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The biotransformation of tritiated 3-dehydroecdysone by crayfish, *Procambarus clarkii*

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Abstract. The injection of 3-dehydroecdysone (3dhE, 5 μg/g), the major ecdysteroid secreted by the Y-organ of crayfish *Procambarus clarkii*, resulted in apolysis within about 5 days. The hormonal response at the molecular level was investigated by injection of the radio-labeled compound; within 3 h of injection of [^{3}H]3dhE, most radio-isotope was found in the extracted epidermal tissues and identified as ecdysone, 20-hydroxyecdysone (20E), and their 3α-hydroxy epimers. The biotransformation was undoubtedly performed in the peripheral area of the Y-organ. Cleavage of the polar conjugates, using an enzyme from *Helix pomatia*, gave all of the above ecdysteroids including 3dhE. It was also found that the biosynthetic site of 3dhE was different from that of ecdysone at the subcellular level of the Y-organ.
Key words. Molting hormone; ecdysteroids; 3-dehydroecdysone; 3α-hydroxyecdysteroids; Y-organ; crayfish; *Procambarus clarkii.*

Previous evidence has demonstrated that the molting glands, Y-organs, of crustaceans secrete not only ecdysone[1] but also other ecdysteroids, 25-deoxyecdysone[2,3] and 3-dehydroecdysone (3dhE)[4]. Additionally, 20-hydroxy-25-deoxyecdysone or ponasterone A and 3dhE have been found in the circulation and are thought to be involved in hormonal function in the insect kingdom[5–7]. However, the physiological significance of 3dhE in crustaceans is so far equivocal. We reported in a previous paper[8] that, 3dhE was the major product of the cultured Y-organs from crayfish *Procambarus clarkii*. The present paper deals with hormonal response and biotransformation of 3dhE, injected at a dose of 5 μg/g live weight of the crayfish. The conversion of 3dhE in the crayfish is also discussed.

Materials and methods

Animals. Juvenile male crayfish, *P. clarkii* (70–90 mm), were purchased and maintained for several days in tap water at the normal seasonal temperature and photoperiod. To activate the Y-organs[8], eyestalks were bilaterally excised (day 0) and the chelae were forced to autotomize on the same day.

Ecdysteroidogenesis of Y-organ and the homogenized Y-organ, in vitro. The Y-organs were dissected in the days after eyestalk removal (day 2–9, 4-organs each) and cultured in medium 199 (4 organs/1 ml) at 25 °C for 12 h to produce ecdysteroids; the culture medium was prepared as previously described[8] except for the addition of antibiotics. The effect of xanthurenic acid, an ecdysone biosynthesis inhibitor[9], on the culture of Y-organs (from 5 donors on day 4) was examined; a pair of Y-organs was divided into two parts and incubated in the medium (5 organs in 1 ml) as mentioned above, in the presence or absence (control) of xanthurenic acid (2×10^{-6} M). The homogenized Y-organ (at 0 °C, 100 μl/Y-organ) was centrifuged for 20 min (at 5 °C, 1000 × *g*), The supernatant and the washings (50 μl × 2/Y-organ) were combined, divided into aliquots (200 μl/Y-organ) and incubated to compare with the Y-organ culture (see above).

HPLC analysis of ecdysteroids. The Y-organ culture, and hemolymph collected by hypodermic needle from the base of the first pereiopod (0.5 ml from each donor) just before the Y-organ dissection, were centrifuged (1000 × *g*) to remove debris. Each supernatant was loaded on Sep-Pak C_{18} (Waters) and eluted with 25% (5 ml) and 60% (10 ml) of aqueous methanol successively. The eluate (60% aq. MeOH) was concentrated and subjected to RP-HPLC (Shimadzu system, LC-9A, SPD-6A, C-R6A, SCL-6B); Cosmosil 5C_{18} (Nacalai tesque), 4.6 × 250 mm, 1 ml/min, 40 °C, 254 nm detection. The ecdysteroids of the Y-organ culture were analyzed under gradient elution, 1%/min, 40–50% (MeOH:CH_3CN; 3:1) in water. Each hemolymph aliquot was fractionated under the above elution condition and then analyzed under gradient elution, 0.5%/min, 35–40% (MeOH:CH_3CN; 3:1) in water. The retention times of authentic compounds were used to confirm the identification. In addition, purities of the elution bands were examined by a diode-array detector in the range of 210–300 nm (Hewlett-Packard's Series II, 1090 L. C.). Ecdysone and 20E were purchased from Sigma, while 3dhE was prepared from ecdysone according to the published method[10].

