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Authors: Haruyuki Sonobe, Hideki Tokushige, Toshihiro Makka, Hibiki Tsutsumi, Noriyuki Hara, et. al.

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Comparative Studies of Ecdysteroid Metabolism between Diapause Eggs and Non-diapause Eggs of the Silkworm, *Bombyx mori*

Haruyuki Sonobe[1]*, Hideki Tokushige[1], Toshihiro Makka[1], Hibiki Tsutsumi[1], Noriyuki Hara[2] and Yoshinori Fujimoto[2]

[1]*Department of Biology, Konan University, Kobe 658-8501, Japan and*
[2]*Department of Chemistry, Tokyo Institute of Technology, Tokyo 152-8551, Japan*

ABSTRACT—2, 22, 25-Trideoxyecdysone (5β-ketodiol) has been shown to be derived from cholesterol in eggs of the silkworm, *Bombyx mori*. In order to investigate the difference in ecdysteroid metabolism between diapause eggs and non-diapause eggs of the silkworm, ^{3}H-5β-ketodiol was microinjected into eggs at several stages of early embryogenesis, and the metabolites were characterized using high-performance liquid chromatography. The injected ^{3}H-5β-ketodiol was metabolized not only to free ecdysteroids, but also to conjugates (phosphoric esters of ecdysteroids), in both diapause eggs and non-diapause eggs. Among these metabolites, 20-hydroxyecdysone, which is considered as the active hormone in silkworm eggs, was detected in non-diapause eggs. However, in diapause eggs, various radioactive putative precursors of 20-hydroxyecdysone, such as 2, 22-dideoxyecdysone, 2-deoxyecdysone and ecdysone, were detectable, but ^{3}H-20-hydroxyecdysone was not found. These results suggest that the 20-hydroxylation of ecdysone, which is catalyzed by ecdysone 20-monooxygenase, may be a rate-limiting step in the formation of 20-hydroxyecdysone from ketodiol in the silkworm eggs.

INTRODUCTION

In order to elucidate the function of egg ecdysteroids, developmental changes in the amounts of the ecdysteroids have been studied during embryonic development in several insect species. The correlation between free ecdysteroid peaks and embryonic cuticular events has been demonstrated in some species, *e.g. Locusta migratoria* (Lagueux *et al.*, 1979) and *Blaberus craniifer* (Bullière *et al.*, 1979), but is not apparent in other species, *e.g. Manduca sexta* (Warren *et al*, 1986), *Calliphora erythrocephala* (Bordes-Alléaume and Sami 1987) and *Bombyx mori* (Ohnishi, 1990; Sonobe *et al.*, 1997). As for the *Bombyx* silkworm, the function of egg ecdysteroids is assumed to be related to embryonic diapause (Ohnishi *et al.*, 1971; Mizuno *et al.*, 1981; Gharib *et al.*, 1981; Coulon, 1988; Ohnishi, 1990).

Recently, we demonstrated the developmental changes in the amounts of nine free ecdysteroids and corresponding conjugated ecdysteroids detected in diapause eggs and non-diapause eggs of the silkworm, and suggested that the metabolism of ecdysteroids may be different between the two types of eggs (Sonobe *et al.*, 1997). Of interest is the observation that the amount of 20-hydroxyecdysone (20E), which is considered to be an active molecule in silkworm eggs (Gharib *et al.*,1981), increased sharply in non-diapause eggs as embryonic development proceeded, but remained at a low level in diapause eggs. However, there is no metabolic information explaining the difference in the amount of 20E between diapause eggs and non-diapause eggs. Two possible metabolic pathways which cause the increase in 20E during embryogenesis may be considered: *de novo* synthesis of 20E, and dephosphorylation of maternal 20E 22-phosphate. In the present study, we focused on characterizing the stage-specific alternations in *de novo* synthesis of 20E concerned with the onset of embryonic diapause. We microinjected [22, 23, 24, 25-^{3}H]-2, 22, 25-trideoxyecdysone (^{3}H-5β-ketodiol) or ^{14}C-cholesterol into silkworm eggs and traced their metabolic fates by analyzing radioactive metabolites by means of high-performance liquid chromatography (HPLC).

