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Metabolism of 3-dehydroecdysone in the crayfish *Orconectes limosus* (Crustacea: Decapoda)

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Ecdysteroid metabolism, 3-dehydroecdysone, Crustacea

Abstract. 3-Dehydroecdysone (3DE) is the major secretory product of crayfish moulting glands. The metabolic fate of 3DE in the crayfish *Orconectes limosus* was investigated after in vivo injection of [^{3}H]3DE and after incubation of various tissues with [^{3}H]3DE in vitro. Metabolites in whole body and tissue extracts were analysed by HPLC. In vivo [^{3}H]3DE is efficiently reduced and hydroxylated to [^{3}H]20-hydroxyecdysone (20E), which is one of the major metabolites observed in our study and also the major circulating ecdysteroid. 3β-reduction and 20-hydroxylation were the predominant enzymatic activities obtained after in vitro incubation of [^{3}H]3DE with various tissues. Under the conditions utilised here, antennal glands and CNS (abdominal ganglia) exhibited highest reductase activity per mg wet weight; epidermis, gonads, midgut gland and hindgut had moderate activities; muscle had the least and hemolymph was inactive. 3DE conversion by Y-organs differed moulting stage dependently. 20-hydroxylation activities were similarly distributed in all organs except for ovaries, in which 20-hydroxylation exceeded the 3β-reduction. Ovaries were also the only tissue containing a significant 3α-reductase activity.

INTRODUCTION

Ecdysteroids**, or moulting hormones regulate fundamental events in the life of arthropods such are growth, moulting and larval development. The moulting glands of several arthropod species secrete 3DE or 25-deoxyecdysone (25dE) in addition to or instead of ecdysone (E) (reviewed in Grieneisen, 1994). *Manduca sexta* and other lepidopteran species produce 3DE in the prothoracic glands (Warren et al., 1988; Kiriishi et al., 1990; Blais & Lafont, 1991) and 3DE is also the major ecdysteroid produced by the Y-organs of several crustacean species. The Y-organs of the crabs *Cancer antennarius* (Spaziani et al., 1989), *Menippe mercenaria* (Rudolph & Spaziani, 1992; Rudolph et al., 1992), the penaeid shrimp *Penaeus vannamei* (Blais et al., 1994) as well as those of the crayfishes *Procambarus clarkii* (Sonobe et al., 1991), *Orconectes limosus* (Böcking et al., 1993), and *Astacus astacus* (Böcking, unpublished results) secrete 3DE in vitro.

In insects, 3DE, after being secreted by the prothoracic gland is rapidly converted into E by 3-oxoecdysteroid-3β-reductases (β-reductases) present in the hemolymph and various other tissues (Blais & Lafont, 1984; Milner & Rees, 1985; Weirich et al., 1989, 1993; Warren et al., 1988, Sakurai et al., 1989; Kelly et al., 1990; Gelman et al., 1991; Kiriishi et al., 1990). Subsequently, 20-hydroxylase transforms E into the physiologically more

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** Abbreviations for ecdysteroids follow the nomenclature suggested by Lafont et al. (1993).

active ecdysteroid 20E. 3DE and its hydroxylated metabolite 3-dehydro-20-hydroxy-ecdysone (3D20E) are also central components of a deactivating system in insects (reviewed in: Lafont & Koolman, 1984; Weirich, 1989; Thompson et al., 1990). Ecdysone oxidase (E.C 1.1.3.16) converts E and 20E in their 3-oxo-counterparts, which are subsequently reduced by a 3-oxoecdysteroid-3α-reductase (α-reductase) to give the hormonally inactive 3α-epimers E' and 20E'.

