Enzymes of ecdysteroid transformation and inactivation in the midgut of the cotton leafworm, *Spodoptera littoralis*: properties and developmental profiles

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In the midgut cytosol of Lepidoptera, ecdysteroids undergo inactivation by transformation via the 3-dehydro derivative to the corresponding 3-epiecdysteroid (3 α -hydroxy) and by phosphate conjugation. The oxygen-dependent oxidase catalyses formation of 3-dehydroecdysteroid, which can be reduced either irreversibly by 3-dehydroecdysone 3 α -reductase to 3-epiecdysteroid, or by 3-dehydroecdysone 3 β -reductase back to the initial ecdysteroid. Furthermore, these ecdysteroids undergo further inactivation by phosphorylation. These ecdysteroid transformations have been investigated in last instar larvae of the cotton leafworm, *Spodoptera littoralis*. The products of the phosphorylation have been characterized as predominantly ecdysteroid 2-phosphate accompanied by smaller amounts of the corresponding 22-phosphate. The phosphotransferases require Mg²⁺ and ATP. Whereas the 3-dehydroecdysone 3 α -reductase has a clear preference for NADPH rather than NADH, the corresponding 3 β -reductase markedly favours NADH. The physiological significance of the latter enzyme is unclear. The

profiles of the various enzymic activities in dialysed midgut cytosol supplemented with appropriate cofactors were determined throughout the last larval instar. All activities were detectable throughout the instar, but the respective enzymes exhibited maxima at different times. Ecdysone oxidase showed a peak early in the instar, with 3-dehydroecdysone 3 α -reductase increasing to a peak as the former activity declined. The 3-dehydroecdysone 3 β -reductase exhibited peak activity late in the instar, a profile similar to that observed for the corresponding haemolymph enzyme involved in reduction of the 3-dehydroecdysone product of the prothoracic glands to ecdysone. Thus, the significance of the midgut 3 β -reductase may be related to production of active hormone. Both ecdysteroid 22- and 2-phosphotransferases showed high activities early in the instar and then declined. The physiological significance of the profiles for the ecdysone oxidase, the 3-dehydroecdysone 3 α -reductase and phosphotransferases is unclear.

INTRODUCTION

A prominent pathway of inactivation of ecdysteroids (insect moulting hormones) in the midgut of Lepidoptera occurs via a two-step epimerization system (Figure 1; [1–4]), with analogous reactions occurring in the case of ecdysone and 20-hydroxyecdysone. The first step is the irreversible oxidation of ecdysteroid to 3-dehydroecdysteroid, catalysed by ecdysone oxidase (EC 1.1.3.16; Figure 1, enzyme 1, (for reviews see [5–7])). 3-Dehydroecdysteroids are regarded as catabolites of low hormonal activity, having only 0.07–0.1 of the activity of the parent compound in the *Calliphora* bioassay [8] and in two different *Drosophila* chromosome puff assays [9].

It is well established that 3-dehydroecdysteroids do not appreciably accumulate in the insect midgut *in vivo*, but are converted into 3 α -hydroxy epimers (3-epiecdysteroids) by a 3-dehydroecdysteroid 3 α -reductase enzyme (EC 1.1.1.1; Figure 1, enzyme 2). Epimerization *in vitro* of ecdysteroids by the midgut cytosol of *Manduca sexta* was first demonstrated by Nigg et al. [1], the 3 α -epimers (3-epiecdysteroids) being much less active than the corresponding 3 β -hydroxyecdysteroids in the *Musca* bioassay [1,10]. Since 3-epiecdysone cannot be converted back into a 3 β -hydroxy metabolite [2,11], 3 α -epimers represent irreversible removal of hormonal activity. However, 3-dehydroecdysteroids can be converted back into their 3 β -hydroxy parent

compounds, catalysed by 3-dehydroecdysteroid 3 β -reductases (EC 1.1.1.1; Figure 1, enzyme 3; [3,4,12]) and 3-dehydroecdysteroids, therefore, could be regarded as transient inactive forms of hormone.

In the cotton leafworm, *Spodoptera littoralis*, the 3 α -hydroxy-forming reductase preferentially uses NADPH, whereas the 3 β -hydroxy-forming one shows a preference for NADH [4]; however, the converse holds for *Manduca sexta* [12]. Thus, further investigation of the cofactor requirements of these reductases is warranted.

The ecdysteroids and their 3-dehydro- and 3-epi-metabolites may be further inactivated by the action of phosphotransferase enzymes (EC 2.7.1.1; Figure 1, enzyme 4; [7,13]). Phosphate conjugates are prominent inactivation products of ecdysteroids in immature stages of several insect species (for reviews see [14,15]). However, in many cases, the products have been incompletely characterized. There is also a paucity of information on the properties of the phosphotransferase enzymes catalysing such reactions [13].

We now report results on characterization of the enzymes which catalyse the enzyme reactions shown in Figure 1 in the midgut of last (sixth) larval instar *Spodoptera littoralis*. This includes co-substrate requisites of the enzymes, with particular emphasis on the 3-dehydroecdysone reductases, characterization of the reaction products (especially the phosphate conjugates),

Abbreviation used: FAB-MS, fast-atom bombardment mass spectrometry.

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Figure 1 Inactivation of ecdysteroids in the midgut of *Lepidoptera*

(1) Ecdysone oxidase, (2) 3-dehydroecdysone 3 α -reductase, (3) 3-dehydroecdysone 3 β -reductase and (4) ATP:ecdysone 2- and 22-phosphotransferases.

and finally, the developmental profile of the activity of the enzymes throughout the instar. Since both ecdysone and 20-hydroxyecdysone undergo the reactions of interest, ecdysone (Figure 2) and its derivatives have been used for convenience in the current work.