* Corresponding author: Tel. +81-78-435-2512;
FAX. +81-78-435-2539.
E-mail: sonobe@ipc.konan-u.ac.jp

MATERIALS AND METHODS

Insects

The bivoltin race (kinshu x showa) of the silkworm, *Bombyx mori*, was used to obtain eggs. The female moths were incubated at 25°C under constant illumination during their embryonic stage. Under these conditions, the moths laid diapause eggs. Some of these eggs were

treated with HCl solution (specific gravity, 1.11 at 15°C) at 20 hr after oviposition in order to avoid diapause initiation (Sonobe and Odake, 1986). In diapause eggs, embryos ceased development at the late gastrula stage (72–96 hr after oviposition), but in eggs which were treated with HCl, larvae hatched 11 days after oviposition. To obtain closely synchronized eggs after oviposition, eggs laid within 3 hr were pooled and kept at 25°C until the use.

Ecdysteroids

^{3}H-5β-Ketodiol (abbreviated as K in figures) was synthesized as described by Haag and co-workers (1984) with a specific activity of 4,292 GBq/mmol. This compound was purified routinely using reverse phase (RP) HPLC (Beydon *et al.*, 1987) before use. 4-^{14}C-Cholesterol (1.92 GBq/mmol) was purchased from NEN Life Science Products (USA). 20E and ecdysone (E) were obtained from Sigma (USA). 2-Deoxyecdysone (2dE), 22-deoxy-20-hydroxyecdysone (22d20E), 3-epi-22-deoxy-20-hydroxyecdysone (22d20E') and 2, 22-dideoxy-20-hydroxyecdysone (2,22d20E) were purified from *Bombyx* ovaries and eggs as described previously (Kamba *et al.*, 1994, 1995; Mamiya *et al.*, 1995). 3-Epiecdysone (E'), 22-deoxyecdysone (22dE), 2, 22-dideoxyecdysone (2, 22dE) and 22, 25-dideoxyecdysone (22, 25dE) were generous gifts from Professor R. Lafont. 20,26-Dihydroxyecdysone (20,26E) and 3-epi-20-hydroxyecdysone (20E') were provided by Dr. M. Wilder and Professor H. Rees, respectively.

Injection of radioactive tracers

The radioactive tracer, ^{3}H-5β-ketodiol or ^{14}C-cholesterol, was dissolved in a 3% (v/v) solution of Tween 80 in acetone, and the solvent was then evaporated under a stream of nitrogen. The labelled tracer and Tween 80 were then mixed with a volume of saline equal to the volume of 3% Tween 80 solution which had been added. Following thorough vortex mixing, the tracer solution was then sonicated for 2 min to form an emulsion. Three nl containing 30,000 dpm were injected into each egg by means of a microinjector (IM-200, Narishige, Japan) and a micromanipulator (WR-89, Narishige) with minor improvements. The radioactive tracer was injected routinely into 300 eggs at each developmental stage. These eggs were incubated in a wet chamber at 25°C for 12 hr. The incubation was stopped by placing the eggs in a freezer (–80°C).

Extraction of ecdysteroids

The procedure for extraction of ecdysteroids from the eggs was essentially the same as that described previously (Kamba *et al.*, 1994; Mamiya *et al.*, 1995). Briefly, 100 eggs which had been injected with radioactive tracer were homogenized in 2 ml of cold 70% aqueous methanol, and the homogenate was centrifuged at 3,500 rpm for 40 min. The pellet was re-extracted twice in the same way and the combined extracts were shaken with an equal volume of hexane to remove lipids. The aqueous methanol layer was evaporated to dryness, dissolved in ethanol, and then stored at 4°C overnight. Precipitated gummy impurities were removed by centrifugation and the concentrated supernatant was applied to a silicic acid cartridge (Varian, USA). The cartridge was first developed with 30% (v/v) methanol in chloroform in order to elute free ecdysteroids. The cartridge was then washed with 100% methanol to elute the conjugated ecdysteroids. The free ecdysteroid fraction was analyzed using HPLC. The conjugated ecdysteroids were hydrolyzed into the free form using *Helix enzyme* (a crude enzyme preparation from *Helix pomatia*, IBF, France) or calf intestine alkaline phosphatase (Grade II, Boehringer Mannheim, Germany) prior to HPLC analysis (Kamba *et al.*, 1994; Mamiya *et al.*, 1995).