In contrast to insects, little is known about the metabolic fate of 3-oxoecdysteroids in crustaceans. Also in this arthropod group 20E (or ponasterone A in 25dE producing species) is by far the predominant circulating ecdysteroid, as has been revealed by whole body and hemolymph extraction and subsequent characterisation of extracted ecdysteroids by different methods (reviewed in Spindler et al., 1980; Lachaise, 1989, 1990; Jegla, 1990). The major hemolymph ecdysteroid of the 3DE producing species *Procambarus clarkii* (Sonobe et al., 1991), *Orconectes limosus* (Böcking, 1991) and *Penaeus vannamei* (Blais et al., 1994) is also 20E. These species contained no or only traces of 3DE and no 3D20E was found. That is indirect evidence for a 3β-reductase in these species. In this regard, the only exception so far examined is the crab *Menippe mercenaria* (Rudolph & Spaziani, 1992; Rudolph et al., 1992), in which after in vivo labelling with [^{3}H]cholesterol 3D20E, 3DE and PonA appear as hemolymphatic ecdysteroids. A recent study on 3DE metabolism in the crayfish *Procambarus clarkii* (Ikeda & Naya, 1993) has shown that 3β- as well as 3α-reduction can occur after injection of tritium labelled 3DE.

At the moment, the sites of 3DE reduction in crustaceans remain unrevealed. As in *Manduca*, the moulting gland itself seems not to be the appropriate tissue since in all examined species, Y-organs reincubated with [^{3}H]3DE produced no or only small amounts of E. But in contrast to insects, no reductase activity was found in the hemolymph of *Procambarus* (Sonobe et al., 1991) and in the serum of *Cancer* (Spaziani et al., 1989) The only crustacean tissue shown to contain significant reductase activity is the epidermis of *Penaeus* (Blais et al., 1994), but beyond this no other tissue has been examined.

To understand more precisely the 3DE metabolism in crustaceans, we were led to study the metabolism of 3DE in vivo and the ability of different crayfish tissues to convert 3DE in vitro.

MATERIALS AND METHODS

Animals

Crayfish, *Orconectes limosus*, were obtained from the Havel river in Berlin and were kept in the laboratory as previously described (Christ & Sedlmeier, 1987). Moult stages were determined according to Willig & Keller (1973). Routinely, animals from early premoult stage (D_0) were taken for the experiments.

Chemicals

All chemicals were of analytical grade.

E and 20E were from Simes (Milan, Italy). 3DE, 3dehydro-20-hydroxyecdysone (3D20E) and 3-dehydro-2-deoxyecdysone (3D2dE) were prepared from the corresponding unoxidized compounds by chemical oxidation with oxygen gas in the presence of platinum as catalyst according to Girault et al. (1989).

Tritiated 2-deoxyecdysone (2dE) was kindly provided by C. Hétru and J. Hoffmann (Strasbourg, France). All other tritium labelled ecdysteroids were prepared from this compound as has been described elsewhere (Modde et al., 1984).

Drying of [^{3}H]3DE solutions in organic solvents was always carried out in the presence of at least 10% water (1% TFA), thus preventing the degradation of 3DE to less polar compounds, which has been frequently observed in previous studies. This degradation is possibly due to imine formation of the 3-oxosteroid with trace amounts of amines present in the organic solvents or biological samples. Similar findings have been reported for vertebrate type 3-oxosteroids (Eriksson et al., 1983, 1984).

Metabolism of 3DE and E in vivo

Approx. 1 µCi (approx. 20 pMol) of [^{3}H]E and [^{3}H]3DE were dissolved in 100 µl Van Harreveld Saline (Van Harreveld, 1936) and injected in male intermoult and premoult crayfish. The animals were kept subsequently in 1 l of well-aerated water. Hemolymph samples were drawn after designated time intervals. At the end of the incubation, animals were bled and dissected. Abdominal muscles, stomach, midgut gland, hindgut, testes, abdominal nerve chain and antennal glands were totally removed. Integument was from the dorsal part of the thorax and the abdomen. The tissues were rinsed with saline and the wash fluid was pooled with the hemolymph. Tissues as well as the remainder of the crayfish from now on referred to as carcass were weighed and homogenised in 6 volumes of methanol using an ultraturrax homogenizer. The extracts were centrifuged for 30 min at 20,000 g, the pellets were 2 times reextracted with methanol and the combined supernatants were evaporated to dryness after liquid scintillation counting of an aliquot. The dried matter was redissolved in distilled water, and after centrifugation and chloroform partitioning, the ecdysteroids in the aqueous phase were extracted by SepPak C18 chromatography as has been detailed in Lachaise & Lafont (1984). Incubation water was directly subjected to SepPak purification. Extracts were dried, redissolved in the appropriate solvent and subjected to HPLC.

