Evidence for the involvement of 3-oxo- Δ^4 intermediates in ecdysteroid biosynthesis

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Although the involvement of 3-oxo- Δ^4 compounds as intermediates in arthropod ecdysteroid biosynthesis has been postulated for a long time, it has not yet been directly demonstrated. In the present study, 3-oxo- Δ^4 -steroids have been synthesized and incubated *in vitro* with dissociated moulting gland cells from the crab *Carcinus maenas*. The tritiated compounds were converted into 3-dehydroecdysone, ecdysone and/or 25-deoxyecdysone, i.e. final ecdysteroids. This means that the 3-oxo- Δ^4 compounds had undergone a 5β -reduction, to give the 5β -conformation of ecdysteroids. Our results suggest

that the 3-oxo- Δ^4 -steroid 4,7-cholestadien-14 α -ol-3,6-dione may be an intermediate in the biosynthetic pathway. The 5β -reduction reaction involves a cytosolic enzyme which requires NADPH as electron donor and seems specific for 3-oxo- Δ^4 substrates. This reaction was the most active in crab Y-organs, as compared with other tissues. The characteristics of the 5β -reductase (subcellular localization, substrate and cofactor requirements) appear similar to those of the vertebrate 3-oxo- Δ^4 -steroid 5β -reductase involved in steroid hormone catabolism and bile acid biosynthesis.

INTRODUCTION

Ecdysteroids are the major steroid hormones of arthropods, and also represent widespread secondary metabolites in plants. Their chemical structure is characterized by a *cis*-junction of A and B cycles, a 7-en-6-one chromophore and a 14 α -hydroxy group. They generally possess the carbon skeleton of cholesterol (C_{27}) or phytosterols (C_{28} , C_{29}), from which they originate. At the present time the biosynthetic pathway of ecdysteroids, which leads to the production of ecdysone, 25-deoxyecdysone (25dE) and/or 3-dehydroecdysone (3DE) in arthropod moulting glands, is only partially known (for reviews, see [1–3]) (Figure 1). The initial conversion of cholesterol into 7-dehydrocholesterol [4] and the three terminal reactions, i.e. the sequential hydroxylations at C-25, C-22 and C-2 of 2,22,25-trideoxyecdysone (3 β ,14 α -dihydroxy-5 β -cholest-7-en-6-one, referred to as 5 β -ketodiol) by cytochrome P-450 enzymes [5], are well established. Between these steps a 'black box' still remains, comprising several reactions that have yet to be elucidated, including the formation of the A/B *cis* ring junction. A sequence for the 'black-box' reactions has been proposed [6], but has remained up to now largely speculative.

Studies with radiolabelled 5 β -ketodiol and its 3-oxo analogue (5 β -diketol; 14 α -hydroxy-5 β -cholest-7-ene-3,6-dione) have been performed using moulting glands producing either ecdysone or 3DE, and led to the conclusion that the formation of 5 β -diketol precedes that of 5 β -ketodiol, at least in 3DE-producing species (e.g. the Lepidopteran *Pieris brassicae* [7], the crayfish *Orconectes limosus* [8]). Further upstream elucidation of the biosynthetic pathway required the preparation of new labelled molecules, the choice of which was based on the hypothetical scheme of Grieneisen et al. [6] and a careful examination of available literature. (1) The formation of cholesterol derivatives with an A/B *cis* ring junction proceeds through 3-oxo- Δ^4 intermediates.

This has been well documented with regard to steroid hormone catabolism and bile acid synthesis in vertebrates (e.g. see [9]) and cardenolide biosynthesis in plants [10]. Indeed, in insects, the specific elimination of 3 α - and 4 β -hydrogens from tritiated cholesterol during its conversion into ecdysteroids suggested the involvement of analogous 3-oxo- Δ^4 intermediates early in the biosynthetic pathway of ecdysteroids [11]. (2) Ecdysteroid-like natural products bearing a Δ^4 moiety have been isolated from several marine invertebrates and plants. Steroidal 4,7-dien-3,6-diketones have been described in the marine sponge *Rhaphidostila incisa* [12] and the fungus *Ganoderma lucidum* [13], and it has been demonstrated that fungi can synthesize ecdysteroids [14].