HPLC analysis of ecdysteroids

Free ecdysteroids (or hydrolyzed products in the case of conjugated ecdysteroids) were analyzed using HPLC (LC-9A system, Shimazu, Japan) equipped with a RP-column (Wakosil 5C18, 4.6×250 mm, Wako, Japan) or normal phase (NP) column (NUCLEO SIL 100–5, 4.6×150 mm; Chemco, Japan). The RP-column was eluted with either of the following two solvent systems at 40°C. (1) solvent system 1: an increasing linear gradient (10–100% over 34 min) of 10% acetonitrile:isopropanol (10:4, v/v) in 0.1 % TFA, at a flow rate of 1ml/min (Böking *et al.*, 1993). (2) solvent system 2: isocratic elution with 45% aqueous methanol at a flow rate of 1ml/min. The NP-column was eluted with either of the following two solvent systems at 40°C. (3) solvent system 3: an increasing linear gradient (0–100% over 40 min) of isooctane:isopropanol:water (100:40:3, v/v) in isooctane:isopropanol:water (660:70:2, v/v) (Descamps and Lafont, 1993) at a flow rate of 0.75 ml/min. (4) solvent system 4: isocratic elution with methylene chloride:methanol (93:7, v/v) at a flow rate of 1ml/min (Ikekawa *et al.*, 1980). Fractions were collected every 0.5 min for monitoring radioactivity.

Ecdysteroids were identified by comparison of retention times with those of authentic compounds (detection at 254 nm). Epimerized ecdysteroids which could not be obtained as standard compounds, *e.g.* 20, 26E', were identified according to the method of Professor R. Lafont (personal communication). In this, the retention time of the epimer was calculated from the retention times of 20E, 20E' and 20, 26E (which are available as references) using NP-HPLC with cyclohexane:isopropanol: water (100:40:3, v/v) at a flow rate of 1 ml/min and employing the retention time relatiopship: 20, 26E'=20, 26E×20E' / 20E. Using the same procedure, 22dE' and 2, 22dE' were also identified.

RESULTS

Developmental changes in ecdysteroids formed from ^{3}H-5β-ketodiol during early development

In order to compare the mode of metabolism of ^{3}H-5β-ketodiol between diapause eggs and non-diapause eggs, ^{3}H-5β-ketodiol was injected into the two types of eggs at five different stages during early embryogenesis: (1) 3 hr after oviposition (syncytial blastoderm: before the onset of embryonic diapause), (2) 24 hr after oviposition (early gastrula stage in both diapause eggs and non-diapause eggs), (3) 48 hr after oviposition (the late gastrula stage in non-diapause eggs, middle gastrula stage in diapause eggs), (4) 72 hr after oviposition (segmented germ-band stage in non-diapause eggs, late gastrula stage in diapause eggs), and (5) 96 hr after oviposition (organogenesis stage in non-diapause eggs, late gastrula stage in diapause eggs). Further details of morphological changes in embryogenesis of the silkworm were described in the previous article (Sonobe *et al.*, 1986). After 12 hr incubation, eggs were extracted and 100,000 dpm of the free ecdysteroid fraction, as well as the free ecdysteriod fraction liberated by hydrolysis from the conjugated ecdysteroids, were applied to RP-HPLC (Figs 1 and 2).

Fig. 1 shows radiochromatograms of free ecdysteroid fractions. In 3-hr-old eggs, ^{3}H-5β-ketodiol was converted into small amounts of 22, 25dE, 2, 22dE' and 2, 22dE, but most ^{3}H-5β-ketodiol remained unmetabolized. From 24 hr to 96 hr after oviposition, a small amount of 22dE' was detected in 24-, 48- and 72-hr-old diapause eggs, but there were no significant differences in the components of metabolites in diapause eggs. In contrast, ^{3}H-5β-ketodiol was transformed into more polar compounds in non-diapause eggs as embryogenesis proceeded: 22d20E (and/or 22d20E') and 20E (and/or 20E') appeared from 48 hr after oviposition, and furthermore 20,