Hormonal response and biotransformation. a) The three experimental groups (ca. 20 g crayfish, n = 7–12 in each) were injected with 5 μg/g[11] of each of the three ecdysteroids (3dhE, ecdysone, and 20E) dissolved in medium 199 containing 10% ethanol (300 μl). Controls

received either 300 µl of the medium or cholesterol (5 and 20 µg/g) in medium, respectively. Each sample was injected into the hemocoel, at the base of the first pereiopod. After injection, the animals were exposed to air for 1 h and then returned to the aquarium at 25 °C on a photophase 16:8 (L:D) to observe hormonal response for several days.

b) [^{3}H]3dhE (851 MBq/mmol) dissolved in the medium was also given to animals (5 µg/g). Radio-labeled 3dhE was prepared from [23,24-^{3}H]ecdysone (3064 GBq/mmol, Du Pont/NEN Research Products) in a similar manner as 3dhE[10]. The hemolymph was sampled 1, 3, 12, and 72 h after injection (200 µl each), and all excreta were also collected at the same time. The hemolymph donors (n = 4) were killed for the analysis of the radiolabel distribution. The lyophilized excreta, hemolymph, tissues of epidermis with carapace, muscle, hepatopancreas, and all remaining tissues were extracted with methanol and centrifuged before liquid scintillation analysis (Packard, TRI-CARB 460C). After concentration, each extract was also subjected to a preparative RP-HPLC (Beckman, System Gold 110B, 166); Cosmosil $5C_{18}$, 4.6 × 250 mm, 0.8 ml/min, isocratic: 25% (MeOH:CH_3CN; 3:1) in water for 20 min and 50% for 25 min, 40 °C, 254 nm detection. The elution was collected (0.4 ml each), and a part of the eluate was mixed with liquid scintillation solution (Du Pont, AQUASOL 2). Radioactivities were counted with a liquid scintillation counter (Packard) to give the radio-profile represented in figure 4. The rest of the eluate corresponding to each peak (see fig. 4) was concentrated and purified by use of a preparative NP-HPLC; APS-Hypersil (Shadon Southern Product), 4.6 × 250 mm, 2 ml/min (isopropanol : MeOH : CH_2Cl_2 : AcOH : $(Et)_3N$; 1 : 4 : 95 : 0.05:0.05). The peaks were identified as 20E, 20E′ 3dh20E, E, E′, and 3dhE from their retention times (RP- and NP-HPLC). The eluate denoted A in figure 4 was also collected. After evaporation of the solvent, the residue was dissolved in 2 ml of sodium acetate buffer (pH 5.2, 100 mM) containing the digestive juice of *Helix pomatia*[12] (ca. 2400 units of β-glucuronidase, Type H-2, EC 3.2.1.31, from Sigma) and kept at 37 °C over night. The reaction mixture was evaporated and then suspended in MeOH. After centrifugation, the supernatant was subjected to RP-HPLC under the isocratic condition mentioned above. For comparison of retention time, 3α-hydroxyecdysone (E′)[10] was prepared by sodium borohydride ($NaBH_4$) reduction of 3dhE. In a similar manner, 3α,20-dihydroxyecdysone (20E′)[10] was prepared from 20E via 3-dehydro 20-hydroxyecdysone (3dh-20E)[10].