It appeared, therefore, reasonable to assume that, in the ecdysteroid biosynthetic pathway, 5 β -diketol is formed by the reduction of a 3-oxo- Δ^4 precursor, which was indeed postulated in [6] but apparently not tested experimentally. In order to investigate the involvement of a 5β -reductase in ecdysteroid biosynthesis (Figure 1), we synthesized 3-oxo- Δ^4 -steroids in both radiolabelled and non-labelled forms. These molecules were assayed *in vitro* using moulting glands (Y-organs) of the crab *Carcinus maenas*, which are known to synthesize three different ecdysteroids: ecdysone, 25dE [15] and minor amounts of 3DE [16]. Our present work provides the first direct evidence for the involvement of a 5β -reductase in the ecdysteroid biosynthetic pathway.

EXPERIMENTAL

Animals

Shore crabs (*Carcinus maenas*) were supplied by the Biological Station, Roscoff, France, and kept in the laboratory as previously described [17]. Intermoult animals were used for most experiments.

Abbreviations used: 25dE, 25-deoxyecdysone; 3DE, 3-dehydroecdysone; compound **1a**, 14 α -hydroxycholesta-4,7-diene-3,6-dione; compound **1b**, 14 α ,25-dihydroxycholesta-4,7-diene-3,6-dione; NP-HPLC, normal-phase HPLC; RP-HPLC, reverse-phase HPLC.

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Figure 1 Biosynthetic pathways of ecdysteroids in arthropods

Reactions enclosed in boxes are at present unknown and hypothetical. The 5β-reduction of a putative 3-oxo-Δ⁴ intermediate, shown in the larger box, is investigated in the present study. 14α-Hydroxycholesta-4,7-diene-3,6-dione is referred to as compound **1a** in the text.

Synthesis of putative ecdysteroid precursors

Several molecules were synthesized, firstly as non-labelled forms to serve as reference compounds. They were characterized by their UV, mass and proton NMR spectra. UV spectra were taken in methanol using a Varian DMS 100 spectrophotometer. NMR spectra were recorded on a Bruker AMX500 spectrometer using standard programs. Electron-impact and chemical-ionization (using ammonia as the reagent gas) mass spectra were obtained with a Riber 10-10B apparatus (Nermag S. A.) using a direct inlet probe.

(a) Synthesis of 14α-hydroxycholesta-4,7-diene-3,6-dione (compound **1a**, Figure 2)

This molecule was synthesized by a three-step process from 7-dehydrocholesterol. The two first steps (performed as in [18]) yielded cholesta-4,7-diene-3,6-dione, which was then treated with a solution of selenium dioxide in dioxane (70 °C, 30 min) in order

Figure 2 Structures of compounds **1a** and **1b**, putative 3-oxo-Δ⁴ intermediates of ecdysteroids

Positions of ³H in labelled molecules are indicated by *.

to introduce the 14α-hydroxy group. Pure structure **1a** was obtained from preparative normal-phase (NP)-HPLC, with Du Pont equipment, on a Zorbax-SIL column (250 mm × 21.2 mm internal diam.) eluted with methylene chloride/propan-2-ol/water (1900:40:1, by vol.). Its spectral characteristics were as follows. UV λ_{max} (in methanol), 276 nm; chemical-ionization MS: *m/z* 430 (M + H + NH₃)⁺, 413 (M + H)⁺, 395 (M + H - H₂O)⁺; NMR data are given in Table 1. The structure of **1a** is clearly established from the UV absorbance of the chromophore and several characteristic features of the NMR spectrum: the disappearance of 3-H and 5-H signals, and the appearance of a new ethylenic singlet signal at δ 6.45 p.p.m. (4-H). These values are in agreement with previous studies [18].