EXPERIMENTAL

Materials

Ecdysone was purchased from Simes, Milan, Italy; [23,24-³H₂]ecdysone (89.0 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. Authentic 3-dehydroecdysone and 3-epiecdysone were chemically synthesized according to the method of Dinan and Rees [16] and characterized by negative-ion fast-atom bombardment mass spectrometry (FAB-MS) and ¹H-NMR. [23,24-³H₂]3-Dehydroecdysone was enzymically prepared from [23,24-³H₂]ecdysone by incubation with a dialysed post-microsomal midgut preparation of *S. littoralis* at 37 °C for 18 h. Authentic ecdysone 2- and 22-phosphates, isolated from embryonated or newly laid *Schistocerca gregaria* eggs [17,18],

were characterized by FAB-MS and NMR spectra by Dr. M. Kabbouh, (University of Liverpool, U.K.). Standard ecdysone 2,3-acetonide was prepared according to the method of Galbraith and Horn [19] and characterized using FAB-MS, by Dr. R. M. Magee (University of Liverpool, U.K.).

Culture of *Spodoptera littoralis*

Sixth instar *S. littoralis* were reared on an artificial diet [20] under a photoperiod of 18 h light:6 h dark at 28 °C and 70 % relative humidity. Under these conditions, the final (sixth) larval instar lasted 5 days. Insects were synchronized at the 5th to 6th larval instar moult by selection of animals in which the head and mouthparts had fused during the scotophase. The newly moulted sixth instar larvae were designated $t = 3 \pm 3$ h, assuming an average moult midway through the dark period.

Enzyme preparation

Approximately 20 *S. littoralis* larvae were routinely dissected in ice-cold Insect Ringer solution [21] and the midguts excised. Cytosol was prepared according to the method of Milner and Rees [4]. The supernatant was dialysed against 100 vol. of 10 mM Tris/HCl buffer, pH 7.5, for 18 h, to remove endogenous cofactors.

Enzyme assays

Enzyme assays consisted of enzyme preparation (100 μ l; generally 0.5–1 mg of protein) and the required cofactor in 0.2 M sodium phosphate buffer, pH 7.4 (100 μ l). After pre-incubation for 5–10 min at 37 °C, the reaction was initiated by addition of the [23,24-³H₂]ecdysteroid substrate (8.4 μ M; 0.1 μ Ci) in the same buffer (50 μ l) and incubation continued for 60 min (total incubation volume, 250 μ l).

Reactions were terminated by addition of chilled ethanol (400 μ l). The mixture was then centrifuged for 10 min at 9000 g at 4 °C, the supernatant collected, the pellet re-extracted with

Figure 2 Structure of ecdysone

methanol and the combined alcoholic extracts evaporated to dryness under vacuum in a centrifugal evaporator (Gyrovap, Uniscience). The reaction products were analysed by HPLC with detection of radioactivity using an on-line monitor (Radiomatic A-200 Flo-One Beta; Canberra Packard, Pangbourne, Berks., U.K.). Enzymic activities (nmol/h per mg of protein) are the means (with S.E.M.) of two independent experiments, with enzymic assays in duplicate. Apparent transformations observed in parallel 'control incubations' of boiled enzyme preparation have been subtracted from enzymic activities.

For the characterization of the enzymic product(s), activity was assayed using 50 μ g of ecdysteroid. Following HPLC, the reaction products were detected by UV absorbance at 254 nm.

To study the effects of anaerobic conditions, mixtures containing dialysed enzyme preparation (100 μ l; 0.5–1.0 mg of protein), the appropriate cofactor (0.5 mM NADH, or 0.5 mM NADPH, or 2 mM ATP/10 mM $MgCl_2$) in 0.2 M sodium phosphate buffer, pH 7.4, and reagents of the glucose oxidase–peroxidase system (below) were transferred to a heating block set at 37 °C. Vials were stoppered using 'Suba' seal stoppers and two 19G needles inserted into each. One of the needles was attached, via a three-way tap, to a vacuum pump and a nitrogen gas inlet, the latter two valves being mutually exclusive. The vials were repeatedly evacuated for 1 min, then filled with nitrogen gas for 1 min, throughout the 10 min equilibrium period. The reactions were initiated by injection of [3H]ecdysteroid substrate (0.1 μ Ci; 8.4 μ M) via the second needle. During the reaction, the mixtures were continuously flushed with nitrogen. Any remaining dissolved oxygen was removed by the glucose oxidase–peroxidase system [22], bought as a kit from Boehringer–Mannheim GmbH (Germany; ABTS dye [2,2'-azino-bis(ethylbenzthiazoline-6-sulphonic acid)] (1.0 mg/ml), glucose (0.51 mM), peroxidase (0.4 unit), glucose oxidase (5.0 units), in 100 mM phosphate buffer, pH 7.0). Reactions were terminated after 1 h (the maximal velocity of the glucose oxidase–peroxidase system was known to be attained after 15 min). Control incubations contained (i) boiled enzymes, with the reaction being performed in the presence or absence of nitrogen, or (ii) no glucose oxidase–peroxidase system, again, being carried out in the presence or absence of nitrogen.

Protein estimation

Protein content was estimated by the Folin-phenol method using BSA as the protein standard [23].

HPLC analysis

Samples containing free ecdysteroids were analysed using a C_{18} Nova-Pak cartridge (10 cm $\times$ 8 mm, 10 μ m particle size, Waters Associates), linked to a Waters model 440 UV detector set at 254 nm with radioactivity being monitored by an on-line detector. An isocratic mobile phase of 0.1 % (v/v) trifluoroacetic acid in water:acetonitrile (78:22, v/v) was employed at a flow rate of 1.0 ml/min (system 1). Adsorption HPLC was carried out using a μ Bondpak NH_2 Nova-Pak cartridge (10 cm $\times$ 8 mm, 10 μ m particle size, Waters Associates) eluted with 8 % (v/v) methanol in 1,2-dichloroethane at a flow rate of 1.0 ml/min (system 2).