Results and discussion

All experimental animals receiving injections of 3dhE, ecdysone and 20E underwent apolysis over 3–5 days. Although all died before completion of ecdysis, separation of the epidermis from the old cuticle and formation of gastroliths in the stomach were observed in accord with previous reports[13]. Krishnakumaran et al.[11] postulated that the abnormal molt probably resulted from the delivery of a large amount of hormone in one dose, in contrast to the normal gradual release of hormone in situ. In our experiment, effects of ecdysteroids (5 µg/g each) were apparent by day 3 with 20E, day 4 with ecdysone, and days 3–5 with 3dhE. Doses up to 20 µg/g of cholesterol showed no effect. The time required for response to 3dhE was almost comparable with that of 20E and ecdysone; a statistical study is necessary to determine the lag phase that may be due to enzymatic activities in situ. Thus, we postulated that 3dhE was involved in the molting process as a precursor of ecdysone and 20E. It is also possible that 3dhE has a hormonal function of its own as demonstrated in puffing[14].

The Y-organs were activated to produce ecdysteroids by de-eyestalking[15] of randomly-chosen donors; removal of eyestalks effectively synchronizes animals to bring them into premolt. Ecdysteroidogenesis in the activated Y-organs in vitro was followed as a function of days after eyestalk removal for periods up to 9 days. Production of 3dhE was dominant, with a peak (day 4) prior to that of ecdysone (day 7), even when total ecdysteroid production was rather low as seen in Type-2 animals; ecdysteroidogenesis per Y-organ is schematically illustrated in figure 1A and the HPLC profiles at day 0, day 8 and day 9 are shown in figure 1C. Type-1 (March) and Type-2 (October) show clear differences, due to differences in the potency of ecdysteroidogenesis that depend on the molt cycle and/or season. Only 20E was detected in the hemolymph and peaked at day 8 in both types (fig. 1B); chromatograms are depicted for day 7, 8 and 9 (fig. 1D-1: preparative; fig. 1D-2: quantitative, re-chromatography of a specific eluate). The weight of a pair of gastroliths from each corresponding donor increased with time after eyestalk removal[15]. According to previous reports[16, 17], increasing amounts of 20E, reflecting ecdysteroidogenesis, may be characteristic of the molt stages of crayfish. Our data revealed that 20E titers in the hemolymph (max. 150 ng/ml in both Type-1 and Type-2 animals, figs 1A, 1B) were not derived solely by ecdysteroidogenesis of the Y-organs. Eyestalk removal was responsible not only for the stimulation of the Y-organ but also for the release of 20E from an ecdysteroid source other than Y-organ[13]. A substrate which gives ecdysteroid on enzymatic hydrolysis may be present.

It should be noted that ecdysone was the only product when the Y-organs were homogenized[9] and cultured with medium; the homogenate culture failed to produce 3dhE. It is suggested that biosynthesis of 3dhE was mediated by the membrane network in association with