(b) Synthesis of 14α,25-dihydroxycholesta-4,7-diene-3,6-dione (compound **1b**, Figure 2)

This structure differs from **1a** by the presence of a 25-hydroxy group, and was obtained using the same procedure but starting

Table 1 Proton NMR data of compounds **1a** and **1b**

Multiplicity of signals: s, singlet; d, doublet. 7-HA,

Signal	δ (p.p.m.)	
	Compound 1a	Compound 1b
4-H	6.45 (s)	6.45 (s)
7-H	6.14 (d, 2.5)	6.14 (d, 2.5)
9-Ha	2.92 (ddd, 10.4, 7.2, 2.5)	2.92 (ddd, 10.4, 7.2, 2.5)
18-Me	0.73 (s)	0.73 (s)
19-Me	1.27 (s)	1.28 (s)
21-Me	0.92 (d, 6.5)	0.94 (d, 6.5)
26-Me	0.85 (d, 6.5)	1.20 (s)
27-Me	0.86 (d, 6.5)	1.20 (s)

from 7-dehydro-25-hydroxycholesterol. The final step of purification was performed by preparative NP-HPLC with chloride/propan-2-ol/water (1800:50:1, by vol.) as solvent. Its spectral characteristics were as follows. UV λ_{max} (in methanol), 275 nm; chemical-ionization MS: m/z 446 ($M+H+NH_3$)⁺, 429 ($M+H$)⁺, 411 ($M+H-H_2O$)⁺, 393 ($M+H-2H_2O$)⁺; electron-impact MS: m/z 428 [(M)⁺, 4], 410 [($M-H_2O$)⁺, 25], 395 [($M-H_2O-CH_3$)⁺, 6], 392 [($M-2H_2O$)⁺, 13], 59 (100); NMR data are given in Table 1. The structure of **1b** was established using the same criteria as for **1a**. The only difference in the NMR signals concerns the side chain, due to the presence of the 25-hydroxy function.

(c) Preparation of tritiated compounds

Labelled **1a** was prepared from [$1\beta,2\beta,24,25\text{-}^3\text{H}_4$]-5 β -cholest-7-ene-3,6-dione, initial specific radioactivity 9.2 TBq/mmol (250 Ci/mmol) [19], upon treatment with selenium dioxide in dioxane (70 °C, 1 h), which introduced both the 14 α -hydroxy and the 4-ene in a single step. Labelled **1b** was prepared from [$22,23\text{-}^3\text{H}$]2,22-dideoxyecdysone, initial specific radioactivity 2.2 TBq/mmol (60 Ci/mmol) [20], which was first converted into its 3-oxo derivative by mild oxidation with pyridinium chlorochromate in methylene chloride; this was then treated with selenium dioxide in dioxane to give the desired molecule.

Another tritiated reference compound, 3 β ,14 α -dihydroxycholesta-4,7-dien-6-one (Δ^4 -ketodiol), was obtained by chemical reduction of labelled **1a** with sodium borohydride in anhydrous tetrahydrofuran (room temperature, 15 min). The synthesis of the non-labelled form was performed according to [21].

Labelled compounds were purified by NP-HPLC using a Zorbax-SIL column (250 mm $\times$ 4.6 mm internal diam.) and cyclohexane/propan-2-ol/water (800:20:0.5, by vol., for **1a**; 400:20:0.5, by vol., for **1b** and Δ^4 -ketodiol) as solvent. They were then stored at -20 °C in HPLC solvent until use. These conditions allowed the compounds to remain undegraded for at least 2 months.

Chemicals

Commercial sources and synthesis of reference unlabelled ecdysteroids were as previously described [8]. 14 α -Hydroxy-5 α -cholest-7-en-3,6-dione (5 α -diketol) was prepared upon equilibration of 5 β -diketol under alkaline conditions [22]. All other chemicals were of analytical grade or higher.

Incubation of Y-organ cells

Cells from *C. maenas* Y-organs were enzymically dissociated as previously described [17]. Aliquots corresponding to 5–10 Y-organ equivalents were incubated in borosilicate tubes in 500 μ l of modified medium 199 [17] complemented with 10 % foetal calf serum for various times at room temperature and under constant agitation. The radioactive compounds (routinely approx. 0.5 μ Ci/sample) were evaporated under nitrogen in incubation vials, then redissolved in ethanol (1 % of final volume) before adding medium and cell aliquots.