Polar ecdysteroids (conjugates) were identified by chromatography on the foregoing reversed-phase column, using two solvent systems: (i) a linear 30 min gradient of 20–70 % (v/v) methanol in 20 mM citrate buffer, pH 6.5, at 1.0 ml/min (system 3); and (ii) a linear 60 min gradient of 8–40 % (v/v) acetonitrile in 20 mM Tris-perchlorate buffer, pH 7.5, at 1.0 ml/min (system 4).

Radioimmunoassay (RIA)

Ecdysteroids were quantified by RIA [24], with H-22 antiserum supplied by Professor L. I. Gilbert (University of North Carolina, U.S.A.). H-22 antiserum is specific for the ecdysteroid nucleus and was, therefore, used to estimate haemolymph ecdysone and 20-hydroxyecdysone during the last larval instar [25,26]. All assays used ecdysone as standard, with the bound and unbound [$^{23,24-3}H$]ecdysone being separated by ammonium sulphate precipitation.

MS analysis

Negative-ion FAB-MS spectra were obtained on a VG 7070H mass spectrometer, using a primary atom beam of xenon and glycerol as the probe solvent. (For conditions see [27]).

Preparation of enzymic products for characterization

The enzymic assays were carried out as described, except that incubations included 50 μ g (0.43 mM) of the appropriate unlabelled ecdysteroid, appropriate cofactors and enzyme preparation (approx. 3–5 mg of protein) in 0.2 M sodium phosphate buffer, pH 7.4 (650 μ l total volume). Extracted reaction mixtures were desalted on C_{18} Sep-Pak cartridges. For this, samples were loaded in 10 % (v/v) methanol in water (2 ml) and salts and proteins eluted with 2 ml of the same solvent before elution of polar conjugates with 30 % (v/v) methanol in water (4 ml) and of the free ecdysteroids with 60 % (v/v) methanol in water (6 ml). Analysis of the reaction products was by HPLC using UV detection.

For identification of the ecdysteroid phosphate esters, hydrolysis of the conjugates was carried out using a crude so-called 'arylsulphatase' preparation from *Helix pomatia* (Sigma) [27]. This enzyme preparation has been shown to contain appreciable phosphatase activity and to hydrolyse ecdysteroid 2- and 22-phosphates [17,18]. Acetonides of the conjugates were prepared by the method of Galbraith and Horn [19].

RESULTS

Characterization of the enzymic products

For characterization of the products of the enzyme reactions, larger amounts of unlabelled substrates (approx. 50 μ g) were used in the incubations. After incubation, the products were desalted on C_{18} Sep-Pak cartridges and applied to HPLC with UV detection for analysis. In addition, samples from the reductase and ecdysone oxidase incubations were subjected to negative-ion FAB-MS, for molecular mass determination.

(a) Product of the ecdysone oxidase assay

Putative 3-dehydroecdysone co-chromatographed with an authentic sample on both reversed-phase and adsorption HPLC (systems 1 and 2). Furthermore, a negative-ion FAB-MS spectrum revealed a prominent ion at m/z 461 [$M-H$] $^-$, corresponding to a relative molecular mass of 462.

To substantiate the identity of the product, approx. 5.0 μ g of the recovered putative 3-dehydroecdysone was chemically reduced using sodium borohydride [16]. The major reduction product (38.4 % of reduced products) co-chromatographed with authentic 3-epiecdysone on reversed-phase HPLC (system 1) accompanied by a small peak corresponding to ecdysone (8.1 %).

Figure 3 Acetonide reaction

(b) The 3-dehydroecdysone 3 α -reductase assay (with NADPH as cofactor)

Using reversed-phase and adsorption HPLC (systems 1 and 2), ecdysone and 3-epiecdysone are readily separated and the major product (81.5% of reduced metabolites) of the 3-dehydroecdysone 3 α -reductase assay (with NADPH as cofactor) co-chromatographed with authentic 3-epiecdysone (retention times; system 1: ecdysone, 9.9 min; 3-epiecdysone, 10.8 min; 3-dehydroecdysone, 15.2 min; system 2: ecdysone, 18.2 min; 3-epiecdysone, 15.1 min; 3-dehydroecdysone, 8.8 min). Negative-ion FAB-MS confirmed that the product had a relative molecular mass of 464, since a peak at m/z 463 [$M-H$]⁻ was obtained.

A minor peak corresponding to ecdysone on HPLC system 1 was also apparent (18.5%).

(c) The 3-dehydroecdysone 3 β -reductase assay (with NADH as cofactor)

Evidence that the major product (89.4% of reduced products) corresponded to ecdysone was provided by reversed-phase and adsorption HPLC (systems 1 and 2), and by negative-ion FAB-MS, which revealed an ion at m/z 463 [$M-H$]⁻, confirming a relative molecular mass of 464.

A minor product co-eluted with authentic 3-epiecdysone (10.6%), using reversed-phase HPLC (system 1).

(d) The products of the ATP: ecdysone 2- and 22-phosphotransferase reactions

ATP and MgCl₂ were used as the cofactors in an assay containing unlabelled ecdysone. The reaction products were collected and re-analysed using HPLC systems 3 and 4. In both cases, the most polar metabolite (7.3% of total ecdysteroid phosphates) co-chromatographed with ecdysone 22-phosphate and the less polar one with ecdysone 2-phosphate (20.9%). However, this was not a complete confirmation of the positional isomers of the phosphates. This was achieved by the chemical synthesis of ecdysteroid acetonides, since it was not practical to obtain sufficient for mass spectrometric or NMR analysis. The strategy adopted involved enzymic preparation of unlabelled ecdysone and 20-hydroxyecdysone phosphates, using dialysed cytosol, supplemented with MgCl₂ and ATP. The putative phosphates were separated by HPLC system 3, before being subjected to chemical acetonide formation. Acetonide formation (Figure 3) renders the compounds less polar.