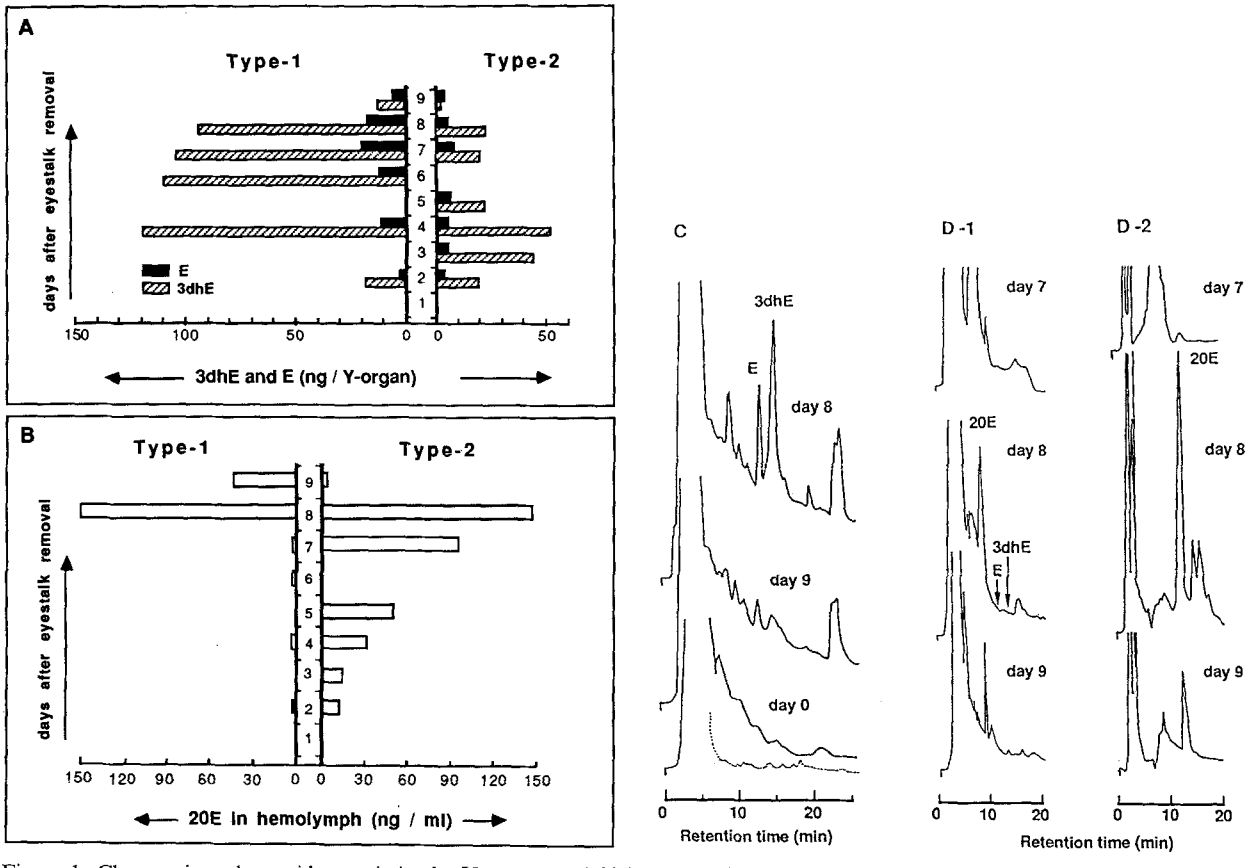

Figure 1. Changes in ecdysteroidogenesis in the Y-organs and 20-hydroxyecdysone in the hemolymph induced by eyestalk removal. Eyestalks were bilaterally excised (day 0). Animals (Type-1, 8–9 cm and Type-2, 7–8 cm in length) were distinguishable by the potency of ecdysteroidogenesis (see text).

A) Ecdysteroidogenesis by the Y-organ incubation (ng/Y-organ). Dissected Y-organs (4 each throughout day 2–9) were cultured in medium 199 at 25 °C for 12 h. Ecdysteroids are shown against days after eyestalk removal (vertical axis) by HPLC analysis for ecdysone and 3dhE (see *C*). Hatched bars, 3dhE (3-dehydroecdysone); closed bars, E (ecdysone).

B) Change in 20E (ng/ml) in the hemolymph (see *D*) against days after eyestalk removal (vertical axis). Hemolymph was collected just before the Y-organ dissection. Open bars, 20E (20-hydroxecdysone).

C) HPLC profiles of the ecdysteroids produced by Y-organs (Type-1) at days 0, 8 and 9 are depicted. The background of medium 199, after the Sep-Pak treatment, is shown by a dotted line. The amounts of E and 3dhE were estimated by UV detection at 254 nm, based on the authentic compounds.

