondria. To verify these interconversions, the 26-hydroxyecdy-

Table 2 Effects of actinomycin D and cycloheximide on induction of 26-hydroxylase

Inhibitors were administered along with RH 5849 (5 μg/injection) into head-ligated *S. littoralis* larvae (for details, see the text), and the ecdysone 26-hydroxylase activity in fat-body mitochondria was determined under standard assay conditions. Values represent means (with range) for two independent experiments.

Treatment	Ecdysone 26-hydroxylase activity (pmol/min per mg of protein)
RH 5849	29.5 ± 8.3
RH 5849 + actinomycin D (12.5 μg/injection)	0.8 ± 0.1
RH 5849 + cycloheximide (50 μg/injection)	7.1 ± 0.9

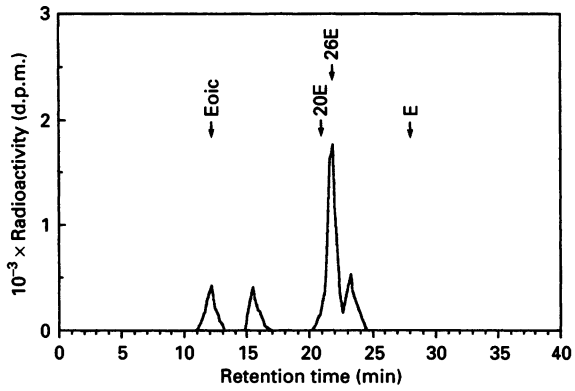
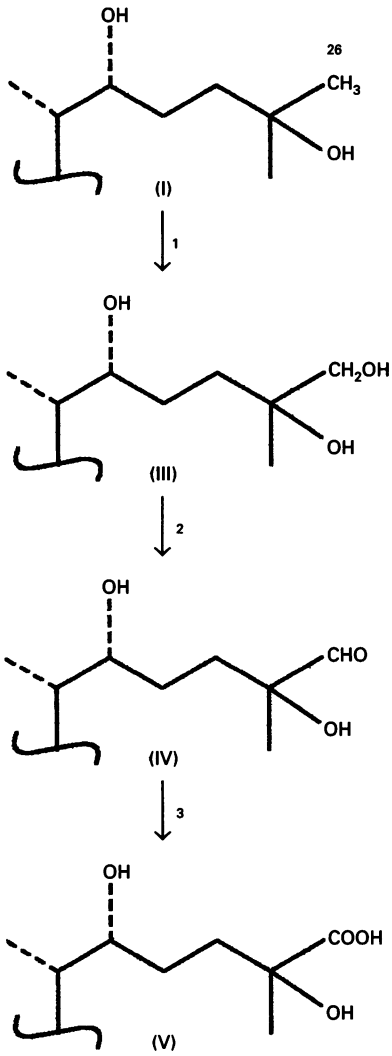


Figure 6 Reversed-phase h.p.l.c. radiochromatogram of the 26-hydroxyecdysone metabolites formed by fat-body mitochrondria from RH 5849-induced larvae

[^{3}H]26-Hydroxyecdysone purified by reversed-phase h.p.l.c. was incubated under the standard assay conditions with fat-body mitochondria from RH 5849-induced *S. littoralis* larvae and the reaction products were analysed by reversed-phase h.p.l.c. (system 1). The positions of elution of authentic ecdysone (E), 20-hydroxyecdysone (20E), 26-hydroxyecdysone (26E) and ecdysonoic acid (Eoic) are shown.



Scheme 1

sone and the 26-aldehyde derivative formed in the standard assay were purified by reversed-phase h.p.l.c., incubated separately with RH 5849-induced fat-body mitochondrial preparations and the products analysed by h.p.l.c. As can be seen in Figure 6, 26-hydroxyecdysone was converted into the 26-aldehyde derivative (retention time 23.4 min) and further into ecdysonoic acid, with an additional unknown compound being eluted at 15.6 min. The induced enzyme preparation, however, did not convert the 26-aldehyde derivative of ecdysone into ecdysonoic acid (results not shown). This may not be surprising if a multifuntional enzyme is involved in the interconversion (see the Discussion section).