Since acetonide production requires a *cis*-vicinal diol, the presence of a phosphate at C-2 or C-3, or in the case of 20-hydroxyecdysone, at C-20 or C-22, would block derivative formation. The various possible events are outlined below.

(i) 20-Hydroxyecdysone 2-phosphate $\rightarrow$ ecdysone 20,22-acetonide 2-phosphate

(ii) 20-Hydroxyecdysone 22-phosphate $\rightarrow$ 20-hydroxyecdysone 2,3-acetonide 22-phosphate

(iii) Ecdysone 22-phosphate $\rightarrow$ ecdysone 2,3-acetonide 22-phosphate

(iv) Ecdysone 2-phosphate $\rightarrow$ unable to form acetonide (blocked at C-2)

The products were analysed by reversed-phase HPLC (system 3).

The ecdysteroid phosphate-esters, upon acetonide formation, were converted into more hydrophobic compounds, as revealed by reversed-phase HPLC (Table 1). The retention time of the putative ecdysone 2-phosphate, after being subjected to the acetonide formation reaction, remained the same, since no acetonide could be formed. This indicates that the molecule must be blocked (phosphorylated) at either C-2 or C-3.

The ecdysteroid phospho-acetonides were hydrolysed using a crude so-called arylsulphatase preparation from *Helix pomatia*, to give the acetonides. The expected reactions were:

(i) ecdysone 20,22-acetonide 2-phosphate $\rightarrow$ ecdysone 20,22-acetonide

(ii) 20-hydroxyecdysone 2,3-acetonide $\rightarrow$ 20-hydroxyecdysone 2,3-acetonide

(iii) ecdysone 2,3-acetonide 22-phosphate $\rightarrow$ ecdysone 2,3-acetonide

Samples were analysed using reversed-phase HPLC (system 3; Table 1). It appears that (a) 20-hydroxyecdysone 20,22-acetonide is formed, suggesting that either the C-2 or C-3 hydroxyl group is blocked; and (b) 20-hydroxyecdysone 2,3-acetonide is also produced, showing that acetonide formation does not occur at the 20,22 positions. This suggests that the C-22 hydroxyl group is blocked by phosphorylation, since such conjugation at the tertiary C-20 is unlikely. Furthermore, since ecdysone does not have a C-20 hydroxyl group, the only possible phospho-acetonide product in that case would be ecdysone 2,3-acetonide 22-phosphate, which would be hydrolysed to ecdysone 2,3-acetonide. The fact that no di-acetonide of 20-hydroxyecdysone was produced suggested that there were no phosphates at positions other than C-2, -3, -20 or -22.

The products formed in these transformations were those expected. Hence, the parent ecdysteroid is phosphorylated at either C-22 or C-2/C-3. However, an ecdysone metabolite produced in the phosphotransferase reaction co-chromatographed with ecdysone 2-phosphate. Furthermore, by analogy with ecdysteroid 2- and 3-acetates [27], ecdysone 3-phosphate would be expected to be more polar than the 2-phosphate and would be resolved by reversed-phase HPLC. The evidence is, thus, consistent with phosphorylation at C-2, but not C-3.

Further investigation showed that ecdysteroids altered at the C-3 position could be enzymically phosphorylated. For this, metabolites of the epimerization system, namely 3-dehydroecdysone and 3-epiecdysone, were used as potential substrates and the products compared with ecdysone phosphates by reversed-phase HPLC (system 3). In both cases, two products were formed. The putative phosphates of 3-epiecdysone and 3-dehydroecdysone were slightly less polar than the ecdysone 2- and 22-phosphates (0.6 min difference between the two 3-

Table 1 Comparison of retention times (RT) for ecdysteroid derivatives when analysed by reversed-phase HPLC (system 3)

The enzymically produced ecdysteroid phosphates were subjected to acetonide formation. Following this, the phospho-acetonides were hydrolysed with a crude arylsulphatase preparation (from *Helix pomatia*) to yield the ecdysteroid acetonide derivative. Retention times were compared with authentic standards: ecdysone, 27.1 min; 20-hydroxyecdysone, 24.7 min.

Putative phosphotransferase products	RT (min)	Phospho-acetonide	RT (min)	Acetonide	RT (min)
(a) 20-Hydroxyecdysone 22-phosphate	15.6	20-Hydroxyecdysone 2,3-acetonide 22-phosphate	18.0	20-Hydroxyecdysone 2,3-acetonide	28.6
(b) 20-Hydroxyecdysone 2-phosphate	21.7	20-Hydroxyecdysone 20,22-acetonide 2-phosphate	23.2	20-Hydroxyecdysone 20,22-acetonide	33.1
(c) Ecdysone 22-phosphate ^a	17.1	Ecdysone 2,3-acetonide 22-phosphate	18.6	Ecdysone 2,3-acetonide ^a	41.9
(d) Ecdysone 2-phosphate ^a	23.3	(none)		(none)	

^aCo-chromatography with authentic material.

Table 2 Cofactor requirements for conversion of ecdysone into ecdysone phosphates

Approximately 50 µg of unlabelled ecdysone was incubated with either non-dialysed or dialysed cytosol, supplemented with the various cofactor combinations listed below. The mean percentage conversions from two individual experiments into 3-dehydroecdysone (3DE), 3-epiecdysone (E'), ecdysone 2-phosphate (E2P) and ecdysone 22-phosphate (E22P) are recorded. Abbreviation: ND, not determined.