Metabolism of 3DE in vitro

Tissues were dissected under ice-cold Van Harreveld saline (Van Harreveld, 1936) minced, and incubated in culture medium [approx. 20 µl/ mg wet weight of medium 199 modified according to Keller & Schmid (1979)] with the tritiated 3DE (approx. 100,000 cpm) added in ethanolic solution (final concentration 1%, v : v).

Conversion of 3DE in homogenates was tested by adding [^{3}H]3DE (200,000 cpm) to a homogenate of 250 mg tissue in 1 ml of potassium phosphate buffer (50mM, pH 7.4) which contained 0.2 mg/ml NADP, 0.5 mg/ml glucose-6-phosphate and 1 U/ml of G6P-dehydrogenase from *Leuconostoc mesenteroides* (Sigma).

At the end of the incubation, the reaction was stopped by adding 4 vol. of acetonitrile. The mixture was vigorously vortexed and centrifuged for 10 min at 10,000 g. The supernatant was dried, redissolved in acetonitrile and subjected to RP-HPLC.

HPLC

All HPLC analyses were performed on a two pump system with a model 720 gradient controller (Waters). Four different systems were employed.

System 1: For routine RP-HPLC analysis of metabolites in vivo we used a Spherisorb ODS 2 column at a flow rate of 1 ml/min. Ecdysteroids were eluted 5 min isocratically with 20% of acetonitrile/isopropanol (5 : 2, v : v) in water/TFA (0.1%, v : v), followed by a hyperbolical gradient (curve 7) ranging from 20 to 60% of the organic solvent in 15 min and a linear gradient from 60 to 100% in 10 min.

System 2: To analyse highly polar metabolites, peaks from RP-HPLC were collected, dried, redissolved, in 1 ml sodium acetat buffer (100 mM, pH 5.0) and divided in two aliquots, one of which was hydrolysed with glucoronidase Type H2 (Sigma) for 24 h at 37°C. Hydrolysed products and non hydrolysed references were analysed by RP-HPLC on a Spherisorb ODS-2 column with a linear gradient ranging from 8 to 40% (v : v) Tris-$HClO_4$ in acetonitrile in 60 min.

System 3: NP-HPLC was performed on a Zorbax-Sil column eluted with dichloromethane/isopropanol/water (125 : 30 : 2, v : v : v) at a flow rate of 2 ml/min.

System 4: Metabolites after in vitro experiments were routinely analysed by RP-HPLC-analysis on Spherisorb ODS 2 column and isocratic elution with 23% acetonitrile in water (0.1% TFA). Typical retention times were 5.2, 5.6, 6.8, 9.8, 10.4, and 13.6 min for 20E, 20E', 3D20E, E, E', and 3DE respectively, but variations up to 15% were observed if the composition of the solvent was slightly changed.

Radioactivity in RP-HPLC was measured continuously by a PC-controlled Flo-one beta A-200 monitor (Radiomatic). In NP-HPLC 0.4 ml fractions were collected, aliquots were dried, and radioactivity was

determined by liquid scintillation counting. Radioactive conversion products were identified by comigration with unlabelled reference compounds coinjected with each HPLC run. Conversion rates were calculated by peak integration (Program A-250, Radiomatic).

RESULTS

Metabolism of 3DE in vivo

[^{3}H]3DE or [^{3}H]E were injected into crayfish. We used 4 animals for each ecdysteroid. Whole animals or dissected tissues and hemolymph samples were extracted in methanol. Extracts were prepurified by SepPak chromatography and ecdysteroids were analysed by HPLC.