DISCUSSION

The present study clearly demonstrates that enzymes responsible for the inactivation of ecdysteroids in *Spodoptera littoralis* can be induced by administration of both ecdysteroids and the ecdysteroid agonist RH 5849. The induction of ecdysone 26-hydroxylase activity, by administration of either ecdysteroids or RH 5849, has been shown in both early-sixth-instar and in older larvae. 20-Hydroxyecdysone was a more effective inducer than ecdysone, suggesting that ecdysone must first undergo 20-hydroxylation in order for it to exhibit inductive activity. The effectiveness of RH 5849 may, in part, be a consequence of its lower metabolism and/or excretion (Wing, 1988; Wing et al., 1988).

Evidence has been furnished for the intermediacy of the aldehyde (IV) in the conversion of 26-hydroxyecdysone (III) into the corresponding ecdysonoic acid (V) as indicated in Scheme 1.

Induction of enzymes involved in conversion of 26-hydroxyecdysone into the corresponding 26-oic acid was not observed by any of the inducing compounds in early sixth-instar larvae. In older larvae, this induction was observed only with RH 5849. It may be that the 26-aldehyde (IV) and acid (V) are detectable only when there is appreciable 26-hydroxyecdysone formed to serve as substrate for reactions 2 and 3 in Scheme 1. When 26-hydroxyecdysone (III) was used as substrate with RH 5849-induced mitochondria, both the 26-aldehyde (IV) and acid (V) were detectable (Figure 6). However, use of the 26-aldehyde (IV) in such an induced system did not yield the corresponding acid (V); the reason for this is unclear.

The induction of an inactivating steroid hydroxylase activity by its substrate is reminiscent of induction of catabolism of

1,25-dihydroxycholecalciferol (1,25-dihydroxyvitamin D_3) by the hormone itself in mammalian tissues (Napoli et al., 1991). The transformations in Scheme 1 are also analogous to those occurring during sterol side-chain modifications involved in bile acid synthesis (Björkhem, 1985). Three systems have been proposed to account for this oxidative sequence (Dahlback and Holmberg, 1990): (i) according to the classical scheme (Dahlback and Holmberg, 1990), oxidation of 26-hydroxysteroid via the aldehyde to the acid is catalysed by the sequential actions of a cytosolic isoenzyme of alcohol dehydrogenase (Okuda and Okuda, 1983) and an NAD^+-dependent aldehyde dehydrogenase (Okuda et al., 1973); (ii) an NAD^+-dependent mitochondrial enzyme system from rabbit liver has been shown to catalyse the transformation of 26-hydroxysteroid to the acid; inhibition of 26-hydroxy group oxidation in both this and the cytosolic system (i), with the potent ethanol dehydrogenase inhibitor 4-heptylpyrazole, indicates that these two systems share some common properties (Dahlback et al., 1988); in this context, it is also relevant that superfamilies of aldehyde dehydrogenases, the members of which include non-specific cytosolic, mitochondrial and microsomal enzymes, have been recognized (Kedishvili et al., 1992; Johansson et al., 1988); (iii) interestingly, a mitochondrial cytochrome $P\text{-}450_{26}$, which catalyses 26-hydroxylation during bile acid synthesis, has recently been shown to further transform the 26-hydroxysteroid into the corresponding acid; a mechanism has been proposed, involving a 26-aldehyde intermediate (Cali and Russell, 1991). This enzyme requires ferredoxin, ferredoxin reductase and NADPH for catalytic activity (Cali and Russell, 1991). If this latter mechanism was operative in ecdysteroid 26-oic acid formation, it may explain why we were unable to demonstrate conversion of exogenous ecdysone 26-aldehyde into the acid.