D-1) HPLC profiles of hemolymph (Type-1) at days 7, 8 and 9. The retention times of the authentic compounds are indicated in the figure.

D-2) Quantification of 20E by re-chromatography of a specific eluate from a previous HPLC. The amount of 20E estimated by UV detection at 254 nm, based on the authentic compound.

enzymes. According to the biosynthetic scheme proposed by Lachaise et al.[18], the homogenization of the Y-organ may have caused an increase in the inherent β-reductase [3βR] activity and made it possible to convert all the available substrate into ecdysone. It is also worth noting that the basal production of 3dhE (30–35 ng) was markedly higher than that of ecdysone (4–5 ng); ecdysteroidogenesis was reduced in the presence of ecdysone biosynthesis inhibitor (EBI), xanthurenic acid (XA, 2×10^{-6} M)[9,19,20] (fig. 2). The large inhibition ratio in Type-1 was due to strong ecdysteroidogenesis.

The above observation led us to see whether 3dhE might be an immediate precursor of ecdysone and eventually of 20E. When each excised Y-organ, activated by de-eyestalking (day 4) or without activation (one day before day 0), was incubated with the radio-labeled ecdysone or 3dhE in the presence or absence of hemolymph[8], scintillation spectrometry showed no significant interconversion between the ecdysteroids. The conversion reaction of the radio-labeled 3dhE in vivo was examined as follows.

When [^{3}H]3dhE (851 MBq/mmol) was injected into crayfish (100 µg per animal), ca. 70% of the total radiolabel was excreted within 1 h as unchanged 3dhE, and ca. 30% was retained in the body for 3 h after injection. The distribution patterns of the radio-activity in the body changed with time (see fig. 3). At 3 h, the radioactivities were less in the hemolymph and more significant in epidermis than at 1 h. Rapid reduction of 3dhE with a short half-life (ca. 30 min) into ecdysone in vivo was in agreement with the results from insects *Calliphora vicina*[5], *Manduca sexta*[21] etc.

A typical profile of radio-active ecdysteroids from an extract of epidermis (with carapace) is shown in figure

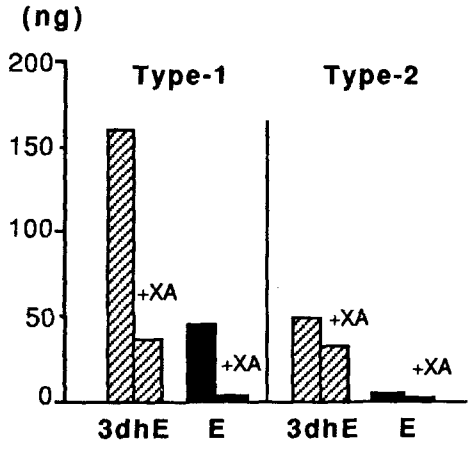

Figure 2. Inhibition of ecdysteroidogenesis in Y-organ by xanthurenic acid in vitro. 4 days after eyestalk removal, the Y-organs were excised from two types' of donors. Type-1 and -2 (n = 5 each) were as noted in figure 1; Type-1 fully induced the capacity of ecdysteroidogenesis but Type-2 did not. A pair of Y-organs was divided into two parts and incubated under the normal culture conditions in the presence or absence of xanthurenic acid. HPLC (see text) was used for quantitative analysis as noted in figure 1. 3dhE: 3-dehydroecdysone; E: ecdysone; +XA: addition of xanthurenic acid.