Subcellular fractionation

Y-organs were dissected in modified medium 199, minced with scissors and homogenized with a glass/glass grinder in 10 vol. (vol./fresh wt.) of 100 mM potassium phosphate buffer (pH 7.4) containing 500 mM sucrose, 1 mM EDTA and 1 mM 2-mercaptoethanol (homogenization buffer). Differential centrifugation was performed at 4 °C: 1000 g for 10 min, followed by two centrifugations at 10000 g for 20 min, then 100000 g for

1 h. Pellets were washed and resuspended (100 μ l per 10 Y-organ equivalents) in incubation buffer (homogenization buffer minus sucrose). The final incubation volume was 500 μ l or 1 ml (100000 g supernatant). In some cases, NADPH or NADH (Sigma) was added in buffer to give a final concentration of 0.5 mM. Subcellular fractions were incubated under the same conditions as dissociated cells. Protein concentrations were determined using Bio-Rad reagent and catalase as standard. The amounts of proteins recovered in each fraction per Y-organ equivalent were 4.4 ± 0.4 μ g for the 10000 g pellet, 3.7 ± 0.3 μ g for the 100000 g pellet and 27.6 ± 2.7 μ g for the 100000 g supernatant (means $\pm$ S.D. of 10 independent measurements; see [16]).

Extraction procedures

At the end of incubation period, 3 vol. of ethanol was added to the medium; the mixture was centrifuged and the supernatant was dried. The 100000 g supernatants were processed in a different way. Samples were placed on ice and diluted with 3 vol. of cold water. They were then adsorbed on to a C18 Sep-Pak cartridge (Waters) and eluted with ethanol (5 ml), which was then evaporated. In all cases, trifluoroacetic acid (0.1 % final concentration) in water was added before drying [23]. Dried samples were redissolved in the appropriate solvent for further HPLC analysis.

HPLC analyses

For analytical purposes, HPLC was performed on a Thermo Separation Products apparatus (pump and gradient former, Spectra P200) at a flow rate of 1 ml/min, using several sets of conditions. For reverse-phase HPLC (RP-HPLC) we used a Spherisorb 5-ODS2 column (250 mm $\times$ 4.6 mm internal diam.) eluted with different solvent systems, as follows (1) A multistep gradient of solvent B (acetonitrile/propan-2-ol, 5:2, v/v) in 0.1 % trifluoroacetic acid in water: 15–25 % B in 2 min, 25 % B for 6 min, 25–75 % B in 20 min, and then 75–90 % B in 7 min (RP1a) or 75 % B for 25 min (RP1b). (2) Isocratic conditions, comprising 80 % acetonitrile/0.1 % trifluoroacetic acid in water (RP2). NP-HPLC was performed on a Zorbax-SIL column eluted with cyclohexane/propan-2-ol/water in the proportions of (by vol.) 100:30:1 (NP1), 660:70:2 (NP2) or 800:20:0.5 (NP3), depending on the polarity of the compounds to be separated. Radioactivity was measured on-line by a Flo-one A250 monitor (Radiomatic; Packard) equipped with a 0.5 ml cell and using a 3:1 ratio of scintillation solution (Ecoscint A; National Diagnostics) to HPLC solvent. The radioactive metabolites were identified by comparing their retention times with those of known standards, detected by UV absorption at 254 nm.

RESULTS

In vitro metabolism of compound **1a** by dissociated Y-organ cells

Compound **1a** was stable for several months when kept in organic solvents at -20 °C. However, it appeared highly unstable when incubated at room temperature in aqueous buffers. Half of compound **1a** was degraded after only about 2.5 h of incubation, giving a mixture of more polar unidentified products, none of which co-migrated with ecdysone, 3DE or 25DE, or any known ecdysteroid intermediate (Figure 3A). Dilution with unlabelled compound **1a** (final concentration 1 μ M) did not change the degradation profile (results not shown). Incubations were therefore shortened in order to obtain both minimum degradation of the precursor and significant levels of metabolites, in particular when using dissociated cells (see below).