14 h after the injection of radiolabelled ecdysteroids most of the radiolabel was found in abundant tissues such as muscle, hemolymph and integument. Only 3–4% of radiolabel was in the water. If expressed as radiolabel/mg wet weight, it seems that radioactivity is accumulated in antennal glands, hindgut and testes (Fig. 1).

HPLC-analysis of extracts from whole animals or dissected tissues showed that both ecdysteroids are nearly completely metabolised after the 14 h incubation (Fig. 2a,b), the pattern of metabolites being nearly identical. 3DE was never found and E only in traces in one of the test animals. The most abundant metabolite was 20E followed by 20,26E and highly polar products. The latter were identified as polar conjugates of 20E and 20,26E by hydrolysis with *Helix* juice. Rechromatography of isolated peaks by RP-HPLC with Tris/$HClO_4$ as the aqueous solvent and NP-HPLC assessed the identity of 20E and 20,26E as major metabolites. We found also small quantities of Eoic and 26 E (not shown).

Specified by tissues, 20E was the major metabolite in carcass, integument and muscle, whereas hemolymph and antennal glands contained high amounts of polar metabolites (20,26E and polar conjugates). Considerable amounts of apolar conjugates were found in the midgut gland and the stomach. Unconverted 3DE or E contributed to more than 70% of the small amount of radioactivity that was found in the water. Most probably they had leaked from the site of injection.

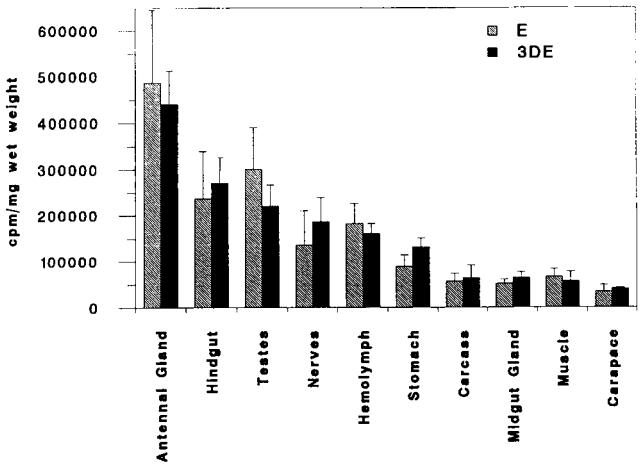

Fig. 1. Tissue distribution of radiolabel normalized to tissue weight 14 h after injection of [^{3}H]3DE or [^{3}H]E respectively. Values represent means ± SD (n = 2).