Conditions	Conversion into product (%)			
	3DE	E'	E2P	E22P
(i) Non-dialysed cytosol without cofactors	0	30.5	0	7.5
(ii) Dialysed cytosol without cofactors	15	0	0	0
(iii) Dialysed cytosol + 10 mM MgCl ₂	10.5	0	0	0
(iv) Dialysed cytosol + 2 mM ATP	ND	0	6.6	1.8
(v) Dialysed cytosol + 10 mM MgCl ₂ + 2 mM ATP	ND	0	21.9	6.1

epiecdysone phosphates and 1.6 min difference between the two 3-dehydroecdysone phosphates). By analogy with the ecdysone phosphates, it could be argued that these products represent esterification at C-2 and C-22 hydroxyl groups; phosphorylation cannot take place at C-3 in 3-dehydroecdysone. Relative conversions of the various substrates (percentage of recovered products) were (mean of two incubations): 3-epiecdysone 22-phosphate (8%), 3-epiecdysone 2-phosphate (16%), ecdysone 22-phosphate (6%) and ecdysone 2-phosphate (14%); 3-

dehydroecdysone 22-phosphate (7%) and 3-dehydroecdysone 2-phosphate (12%).

Upon hydrolysis of these putative phosphates with a crude arylsulphatase preparation, the parent ecdysteroid moieties were recovered.

Cofactor requirements

The cofactor requirements for the 3-dehydroecdysone reductase, phosphotransferase and ecdysone oxidase enzymes were determined separately as follows.

(a) Reductases

Standard enzyme assays contained: (i) no cofactor; (ii) 0.5 mM NADPH; or (iii) 0.5 mM NADH. Analysis of the products, by reversed-phase HPLC (system 1), revealed the following: (i) no conversion of 3-dehydroecdysone was observed in the absence of cofactor; (ii) when 0.5 mM NADPH was added to the incubation mixture, 3-dehydroecdysone 3 α -reductase enzyme action yielded a product which co-chromatographed with 3-epiecdysone (80.2% of the products formed), a lower quantity of (putative) ecdysone was also formed (19.8%) by 3-dehydroecdysone 3 β -reductase action; and (iii) when NADH was cofactor, the major product was (putative) ecdysone (85.3% of the products formed) and a smaller amount (19.8%) of (putative) 3-epiecdysone.

(b) Phosphotransferases

Standard incubations were set up containing: (i) non-dialysed cytosol preparation plus no cofactor; (ii) dialysed cytosol preparation plus no cofactor; (iii) dialysed cytosol preparation plus

Table 3 Effects of anerobic conditions on enzymic activities

Incubations were carried out in the presence of air (control reactions) or in the absence of oxygen. Oxygen was removed by a combination of evacuation during the pre-incubation period, flushing out the incubations with nitrogen and eliminating dissolved oxygen by using a two-step enzymic reaction (see the text for details). The data represent the means of two experiments ($\pm$ S.E.M.), with each incubation performed in duplicate.

Enzyme	Specific activity (nmol/h per mg)		
	Aerobic	Anaerobic	Change (%)
Ecdysone oxidase	4.62 $\pm$ 0.16	0.85 $\pm$ 0.05	- 82
3-Dehydroecdysone 3 β -reductase	8.64 $\pm$ 0.27	8.83 $\pm$ 0.36	+ 2
3-Dehydroecdysone 3 α -reductase	9.19 $\pm$ 0.35	8.68 $\pm$ 0.41	- 5
Ecdysone 2-phosphotransferase	4.65 $\pm$ 0.21	4.43 $\pm$ 0.27	- 5
Ecdysone 22-phosphotransferase	5.12 $\pm$ 0.25	5.23 $\pm$ 0.25	+ 2

Figure 4 Profiles of midgut cytosol enzymic activities and haemolymph ecdysteroid titre during the sixth (final) instar of *Spodoptera littoralis*

Boxes on the abscissa refer to the light and dark phases and insects were synchronized (± 3 h) at the 5th/6th instar moult occurring over a scotophase. Each point represents the mean of three separate investigations with each assay being carried out in duplicate; bars represent the S.E.M. Various developmental markers are shown: ecdysis (E), feeding, gut purge (G), wandering (W), pharate pupae (PP) and pupation (P). Other abbreviations used: (a) 3 α -red, 3-dehydroecdysone 3 α -reductase ($\circ$); EO, ecdysone oxidase ($\bullet$); (b) 3 β -red, 3-dehydroecdysone 3 β -reductase ($\blacksquare$); ecdysteroid, ecdysone equivalents measured by RIA ($\square$); (c) 2P-transf, ATP:ecdysone 2-phosphotransferase ($\blacktriangle$); 22P-transf, ATP:ecdysone 22-phosphotransferase ($\blacktriangledown$).

10 mM MgCl_2 ; (iv) dialysed cytosol preparation plus 2 mM ATP; and (v) dialysed cytosol preparation plus 10 mM MgCl_2 and 2 mM ATP.

Reaction products were analysed by reversed-phase HPLC (systems 1 or 3) and the percentage conversions into ecdysteroid derivatives are summarized in Table 2.

(i) When non-dialysed cytosol preparation was used in the incubation, endogenous cofactors were sufficient to effect 30.5% conversion of ecdysone into a metabolite having the same retention time as 3-epiecdysone (HPLC system 1) and 7.5% conversion into a metabolite having the same retention time as ecdysone 22-phosphate (HPLC system 3). Hydrolysis of the putative ecdysone 22-phosphate with crude so-called 'arylsulphatase' preparation (from *Helix pomatia*) resulted in release of only ecdysone (HPLC system 1).

(ii) However, when a similar reaction was carried out using a dialysed preparation with no additional cofactors, conversion of ecdysone into 3-dehydroecdysone (approx. 15%) was observed.

No phosphorylation was observed in the absence of cofactors (ii) or when only MgCl_2 was added (iii). Surprisingly, if ATP was added as the sole cofactor (iv), then 8.4% conversion into phosphates occurred. However, addition of both ATP and MgCl_2 (v) greatly enhanced phosphotransferase activity (28% conversion), and the products were tentatively assigned as ecdysone 2-phosphate (21.9% of the total) and ecdysone 22-phosphate (6.1% of the total), by co-chromatography with authentic compounds on reversed-phase HPLC (system 3). Therefore, both ATP and MgCl_2 are required for high phosphotransferase activity.