Figure 3 RP-HPLC profiles from *in vitro* incubations of ^3H -labelled compound **1a** (A, B) and 5β - ^3H]diketol (C), either alone (A) or with dissociated Y-organ cells (B, C)

Dissociated Y-organ cells were incubated in modified medium 199 (5.5 Y-organ equivalents per 500 μl ; 0.5 μCi of tritiated precursor) for 3 h. In the blank sample (A), ^3H -labelled compound **1a** was incubated alone for 3 h. HPLC analyses were performed with a multiple step gradient of acetonitrile/propan-2-ol (5:2, v/v) in 0.1% trifluoroacetic acid in water (solvent system RP1b; see the Experimental section). The retention times of standard ecdysteroids are indicated by arrows. Abbreviations: E, ecdysone; 3D2dE, 3-dehydro-2-deoxyecdysone; triol, 22,25-dideoxyecdysone; 3DK, 5β -diketol; $\Delta 43DK$, 14 α -hydroxycholesta-4,7-diene-3,6-dione (compound **1a**).

When incubated with dissociated cells from crab Y-organs, compound **1a** was converted into several more polar products; after a 0.5 h incubation a tritiated compound co-migrating with standard 3DE was detected, but at a low level (about 1% of total radioactivity). A representative HPLC pattern from a 3 h incubation is shown in Figure 3(B). The labelled precursor was totally metabolized giving, as the most polar metabolites, 3DE (6.6% of total radioactivity) and a compound co-migrating with 25dE which was relatively less abundant (2.1% of total radioactivity); no ecdysone was detected after 3 h. The identity of both ecdysteroids was confirmed by their co-migration with authentic standards on NP-HPLC; each peak was collected after RP-HPLC separation and injected on to a NP column. The overall RP-HPLC pattern (Figure 3B) was, in fact, very similar to that obtained when Y-organ cell preparations were incubated with 5β - ^3H]diketol under the same experimental conditions (Figure 3C). The same metabolites appeared, but with different

Figure 4 RP-HPLC profiles from *in vitro* incubations of ^3H -labelled compound **1b** (A, B) and ^3H]3-dehydro-2,22-dideoxyecdysone (C), either alone (A) or with dissociated Y-organ cells (B, C)

Dissociated Y-organ cells were incubated in modified medium 199 (10 Y-organ equivalents per 500 μl ; 0.5 μCi of ^3H -labelled precursor) for 1 h. In the blank sample (A), ^3H -labelled compound **1b** was incubated alone for 1 h. HPLC analyses were performed with a multiple step gradient of acetonitrile/propan-2-ol (5:2, v/v) in 0.1% trifluoroacetic acid in water (solvent system RP1a; see the Experimental section). The retention times of standard ecdysteroids are indicated by arrows. Abbreviations: E, ecdysone; 2dE, 2-deoxyecdysone; 3D2dE, 3-dehydro-2-deoxyecdysone; 3D2,22dE, 3-oxo-2,22-dideoxyecdysone; $\Delta 43D2,22dE$, 14 α ,25-dihydroxycholesta-4,7-diene-3,6-dione (compound **1b**).

relative proportions, as 5β -diketol was converted more slowly than compound **1a**. 3DE and 25dE were detected in low amounts, and all other radioactive peaks were also present in chromatograms of compound **1a** metabolites. We can, therefore, conclude that most of these metabolites were 5β -reduced. The first metabolite to be formed was 5β -diketol, which did not accumulate in 3 h incubations but could be detected during short incubations when using appropriate HPLC conditions (systems RP1b, RP2 or NP2; results not shown).