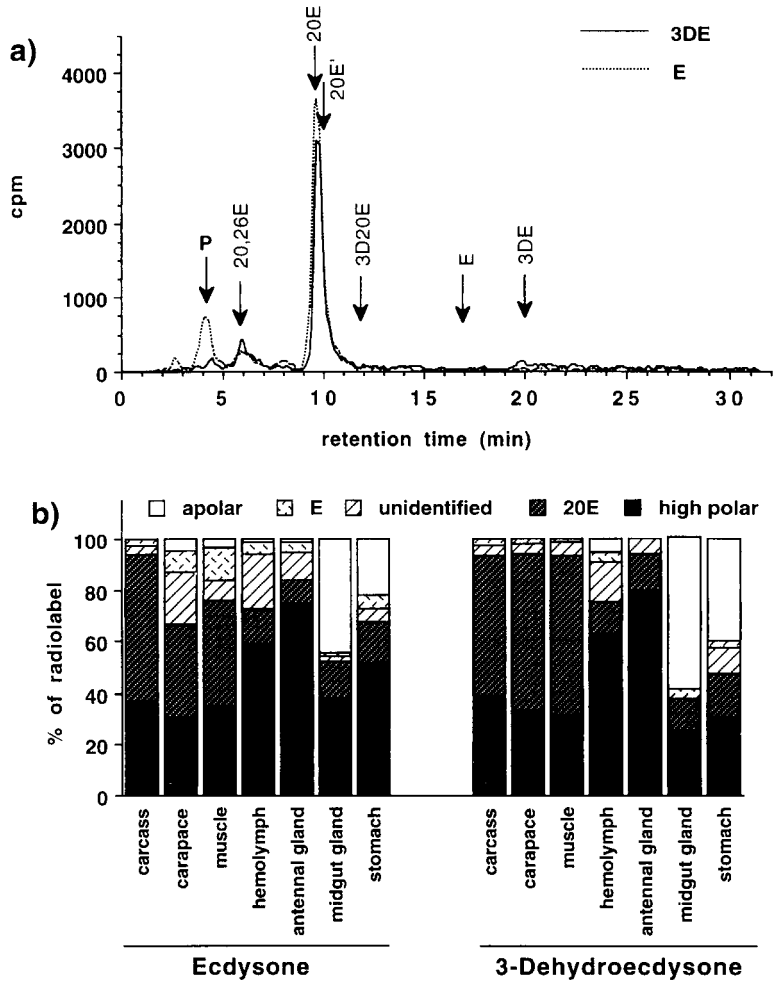

Fig. 2. Metabolic pattern of 3DE and E obtained 14 h after injection in vivo. Either whole animals or dissected tissues were extracted with methanol according to Materials and methods. Aliquots of the extracts were analysed by RP-HPLC. a – representative RP-HPLC analysis (system 1) of whole animal extracts. Retention times of unlabelled reference compounds are indicated by arrows. P marks highly polar compounds [mostly conjugates (see text)] not separated in this system. b – metabolites of 3DE and E 14 h after in vivo injection, specified by tissues ("high polar" includes 20,26E and polar conjugates).

Among the metabolites of 3DE, only traces of E could be detected and 3D20E was never found after one night incubations. 3α-epimers did not appear as metabolites in vivo, regardless of incubation time, tissue or moulting stage.

Fig. 3 shows the time course of 3DE and E metabolism, assayed by HPLC-analyses of hemolymph samples. Both ecdysteroids are rapidly cleared from the hemolymph. 20E becomes predominant already 1 h after injection. Subsequently, 20E decreases leading to 20,26E and highly polar conjugates.

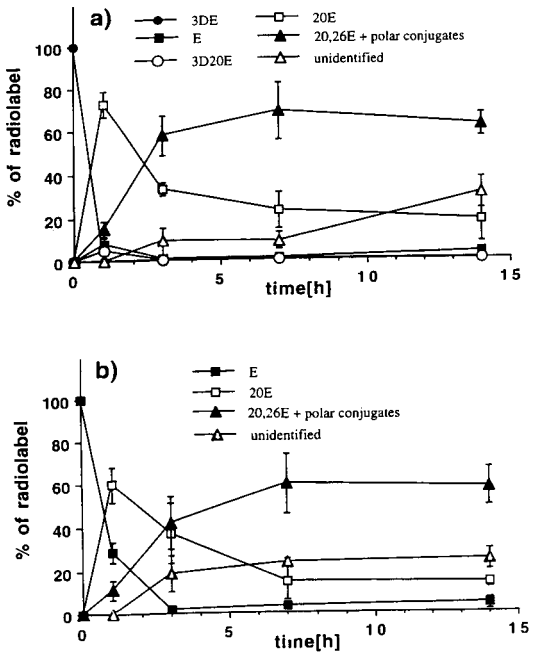

Fig. 3. Time course of metabolite formation in vivo assayed by RP-HPLC analysis of hemolymph samples: a – after injection of [^{3}H]3DE; b – after injection of [^{3}H]E. Values represent means ± SD (n = 2).

The metabolic pattern in pre- and intermoult animals (intermoult data not shown) was essentially the same. There were some small interindividual differences but they had apparently no relation to the moulting stage.

Metabolism of 3DE by tissues in vitro

3DE converting enzyme activity in crayfish tissue was examined by incubating pieces of tissue with [^{3}H]3DE for 16 h and subsequent HPLC-analysis of conversion products. Fig. 4a shows a representative HPLC run obtained after the incubation of neuronal tissue. In vitro, 3DE conversion proceeds more slowly than in vivo. Thus, besides 20E also E and 3D20E, the direct β-reduction and hydroxylation products of 3DE, were detected.