Oxygen requirement

Ecdysone oxidase has previously been reported to require molecular oxygen [12,28]. Thus, the effect of anaerobic conditions on the oxidase and other midgut cytosolic enzymes in *S. littoralis* was examined. Of the enzymes tested (3-dehydroecdysone 3 β - and 3 α -reductases, ecdysone 2- and 22-phosphotransferases and ecdysone oxidase), only the oxidase was affected significantly by the removal of molecular oxygen (Table 3). The ecdysone oxidase had a mean specific activity of 4.62 ± 0.15 nmol/h per mg of protein. Upon removal of oxygen, oxidase activity diminished to 0.85 ± 0.10 nmol/h per mg of protein, a reduction of approx. 82%.

Haemolymph ecdysteroid titre throughout the sixth instar

The haemolymph immunoreactive ecdysteroid titre was assayed throughout the last larval instar. Haemolymph was taken from accurately synchronized sixth instar larvae (± 3 h), at various time-points (a 10 μl pooled aliquot from five larvae at 50 h, 70 h and 115 h was taken for RIA and 1 μl pooled aliquot from five larvae at 80 h, 93 h, 100 h, 105 h, 107 h, and 110 h). Protein was precipitated by addition of chilled ethanol (100 μl) and sedimented (9000 g, 10 min). The supernatants were withdrawn and the pellet re-extracted with ethanol. Alcoholic extracts were combined and dried *in vacuo*. Ecdysteroid content (pg of ecdysone equivalents) of the samples was estimated by RIA using H-22 antiserum. Accordingly, ecdysone and 20-hydroxyecdysone are immunoreactive, having cross-reactivities of 1.0 and 2.05 respectively (J.G. Mercer, personal communication), but 3-dehydroecdysone is known to exhibit little binding (a cross-reactivity of 21.4 compared with ecdysone; [25]). The immunoreactive ecdysteroid profile during the sixth instar is given in Figure 4(b) and exhibits a peak late in the instar.

Developmental profiles of the enzymic activities

Insects were accurately synchronized (± 3 h) during a scotophase at the 5th/6th instar moult and batches of approx. 10 insects were killed at various times into the 6th instar. Dialysed post-

microsomal supernatants of the midgut were obtained and the various enzymes assayed. The 3-dehydroecdysone 3 α - and 3 β -reductases were assayed using NADPH and NADH respectively. The experiment was carried out three times using different generations of insects.

The qualitative and quantitative profiles of the specific activities of the enzymes during the sixth instar are shown in Figure 4 and can be compared with the haemolymph ecdysteroid titre during the last larval instar, (Figure 4b). The ecdysone oxidase activity reaches a peak of 6.09 ± 0.31 nmol/h per mg at approx. 36 ± 3 h (Figure 4a), i.e. at a time when the ecdysteroid titre is negligible. The 3-dehydroecdysone 3 α -reductase (assayed using NADPH) has a rather intriguing profile (Figure 4a). During the first 40 h of the instar, activity is at an intermediate plateau, but reaches a peak at about 55 h. There then follows a steady decline in activity until the end of the instar, when, at 114 h, activity falls to the original level. As for the oxidase, this enzyme reaches a peak in activity when the ecdysteroid titre is negligible.

In contrast, the 3-dehydroecdysone 3 β -reductase (NADH) is highest in the fifth photophase (Figure 4b). However, since the 3-dehydroecdysone 3 β -reductase was assayed in the presence of air, it could be argued that ecdysone oxidase could convert some ecdysone back into 3-dehydroecdysone; yet, under anaerobic conditions, the observed 3 β -reductase activity only increased by 2% (see Table 3). Nonetheless, the profile given for this enzyme is of apparent specific activity values. There is a steady rise in 3 β -reductase activity throughout the instar, until a peak is reached at approx. 107 ± 3 h, just prior to the peak in ecdysteroid content at 110 ± 3 h, after which both titres fall.

The specific activity of the ecdysteroid 22-phosphotransferase enzyme reached a peak early in the instar at approx. 27 ± 3 h (Figure 4c). In the case of the 2-phosphotransferase enzyme, activity was relatively high until about 45 h, when it declined. For both enzymes, activity was maintained at a comparatively low level later in the instar, as the ecdysteroid titre was rising.

DISCUSSION

There is substantial evidence as to the authenticity of the products of the epimerization system. Several physicochemical analyses indicated that the product of the ecdysone oxidase reaction is 3-dehydroecdysone. In the case of the 3-dehydroecdysone reductases, 3-epiecdysone and ecdysone were fully resolved and co-chromatographed with the respective authentic sample on both HPLC systems employed (1 and 2).

By the use of two complementary ion-suppression reversed-phase HPLC systems, the phosphate isomers produced by *S. littoralis* cytosol were shown to co-chromatograph with authentic ecdysone 2- and 22-phosphate, respectively. These identities were corroborated by derivative formation. With all ecdysteroid substrates examined, formation of the 2-phosphate conjugate vastly predominated over the 22-phosphate. It may be significant that both ecdysteroid 2- and 22-phosphotransferases occur in locusts [29,30]. The significance of the occurrence of phosphorylation at two positions (C-2 or C-22) in the ecdysteroid molecule is uncertain. At least in the case of the C-22 position a free hydroxyl group is required for high hormonal activity. Other phosphotransferase activities, such as phosphorylation of *p*-nitrophenol, are known to exist in the midgut cytosol of several insect species, including Lepidoptera [31]. The latter phosphotransferase belongs to the same class of enzymes as those in this study, i.e. those with a secondary alcohol group as acceptor (EC 2.7.1). Whether or not this activity is related to the ecdysteroid phosphotransferases remains to be established. In *Manduca sexta* midgut extracts, incubation of ecdysone with ATP and

MgCl₂ under aerobic conditions yielded four distinct phosphate conjugate peaks, but using 3-epiecdysone, two peaks were observed [13]; these conjugates were not further characterized.