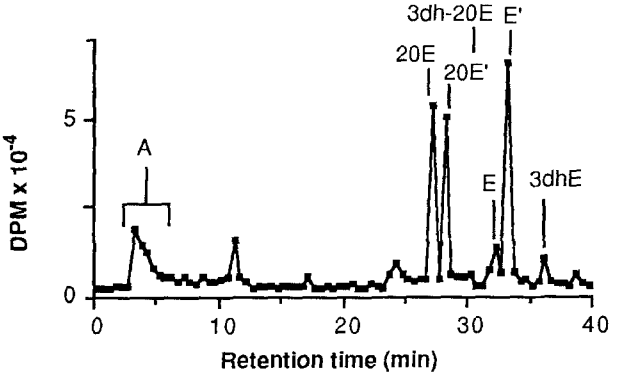

Figure 4. Typical chromatogram of radio-labels derived from [^{3}H]3dhE, in the extraction of epidermis with carapace. 3 h after injection of [^{3}H]3dhE, the extract of epidermis with carapace was subjected to a preparative RP-HPLC, and all fractions were counted by liquid scintillation counter to yield the profile of radioactivity (see text). Arrows show the retention times of the authentic standards. E: ecdysone; E': 3α-hydroxyecdysone; 20E: 20-hydroxyecdysone; 20E': 3α,20-dihydroxyecdysone; 3dhE: 3-dehydroecdysone; 3dh-20E: 3-dehydro 20-hydroxyecdysone; A: a mixture of conjugate.

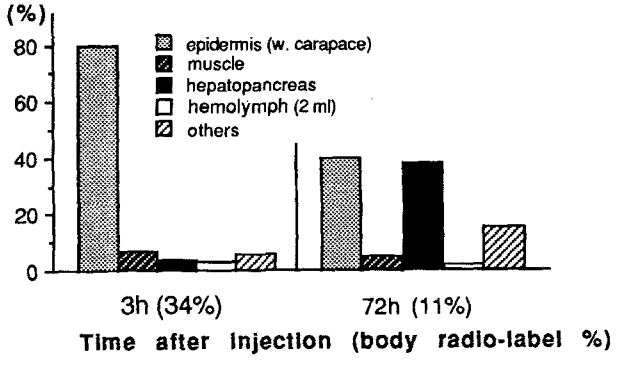

Figure 3. Distribution of the radio-activity in the body after injection of [^{3}H]3dhE. Radio-labeled ecdysteroid secretions were generated in vivo by injecting *P. clarkii* with [^{3}H]3dhE (see text). The radio-isotopes in the target tissues were compared 3 and 72 h after injection. The diagram is based on scintillation counting of each extract from epidermis with carapace, muscle, hepatopancreas, hemolymph, and others (all remaining tissues) as indicated in the figure. The amount of hemolymph per animal was estimated as 2 ml. The majority of injected radio-label was excreted shortly after injection; 66% (3 h) and 89% (72 h).

4. The injected 3dhE was converted to ecdysone, 20E and their 3α-hydroxy epimers and found mainly in the extracted epidermal tissues[22] within 1–3 h, while unchanged tritiated 3dhE alone was found in the hemolymph (fig. 5A). The radio-label was gradually incorporated into more polar compounds including ecdysteroid conjugates (A-fraction in fig. 4) by metabolizing cells[23] or hepatopancreas (3–72 h; cf. fig. 3). The ratio of ecdysone and 20E versus their 3α-hydroxy epimers (E' and 20E') is depicted in figure 5B; the 3α-hydroxy epimers were rather selectively found to be excreted. The mixture of the conjugates, A-fraction in figure 4, were separated by HPLC and subjected to an enzymatic hydrolytic cleavage using *Helix pomatia* enzyme following the published protocol[12]. The HPLC analysis, after usual treatments of the above reaction mixture, revealed the ligands to be 3dhE, ecdysone, 20E and their 3α-hydroxy epimers. It should be noted that the amount of 3dh-20E was quite small.

The animals receiving injections of a high concentration of 3dhE may be forced to excrete rapidly to preserve homeostasis. However, a merit of the injection in high concentration is the synchronization of individual molt cycles and therefore of their precocious molting. It was shown that 3dhE was converted to ecdysone, 20E and also to their 3α-hydroxy epimers, including all of their conjugates in small quantities. The presence of 3α-hydroxyecdysteroids among the conjugates led us to suggest that 3α-reductase is present outside the Y-organ. It is also interesting to find that the converted ecdysteroids were mainly found in the extract of epidermis tissues, which may therefore be a site of ecdysteroid conversion.