Time course studies, exemplarily shown for nerve tissue (Fig. 4b), demonstrate that under our experimental conditions conversion of [^{3}H]3DE became detectable after a lag of 1 h and proceeded linearly for the next 4 hours. From then on, conversion rates decreased, but generally conversion kept on until a total transformation into 20E was achieved. This time course was observed for all tissues with high content of 3DE converting enzyme activity (see below). Conversion was linear for more than 20 h with low activity tissues (see below).

Fig 5. shows a comparison of the 3DE metabolising activities in different tissues, calculated from the conversion rates after 1 night incubations. The amounts of radioactivity in the different peaks obtained by peak integration were normalised to 1 mg wet weight. The radioactivity found in 3β reduced compounds (E and 20E), 3α-reduced ecdysteroids (20E' and E') or 20 hydroxylated metabolites (20E, 20E' and 3D20E) were taken as an expression for 3β-reductase, 3α-reductase or 20-hydroxylase activity respectively. With the exception of hemolymph and Y-organs (see below) all tissues displayed metabolic activity. In these tissues 3β-reduction as well as 20-hydroxylation were observed. The only tissue exhibiting 3α-reduction were the ovaries. In one experiment, we tested also Y-organs from animals in late premoult which displayed 3β-reductase as well as 20-hydroxylase activity (data not shown).

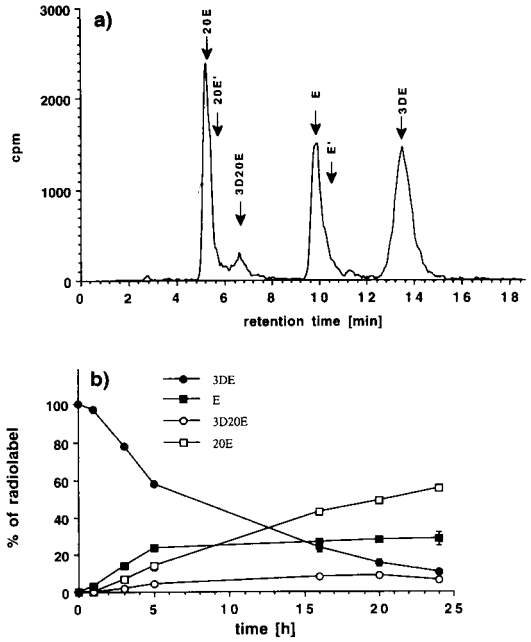

Fig. 4. Conversion of [^{3}H]3DE by nerve tissue in vitro. a – RP-HPLC analysis (system 4) of an extract from culture medium and tissue after 16 h incubation; b – time course of 3DE conversion by nerve tissue in vitro. Given are means ± SD (n = 5).

Highest 3β-reductase activity was found in antennal glands, followed by nervous tissue, the hindgut, the midgut gland, epidermis, and the gonads. Except for the ovaries which contained high hydroxylase activity 20-hydroxylase activities were distributed in the same order. 3β-reductase and 20-hydroxylase activity in muscle was very low. Neither diluted hemolymph nor serum or hemocyte preparations (data not shown) even in presence of a NADPH regenerating system were able to metabolize 3DE.

Metabolism in homogenates

3DE conversion by homogenates and subcellular fractions was completely different from that observed in vivo and with minced tissues in vitro. Using a simple protocol which had been employed in numerous insect studies on ketoreductases, we observed a nearly complete loss of 3β-reduction. Surprisingly some 3α-reduction appeared in homogenates. Fig 6. shows the HPLC profiles of 3DE metabolites obtained with pieces of midgut gland and with midgut gland homoge- nate.

At the moment, we have not succeeded in developping an assay, which avoids this loss of β-reductase and which would allow the precise characterisation of subcellular distribution, kinetic parameters, and substrate specificity of 3DE converting enzymes.