In vitro metabolism of compound **1b**

Tritiated compound **1b** was also unstable when incubated alone in aqueous buffers, and it gave some more polar, unknown,

degradation products (Figure 4A), but in lesser amounts than compound **1a**. When incubated with dissociated Y-organ cells, compound **1b** was very rapidly metabolized into radioactive polar compounds that co-migrated with 3DE and ecdysone, as seen by RP-HPLC analysis after a 1 h incubation (Figure 4B). 3DE was always present at higher levels than ecdysone (respectively 28.4 % and 2.6 % of total radioactivity). Since compound **1b** is hydroxylated on C-25, no 25dE could be formed. We observed a number of intermediates co-migrating with known ecdysteroids on RP-HPLC. Identification of metabolites was confirmed by collecting fractions corresponding to putative ecdysone/3DE and 2-deoxyecdysone/3-dehydro-2-deoxyecdysone, and analysing them by NP-HPLC (NP1 system); each radioactive compound was co-eluted with the corresponding standard ecdysteroid, as in RP-HPLC (results not shown). The major identified metabolites were then 5 β -reduced and could be compared with the metabolites obtained when incubating [3 H]3-dehydro-2,22-dideoxyecdysone as a precursor (Figure 4C). Both metabolite patterns were similar, with a higher proportion of polar ecdysteroids in the case of the Δ^4 compound. The major radioactive peak (retention time 24.2 min) observed in both cases probably corresponded to 3-dehydro-22-deoxyecdysone. Thus 5 β -reduction of compound **1b** was also achieved by Y-organ cells *in vitro*.

Subcellular localization of the 5 β -reduction reaction in Y-organs

Subcellular fractions of Y-organs were incubated with compound **1a** for 1 h or less. 5 β -Reduction was very efficient in the 100 000 g supernatant (cytosolic fraction) in the presence of NADPH; the 5 β -diketol formed represented 70.3 % of total radioactivity after 0.5 h (Figure 5A). Its identity was confirmed by co-migration of the tritiated metabolite with standard 5 β -diketol in RP- and NP-HPLC (Figure 5A), if allowance was made for the isotope effect (see [24]). In addition, the HPLC pattern of the metabolites obtained after reincubation with dissociated Y-organ cells was similar to that obtained with reference 5 β -[3 H]diketol. In the absence of cofactor, or in the presence of NADH, no transformation of the precursor was detected on incubation with cytosolic fractions.

Similarly, precursor **1b** was 5 β -reduced when incubated with the 100 000 g supernatant in the presence of NADPH, giving 3-dehydro-2,22-dideoxyecdysone (82 % of total radioactivity after a 1 h incubation; results not shown).

The results obtained with Y-organ membrane fractions, i.e. 100 000 g pellets ('mitochondria') and 100 000 g pellets ('microsomes'), were rather puzzling. After incubation with labelled compound **1a**, we observed in each case the presence of a radioactive peak co-migrating with standard 5 β -diketol under different RP-HPLC conditions. When samples, incubated in the presence of NADPH, were analysed by an NP-HPLC system allowing the separation of the 5 α - and 5 β -isomers of diketol, two radioactive compounds were detected, which coincided with the two standard molecules if allowance was made for the isotope effect (Figure 5B). The HPLC pattern was very similar for both membrane fractions. No 5 α -diketol, or only a small proportion, was detected when microsomal or mitochondrial fractions respectively were incubated without cofactor. A third metabolite, only detected in the presence of NADPH, was eluted between the two isomer peaks; its chemical nature is unknown at the present time. Reincubation of each radiolabelled putative 5-isomer with dissociated Y-organ cells gave two different HPLC patterns. The 5 β -compound gave the usual profile obtained with 5 β -diketol; the 5 α -compound was transformed at a similar rate (the remaining precursor represented about 12 % of total radioactivity

Figure 5 Subcellular localization of 5 β -reduction in crab Y-organs

NP-HPLC profiles are shown of metabolites formed on incubation of the 100 000 g supernatant (cytosol) (A) and pellet (microsomes) (B). Each fraction (10 Y-organ equivalents) was incubated for 0.5 h in the presence of 0.5 μ Ci of [3 H]14 α -hydroxycholesta-4,7-diene-3,6-dione (Δ^4 3DK; compound **1a**) and NADPH (0.5 mM). The HPLC solvent was cyclohexane/propan-2-ol/water (800:20:0.5, by vol.). The retention times of standard ecdysteroids are indicated by the arrows; 5 β 3DK and 5 α 3DK are the 5 β - and 5 α -isomers of diketol. The shift in retention time between standards and tritiated metabolites is due to the isotope effect.

after a 3 h incubation), but into different, unidentified, metabolites.