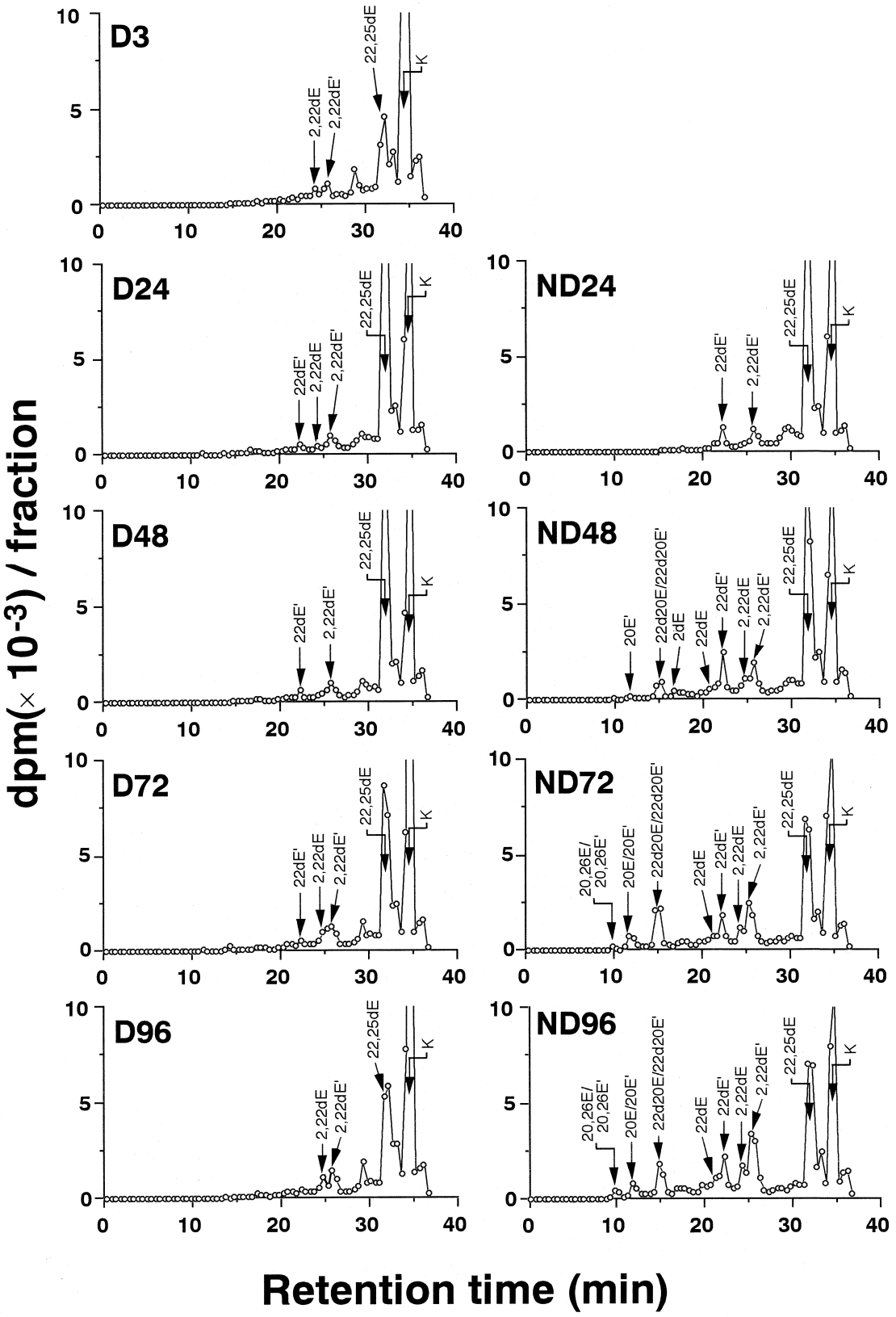

Fig. 1. RP-HPLC analysis of metabolites occurring in the free ecdysteroid fraction after injection of ^{3}H-ketodiol into diapause eggs and non-diapause eggs. Diapause (D) eggs and non-diapause (ND) eggs were injected with ^{3}H-ketodiol at 3, 24, 48, 72 and 96 hr after oviposition, and incubated for 12 hr at 25°C. Free ecdysteroid fraction was prepared, and analyzed using solvent system 1 (see Materials and Methods). Retention times of ecdysteroid standards are indicated by arrows (see text for ecdysteroid abbreviations).