DISCUSSION

Our study on 3DE metabolism in *Orconectes limosus* shows that 3DE, the secretory product of the Y-organs, is efficiently converted into 3β-reduced compounds in vitro as well as in vivo.

Furthermore, the in vivo experiments demonstrate that the metabolites of 3DE are essentially the same as those of E. Since this study focussed on the reduction of 3DE, no great effort was made to identify all the metabolites more polar than 20E, but both the formation of 20,26E and apolar conjugates have been reported for *Orconectes limosus*

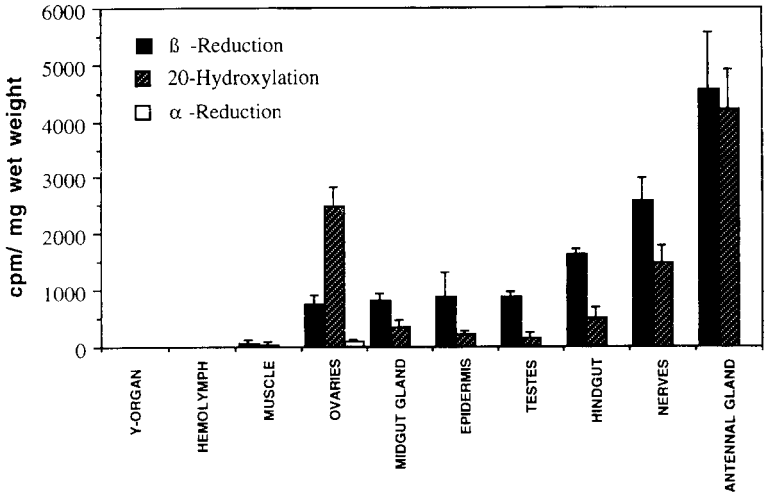

Fig. 5. Comparison of [^{3}H]3DE metabolism by various tissues in vitro (16 h incubations). Radioactive ecdysteroids were extracted from culture media and tissues, and analysed by RP-HPLC (system 4, see Fig. 4a – values for the different enzymes were obtained by calculating the sums of radioactivity comigrating with E + 20E (3β-reduction), E' + 20E' (3α-reduction) or 20E + 20E' + 3D20E (20-hydroxylation) respectively. For comparison, radioactivity was normalised to 1 mg of tissue. Columns represent the mean of n = 4 to 9 incubations from at least two separate experiments ± SD.

(Kuppert et al., 1978) and many other crustaceans [see Snyder & Chang (1992) for detailed bibliography] and they are suggested to be inactivated products.

The in vitro studies, examining the metabolism of 3DE by various tissues revealed the nearly ubiquitous distribution of 3β-reductase in crayfish tissue. Hemolymph was the only tissue without any metabolic activity. With this exception, the ubiquitous occurrence of 3β-reductase has also been found in all tested tissues of *Pieris brassicae* (Blais & Lafont, 1984), *Ostrinia nubilalis* (Gelman et al., 1991), and *Manduca sexta* (Weirich et al., 1993). We found a similar distribution of enzymatic activity for 20-hydroxylase.

The broad distribution of 20-hydroxylase is in good accordance with various other studies on E-metabolism in crustaceans (Lachaise & Feyereisen, 1976; Lachaise & Lafont,

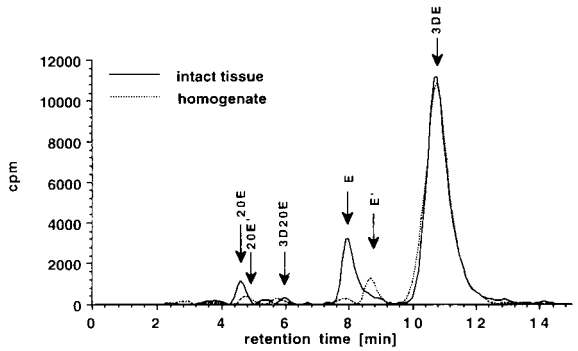

Fig. 6. Comparison of [^{3}H]3DE metabolism by intact tissue and homogenate. 250 mg of midgut gland were either cut in pieces and incubated in culture medium or homogenised and incubated in phosphate buffer containing a NADPH regenerating system. Samples were extracted after 16 h and ecdysteroids were analysed by RP-HPLC (system 4). Retention times of reference compounds are indicated by arrows.