Specificity of 5 β -reduction

To investigate the tissue distribution of 5 β -reduction in crabs, 100 000 g supernatants obtained from homogenates of different tissues (epidermis, muscle, nervous tissue, ovary, testis) were incubated for 20 min in the presence of compound **1a** (0.2 μ M; 0.5 μ Ci); the formation of 5 β -diketol was then analysed by RP-HPLC. Y-organs were clearly the most efficient of all the tissues tested in carrying out 5 β -reduction, being approx. 8 times more active than epidermis, muscle or nervous tissue (activity calculated in pmol/mg of protein). Under these experimental conditions, no [3 H]diketol was detected in the case of testis and ovary.

In order to determine whether the presence of a 3-oxo group is required for the 5 β -reduction reaction in crabs, we incubated a 100 000 g supernatant from Y-organs with a tritiated 3 β -hydroxy- Δ^4 compound, namely 3 β ,14 α -dihydroxycholesta-4,7-dien-6-one. The compound was stable during blank incubations in buffer for several hours. It was poorly metabolized by cytosolic preparations; we detected by NP-HPLC analysis (system NP2) a tritiated metabolite co-migrating with 5 β -ketodiol (the corresponding 5 β -reduced compound), but this represented only approx. 2.5 % of the total radioactivity after 0.5 h and 4 % after 1 h (two independent experiments).

DISCUSSION

The present study shows that crab moulting glands are able to metabolize 3-oxo- Δ^4 precursors into ecdysteroids *in vitro*. This is the first time that the efficient conversion of a 3-oxo- Δ^4 -steroid into ecdysteroids has been observed, and our results provide direct experimental support for an early hypothesis about the formation of 3-oxo- Δ^4 intermediates in the ecdysteroid biosynthetic pathway of arthropods [11]. We can reasonably conclude that the first step is the 5 β -reduction of the 3-oxo- Δ^4 precursor; 14 α -hydroxycholesta-4,7-diene-3,6-dione is reduced to give 5 β -diketol, and metabolism of the 3-oxo- Δ^4 -25-hydroxy precursor gives the corresponding 5 β -compound, 3-dehydro-2,22-dideoxyecdysone, which does not accumulate and is rapidly transformed into more hydroxylated metabolites. Therefore the presence of a hydroxy group at C-25 does not seem to impede the 5 β -reduction reaction. This reaction takes place preferentially (although not exclusively) in Y-organs, the main site of steroidogenesis in crustaceans, and thus appears to be specific to steroidogenic tissues.

In vertebrates, 3-oxo- Δ^4 -steroid 5 β -reductases are involved in the catabolism of steroid hormones and the biosynthesis of bile acids (see [9,25]). A progesterone 5 β -reductase has also been described as part of the biosynthetic pathway of cardenolides in the plant *Digitalis purpurea* [10]. Several properties of the enzyme system responsible for the 5 β -reduction reaction in crab moulting glands appear similar to those of previously analysed enzymes, even at our preliminary stage of biochemical characterization. The substrate requirements appear similar, as all of these enzymes catalyse the reduction of the Δ^4 double bond of substrates having a Δ^4 -3-one structure; indeed, cytosolic preparations of *Carcinus* reduced [3 H]3 β ,14 α -dihydroxycholesta-4,7-dien-6-one only very slowly compared with the 3-oxo molecule, and this result is fully consistent with a previous report that insect prothoracic glands were unable to metabolize this substrate *in vitro* [24]. The 5 β -reductase activity has generally been localized in the soluble fraction, but in a few cases it has also been found in other subcellular fractions (e.g. see [26] for the localization of human hepatic cortisol reductase). In *Carcinus maenas*, 5 β -reductase activity was found in all subcellular fractions of Y-organs. The cytosolic fraction achieved the formation of 5 β -diketol only. This was not the case with the membrane fractions, where other metabolites were formed; one of these co-migrated with a 5 α -diketol reference compound, suggesting the additional presence of a 5 α -reductase. All reported enzymes require NADPH as a cofactor; in *Carcinus*, NADH was ineffective.