The results of the cofactor requirements for dialysed cytosolic 3-dehydroecdysone reductases indicate that the 3 β -reductase shows a preference for NADH and the 3 α -reductase for NADPH, in agreement with previous results for these enzymes from *S. littoralis* [4]. Activity is also detected *in vitro*, when the alternative cofactor is used. Clearly, the ratio [NADH]/[NADPH] in the midgut cytosol is important to the relative significance *in vivo* of the 3-dehydroecdysone 3 α - and 3 β -reductase-catalysed reactions. Although such data are lacking in most biological systems, the proportions of each nucleotide in the reduced form have been determined in rat liver cytosol by an indirect method [32]. In that case, the [NAD⁺]/[NADH] ratio is about 1000, whereas that for the [NADP⁺]/[NADPH] is about 0.01 [33]. If the situation in *S. littoralis* midgut cytosol is similar, it may be presumed that the [NADPH] greatly exceeds the [NADH]. Such a situation would favour the 3 α -reductase reaction and may suggest that the 3-dehydroecdysone 3 β -reductase reaction plays only a minor role *in vivo*. In any case, the significance of the reaction is unclear.

That ATP and Mg²⁺ are required for phosphotransferase activity has been well-documented by several workers [13,27,30,31]. In the case of the ATP:2-deoxyecdysone 22-phosphotransferase in the follicle cells of the desert locust, *Schistocerca gregaria*, GTP was found to be far less effective than ATP. Magnesium was an efficient bivalent cation stimulating phosphotransferase activity in the presence of ATP.

Exclusion of molecular oxygen has been reported to prevent or reduce ecdysone oxidase activity [12,28]. This was confirmed by the anaerobic experiments with the *S. littoralis* enzyme, but an apparent low turnover rate was observed with the exclusion of oxygen. Whether this was due to incomplete removal of oxygen or whether molecular oxygen is not the only species capable of acting as a hydrogen acceptor, cannot be ascertained. It is also possible that there may be a natural cytosolic oxidizing agent, besides oxygen, present in some insects, that accounts for the minimal activity in an oxygen-limited environment [28]. Surprisingly, the ecdysone oxidase of *Pieris brassicae* is unaffected in an anaerobic atmosphere [3].

When ecdysone was added to a non-dialysed cytosol preparation from *S. littoralis*, 3-epiecdysone was the predominant product with a small amount of ecdysone 22-phosphate also being produced (results not shown). It is possible that ecdysone 2-phosphate is below the limits of detection. Interestingly, upon hydrolysis of the ecdysteroid 22-phosphate, only ecdysone was released. This may reflect either that exogenous ecdysone was present in greater quantities than the enzymically formed 3-epiecdysone and was, therefore, phosphorylated to a greater degree or, alternatively, ecdysone is preferentially phosphorylated *in vivo* and exogenous 3-epiecdysone is only phosphorylated in the absence of ecdysone.

The primary faecal ecdysteroid of early last instar *S. littoralis* larvae following [³H]ecdysone injection is 20-hydroxyecdysone 22-phosphate, with a smaller quantity of ecdysone 22-phosphate and even lower levels of ecdysone 2-phosphate, but in later larvae (pre-pupal stage), ecdyson- and 20-hydroxyecdysone-26-oic acids are formed [34]. No 3 α -epimers or their conjugates were detected. This is in agreement with the high phosphotransferase enzyme activities observed early in the instar in the current work (Figure 4). It is possible that the midgut phosphotransferases are important in inactivation of any ingested dietary ecdysteroids.

Comparison of enzyme activity profiles during the last (sixth) larval instar allows possible insight into the interactions of these enzyme systems (Figure 4). The profiles may not quantitatively

represent all individual activities *in vivo*, where there is probably competition for some substrates.

While feeding, the endogenous titre of haemolymph ecdysone and 20-hydroxyecdysone is low and increases to a peak late in the instar ([35]; Figure 4b). The ecdysone oxidase reaches a maximum during this feeding phase and presumably ecdysteroids are inactivated either by production of 3-dehydro metabolites, or possibly by further reduction in activity via epimerization and/or phosphate esterification, since the 3-dehydroecdysone 3 α -reductase and phosphotransferases also exhibit a peak during feeding. It may be significant that the 3 α -reductase activity increases, following maximal ecdysone oxidase activity, suggesting that the product of the ecdysone oxidase, 3-dehydroecdysone, may induce 3 α -reductase activity. Enzymic profiles may be complicated by competition by different cytosolic enzymes for ecdysone and 3-dehydroecdysone substrates. Limited data on the activities of midgut ecdysone oxidase and the two 3-dehydroecdysone reductase systems in *Manduca sexta* suggest that the gross last instar larval development profiles in this species may be somewhat different [36].

That there are peaks of enzymic activity during feeding for the ecdysteroid-inactivating enzymes in *S. littoralis*, when the endogenous pool of active moulting hormone is low, suggests that the enzymes may play a role in the deactivation of ingested ecdysteroids from the diet. However, it would be expected that the enzyme titre would not peak, but plateau, if the activity is linked to feeding.

In Lepidoptera, the prothoracic glands produce appreciable amounts of 3-dehydroecdysone in addition to ecdysone and the former is apparently reduced to ecdysone by haemolymph 3-dehydroecdysone 3 β -reductase [37,38]. At the cessation of feeding and the onset of wandering, the 3-dehydroecdysone 3 β -reductase enzyme in the gut increases, whereas the activity of the other enzymes fall (Figure 4). The increasing 3 β -reductase enzyme activity parallels the profile of the corresponding enzyme in *Spodoptera* haemolymph (T. J. Webb, R. Powls and H. H. Rees, unpublished work). A similar profile for the 3 β -reductase activity has been reported for *Manduca sexta* haemolymph [38]. It is tempting to suggest that the *Spodoptera* midgut 3-dehydroecdysone 3 β -reductase enzyme in some way contributes to the huge surge in 20-hydroxyecdysone levels, since the enzyme activity peaks just prior to the rise in active hormone titre. In this manner, the midgut 3 β -reductase may be important in activation, rather than in the futile cycling of ecdysteroid and 3-dehydroecdysteroid [12]. Since 3-dehydroecdysone 3 β -reductase enzymes are present not only in the haemolymph, but also in various other organs, such as the fat body, salivary glands, suboesophageal ganglia, Malpighian tubules, hindgut, brain [39] and midgut [1,4], it is conceivable that 3-dehydroecdysone is sequestered by the tissues and then re-activated when necessary.