Even though our experiments do not represent normal physiological conditions, it can be suggested that there is a common mechanism and/or significant function of 3dhE[6,7] in controlling the molting hormone titer. Although 3dh-20E was found only in small amounts, the possibility that 3dhE is an active hormone with a physiological significance of its own[4,5] like that of ecdysone[24], still remains.

The conclusions from the present experiments are as follows.

1) Biosynthesis of 3dhE in the Y-organ was intrinsic and independent of that of ecdysone at the subcellular level of the Y-organ, where the degree of inhibition by

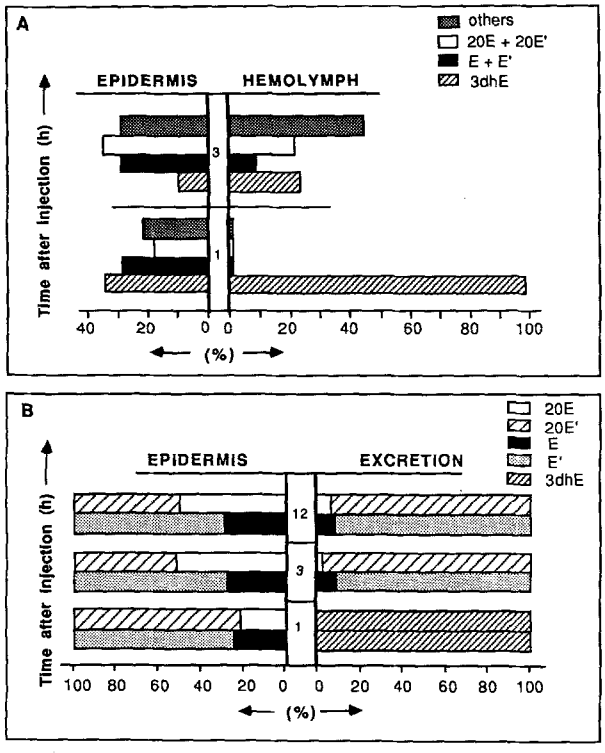

Figure 5. Proportion of the radio-labels derived from injected [^{3}H]3dhE. Each radio-label derived from injected [^{3}H]3dhE was separated and counted as detailed in figure 4. Each compound was identified by co-chromatography on HPLC with the corresponding authentic compound.
A) Illustration of the characteristic proportions of radio-labels in the extract of epidermis with carapace (left) and hemolymph (right); major radio-labels 1 h and 3 h after injection are depicted. 3dhE: 3-dehydroecdysone; E: ecdysone; E': 3α-hydroxyecdysone; 20E: 20-hydroxyecdysone; 20E': 3α,20-dihydroxyecdysone; others: the remaining ecdysteroids including the conjugates.
B) Characteristic change of 3dhE against time after injection; the selective excretions of unchanged 3dhE (1 h) and 3α-hydroxy epimers (E' and 20E'/3 h and 12 h) are depicted. The more efficient hydroxylation of ecdysone into 20E than 3α-E into 3α-20E should be noted.

xanthurenic acid, a common inhibitor of ecdysone biosynthesis[9,19,20], was somewhat different for 3dhE compared to ecdysone.

2) Injected [^{3}H]3dhE was transformed efficiently into ecdysone and 20E, and primarily found in the extract of the epidermis tissues (with carapace). It has also been shown that the presence of 3-oxo-ecdysteroid reductase activity was not restricted to 3β-reductase, and that 3α-reductase was active in releasing E' and 20E'; all 3α-hydroxy-epimers were selectively excreted.

3) The unchanged 3dhE and the converted ecdysteroids, including 3α-hydroxy epimers, were all involved in the conjugated forms.

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