In the migratory locust, *Locusta migratoria*, ecdysteroids have been reported to induce microsomal ecdysone 20-mono-oxygenase activity, the induction being prevented by inhibitors of RNA and protein biosynthesis (Feyereisen and Durst, 1980). Furthermore, mitochondrial 20-mono-oxygenase activity is induced by ecdysone in *Musca domestica* (housefly) larvae (Srivatsan et al., 1987). Similarly, Keogh and co-workers reported that both ecdysteroids and RH 5849 induce mitochondrial ecdysone 20-mono-oxygenase activity in ligated *Manduca sexta* larval midgut, the induction being prevented by actinomycin D and cycloheximide (Keogh et al., 1989; Keogh and Smith, 1991). In order to establish whether different tissues may respond to ecdysteroids and RH 5849 in different ways, we assayed midgut from *S. littoralis* larvae administered with either ecdysteroids or RH 5849. We found that both ecdysteroids and RH 5849 administration leads to the induction of a 26-hydroxylase activity, but not the 20-mono-oxygenase activity (J. H. Chen, M. Kabbouh, M. J. Fisher and H. H. Rees, unpublished work). We also found that the induced 26-hydroxylation activity is present in both mitochondrial and post-mitochondrial fractions of fat body and midgut. Further metabolism of the 26-hydroxyecdysone to the corresponding aldehyde and acid also occurs in these subcellular fractions of both these tissues. However, the pattern of metabolites was somewhat more complex in midgut fractions (J. H. Chen, M. Kabbouh, M. J. Fisher and H. H. Rees, unpublished work).

Although we do not know whether the process of induction differs from species to species, it is possible that erronous conclusions could be reached as a consequence of the very similar chromatographic properties on a given chromatographic system of 20-hydroxyecdysone (the product of the 20-mono-oxygenase-catalysed reaction) and of its isomer, 26-hydroxyecdysone. For instance, in the present study, 26-hydroxyecdysone and 20-hydroxyecdysone chromatograph very closely in the reversed-phase h.p.l.c. system (see Figure 2), while in the adsorption-chromatographic system, ecdysone and its 26-aldehyde derivative co-chromatograph (see Figure 5b).

It is conceivable that hormonal induction of 26-hydroxylation activity may involve either transcriptional and/or post-transcriptional control of gene expression. That induction of 26-hydroxylase activity requires RNA and protein synthesis was demonstrated in experiments involving actinomycin D and cycloheximide. Mitochondrial steroid hydroxylation systems typically consist of an FAD-containing NADPH:ferrodoxin reductase, ferredoxin (an iron–sulphur protein), and cytochrome *P*-450. However, microsomal systems generally consist of only two components, the flavoprotein, NADPH:cytochrome *P*-450 reductase and cytochrome *P*-450, with the possible involvement of NADH:cytochrome b_5 reductase and cytochrome b_5 in some cases (Ortiz de Montellano, 1986). It appears to be a general phenomenon that changes in cytochrome *P*-450-mediated enzyme activities are accompanied by concomitant changes in cytochrome *P*-450 levels (Waterman et al., 1986; Imaoka et al., 1991). During our studies on the mitochondrial ecdysone 20-mono-oxygenase system in fat body of *S. littoralis*, we have demonstrated that antibodies raised against components of vertebrate steroidogenic enzyme systems detect specific immunoreactive polypeptides in fat-body mitochondrial extracts (Chen et al., 1994). This suggests that *S. littoralis* steroid hydroxylation system(s) may contain polypeptide components analogous to those present in vertebrates. Thus, induction of 26-hydroxylase activity might be expected to be a consequence of alterations in cytochrome *P*-450 gene transcription and protein synthesis.

We thank the Science and Engineering Research Council (U.K.) for financial support.

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Received 13 December 1993/26 January 1994; accepted 3 February 1994