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REFERENCES

- 1 Nigg, H. N., Svoboda, J. A., Thompson, M. J., Kaplanis, J. N., Dutky, S. R. and Robbins, W. E. (1974) *Lipids* **9**, 971–974
- 2 Mayer, R. T., Durrant, J. L., Holman, M. G., Weirich, G. F. and Svoboda, J. A. (1979) *Steroids* **34**, 555–562
- 3 Blais, C. and Lafont, R. (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 809–817
- 4 Milner, N. P. and Rees, H. H. (1985) *Biochem. J.* **231**, 369–374
- 5 Koolman, J. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1312–1315
- 6 Koolman, J. (1985) *Methods Enzymol.* **111**, 419–427
- 7 Weirich, G. F. (1989) in *Ecdysone: From Chemistry to Mode of Action* (Koolman, J., ed.), pp. 174–180, Georg Thieme-Verlag, Stuttgart
- 8 Koolman, J. and Spindler, K.-D. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, 1339–1344
- 9 Richards, G. (1978) *J. Insect Physiol.* **24**, 329–335
- 10 Kaplanis, J. N., Thompson, M. J., Dutky, S. R. and Robbins, W. E. (1979) *Steroids* **34**, 333–345
- 11 Lafont, R., Beydon, P., Sommé-Martin, G. and Blais, C. (1980) *Steroids* **36**, 185–207
- 12 Weirich, G. F., Thompson, M. J. and Svoboda, J. A. (1989) *Arch. Insect Biochem. Physiol.* **12**, 201–218
- 13 Weirich, G. F., Thompson, M. J. and Svoboda, J. A. (1986) *Arch. Insect Biochem. Physiol.* **3**, 109–126
- 14 Lafont, R. and Koolman, J. (1984) in *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones* (Hoffmann, J. and Porchet, P., eds.), pp. 196–226, Springer, Berlin
- 15 Lafont, R. and Connat, J.-L. (1989) in *Ecdysone: From Chemistry to Mode of Action* (Koolman, J., ed.), pp. 167–173, Georg Thieme-Verlag, Stuttgart
- 16 Dinan, L. N. and Rees, H. H. (1978) *Steroids* **32**, 629–638
- 17 Isaac, R. E. and Rees, H. H. (1984) *Biochem. J.* **221**, 459–464
- 18 Isaac, R. E., Rose, M. E., Rees, H. H. and Goodwin, T. W. (1983) *Biochem. J.* **213**, 533–541
- 19 Galbraith, M. N. and Horn, D. H. S. (1969) *Aust. J. Chem.* **22**, 1045–1057
- 20 McKinley, D. J. (1970) *Antilocust Research Occasional Report 18*, Antilocust Research Centre, London
- 21 Bodenstein, D. (1946) *Biol. Bull.* **90**, 148–157
- 22 Shimizu, K. (1973) *Arch. Biochem. Biophys.* **125**, 1016–1017
- 23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 24 Mercer, J. G., Munn, A. E. and Rees, H. H. (1987) *Mol. Biochem. Parasitol.* **24**, 203–214
- 25 Soumoff, C., Horn, D. H. S. and O'Connor, J. D. (1981) *J. Steroid Biochem.* **14**, 429–435
- 26 Warren, J. T. and Gilbert, L. I. (1986) *Insect Biochem.* **16**, 65–82
- 27 Rees, H. H. and Isaac, R. E. (1985) *Methods Enzymol.* **111**, 377–410
- 28 Koolman, J. and Karlson, P. (1978) *Eur. J. Biochem.* **89**, 453–460
- 29 Kabbouh, M. and Rees, H. H. (1991) *Insect Biochem.* **21**, 57–64
- 30 Kabbouh, M. and Rees, H. H. (1993) *Insect Biochem. Mol. Biol.* **23**, 73–79
- 31 Yang, R. S. H. and Wilkinson, C. F. (1973) *Comp. Biochem. Physiol.* **46**, 717–726
- 32 Veech, R. L. (1987) in *Pyridine Nucleotide Cofactors* (Dolphin, D., Poulson, R. and Avramovic, D., eds.), pp. 79–104, John Wiley, New York
- 33 Gumaa, K. A., McKlean, P. and Greenbaum, A. L. (1971) *Essays Biochem.* **7**, 39–86
- 34 Milner, N. P. (1984) *PhD. Thesis*, Liverpool, U.K.
- 35 Jarvis, T. D., Earley, F. G. P. and Rees, H. H. (1994) *Insect Biochem. Mol. Biol.* **24**, 531–537
- 36 Weirich, G. F., Feldlaufer, M. F. and Svoboda, J. A. (1993) *Arch. Insect Biochem. Physiol.* **23**, 199–211
- 37 Warren, J. T., Sakurai, S., Rountree, D. B., Gilbert, L. I., Lee, S. S. and Nakanishi, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 958
- 38 Sakurai, S., Warren, J. T. and Gilbert, L. I. (1989) *Arch. Insect Biochem. Physiol.* **10**, 179–197
- 39 Gelman, D. B., DeMilo, A. B., Thyagaraja, B. S., Kelly, T. J., Masler, E. P., Bell, R. A. and Borkovec, A. B. (1991) *Arch. Insect Biochem. Physiol.* **17**, 93–106