In many mammalian species, 5 α -reductase (4-ene-3-oxosteroid 5 α -oxidoreductase; EC 1.3.99.5) is a key enzyme in steroid hormone metabolism, especially androgen activation in peripheral tissues (see [9,27]). This membrane-bound enzyme requires NADPH as coenzyme, and selectively transforms 4-ene-3-oxosteroids into the corresponding 3-oxo-5 α -steroids. In crabs, 5 α -reduction also required the presence of NADPH. However, in arthropods, 5 α -compounds have almost no biological activity [28], and they have never been reported as secretory products of moulting glands. The presence of 5 α -epimers, including 5 α -2-deoxyecdysone, has only been reported once, as cholesterol metabolites in locust ovaries [29], and the authors suggested that they could possibly arise from 5 β /5 α epimerization during sample processing. Our results did not support this hypothesis, as 5 α -epimers were not detected in cytosolic samples. In *Carcinus*, the absence of 5 α -metabolites of 3-oxo- Δ^4 -steroids when using intact cells indicates that, under physiological conditions, these epimers are not formed, possibly due to the compartmentalization of biosynthetic reactions.

5 β -Diketol, the first compound obtained from precursor **1a**, has been considered as a probable intermediate in ecdysone biosynthesis (see [2,3]), in particular in those species where 3DE is a major or even unique secretory product. Upon *in vitro* incubation [3 H]5 β -diketol yields mostly 3DE, in insects [4,7,30] and in crayfish [8]. The same was observed for *C. maenas* in the present study with both 3-oxo precursors. However, the relative abundance of labelled metabolites did not reflect that of endogenous secretory products. A more 'physiological' pattern of labelled metabolites was obtained when Y-organ cells were incubated with [3 H]ketodiol [31]. This means that the secretory pattern must be closely connected with a precise trafficking of intermediates between compartments; any disturbance connected with the use of an unusual precursor, or simply a normal but exogenously supplied precursor, might be able to modify the relative abundance of end-products. This was also observed when using 25-hydroxycholesterol as an early ecdysone precursor [32]. Thus a quantitative difference in the relative abundance of metabolites does not allow us to conclude that an exogenously supplied molecule is not a normal intermediate.

In view of our results and of previous hypotheses, we consider that 14 α -hydroxycholesta-4,7-diene-3,6-dione is a good candidate for representing a true intermediate in the ecdysteroidogenic pathway, even if all the criteria required to confirm it unequivocally as an intermediate [3] have not yet been fulfilled. Further investigations are under way in order to better characterize the 5 β -reductase, and also to identify an immediate precursor of 14 α -hydroxycholesta-4,7-diene-3,6-dione. Our working hypothesis favours cholesta-4,7-diene-3,6-dione, differing only by the absence of the 14 α -hydroxy group. Elucidating the 'black box' reactions indeed represents a difficult challenge, as early intermediates do not accumulate and appear to be highly unstable; it is hoped that the combination of our present 'ascending' strategy with the complementary 'descending' one (used to identify the next step after the formation of 7-dehydrocholesterol; see [33]) will result in significant progress over the forthcoming years.

The ecdysteroid biosynthetic pathway uses enzyme systems that seem to be closely related to those of vertebrates: a set of cytochrome P-450 mono-oxygenases (reviewed in [4]), a 3 β -hydroxysteroid dehydrogenase [16] and a 5 β -reductase (the present work). Thus, although the terminal products are quite different, the steroidogenic systems of vertebrates and invertebrates appear to operate through very similar mechanisms.

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