1984; James & Shiverick, 1984; Soumoff & Skinner, 1988; Snyder & Chang, 1992). The different tissues display 20-hydroxylase activities in a similar order as has been determined for *Homarus americanus* (Snyder & Chang, 1992) and *Panulirus argus* (James & Shiverick, 1984).

With the exception of the ovaries, 3β-reductase activities in *Orconectes* are distributed in the same manner as are 20-hydroxylase activities. Antennal glands and the digestive organs had high metabolic activity in vitro and contained also large amounts of conjugates after in vivo labelling. Thus, the question has to be raised, whether E or 20E produced from 3DE by these tissues, can reenter the hemolymph or whether they are immediately excreted, via polar (antennal glands) and apolar (digestive organs) conjugates. There is some evidence that reduction and hydroxylation may not be necessarily connected to the excretion, because the formation of 26-hydroxylated ecdysteroids and conjugates, even though observed in vitro with these tissues only (data not shown), occurs much more slowly. Moreover, in the in vivo experiments, only a very small percentage of radioactivity was found in the water, containing the urine and feces.

Most of the data presented in our study reflect only enzymatic activities in early premoult stage. Thus, a possible change in activities, correlated with the moult cycle, namely a rise in $D_{2,3}$ at ecdysone peak has not been investigated. This may explain the low activities we found in epidermal tissue. Previous studies showed that 20-hydroxylase activity in crustacean epidermis and other tissues increases during late premoult (Chang & O'Connor, 1978; Snyder & Chang, 1992). Our finding that Y-organs in late premoult contain enzymatic activities whereas those in inter- and early premoult do not, speaks in favour for moulting stage related changes also in *Orconectes*. Moreover, changes of enzyme activies during the vitellogenic cycle, reported for the ovaries of *Carcinus maenas* (Lachaise & Lafont, 1984) and unequal distribution of enzymes in some organs, which had been observed for different parts of the CNS of *Ostrinia nubilalis* (Gelman et al., 1991) may be other important features, which were not detected by our assay.

At first glance, our results are conflicting with a recent study on in vitro metabolism of 3DE in *Procambarus clarkii* (Ikeda & Naya, 1993) a species very closely related to *Orconectes*. In particular, we do not find any evidence neither for 3α-reduction of 3DE nor for an accumulation of radiolabel in the integument nor for a considerable excretion of 3DE. Most of the discrepancies may be explained by the high doses of injected 3DE into *Procambarus* (100 µg vs. approx. 10 ng in the present study). Thus, all the effects described for *Procambarus* may reflect metabolic reactions to a toxic stimulus, whereas our study may be closer to normal physiological conditions. However, the *Procambarus* study showed clearly that 3α-reduction can occur in crustaceans and it is possible that it is an efficient inactivation mechanism. In this regard, the 3α-reductase activity in ovaries found in *Orconectes* may also reflect a physiological protection of maturing eggs against high ecdysteroid titers in the hemolymph.

Surprisingly, the metabolism of 3DE in tissue homogenates was completely different from that observed in vivo and with intact tissues. While E and 20E were the major products obtained with intact tissues, homogenates gave rise mainly to E'. This effect was presumably not due to action of proteases, since trypsin inhibitor and PMSF did not change the metabolic pattern (not shown). The reason for this important loss of 3β-reductase and the significantly increased 3α-reductase activity, which was otherwise observed with

ovarian tissue only, is not clear. Interestingly a change of 3β- : 3α-reductase ratios upon homogenization was shown for the locust midgut (Dinan, 1980) but in this case the homogenization favoured the 3β-reduction. At the moment, we can only speculate that the loss of β-reduction and the rise of 3α-reduction after homogenization may be due to the destruction of intracellular compartiments and transport routes, necessary for the naturally occuring ecdysteroid metabolism.

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