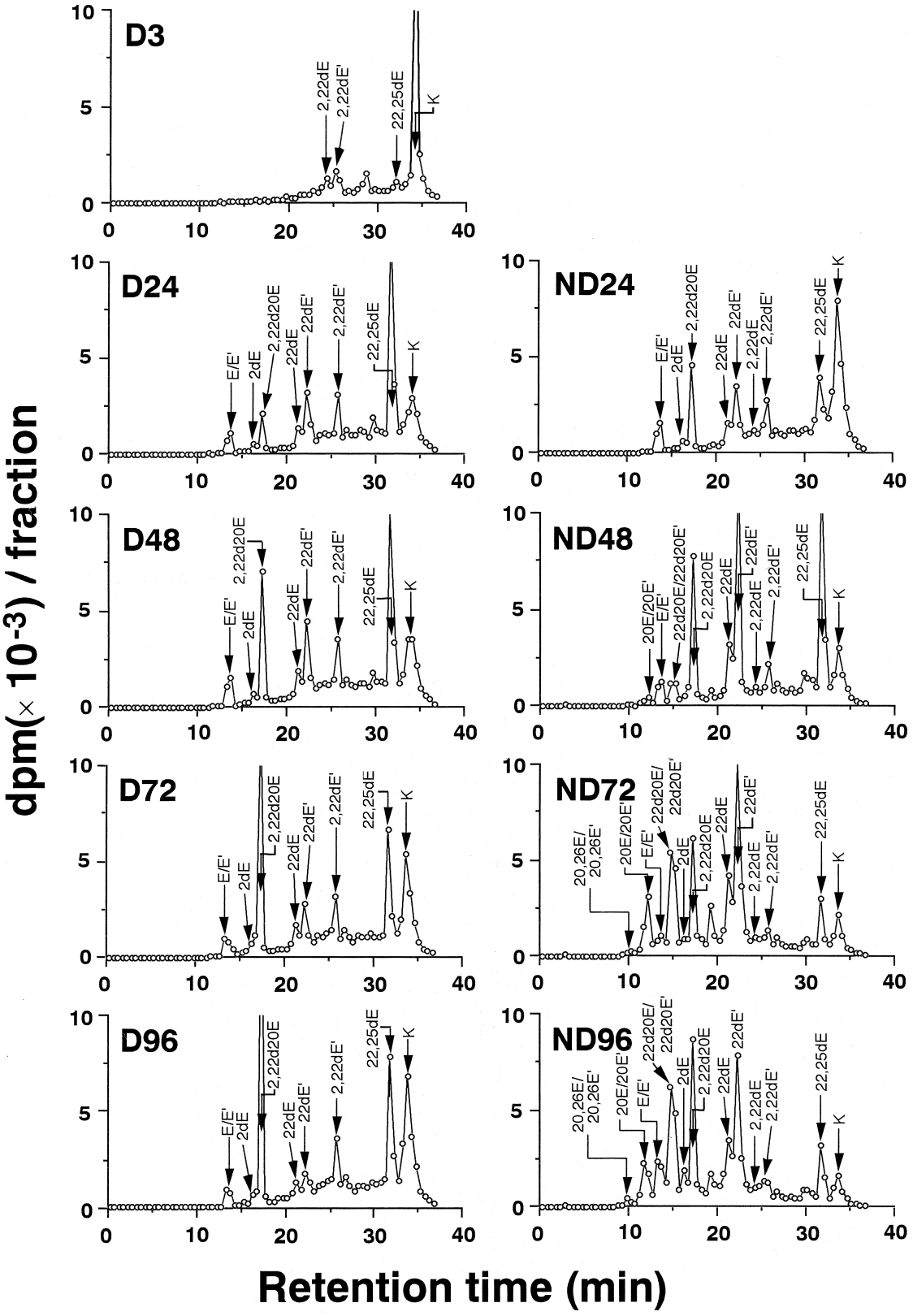

Fig. 2. RP-HPLC analysis of metabolites of ^{3}H-ketodiol occurring in the free ecdysteroid fraction released after *Helix* enzyme hydrolysis of conjugated ecdysteroids. Conjugated ecdysteroids were prepared from the same eggs which were used to prepare the free ecdysteroid fraction in Fig. 1. The free ecdysteroid fraction obtained after treatment with *Helix* enzyme was analyzed using solvent system 1. Retention times of ecdysteroid standards are indicated by arrows (see text for ecdysteroid abbreviations).

26E (and/or 20, 26E') was detectable from 72 hr after oviposition. Fig. 2 shows radiochromatograms of the *Helix* enzyme-hydrolyzable metabolites. In 3-hr-old eggs after oviposition, ^{3}H-5β-ketodiol was scarcely metabolized into polar ecdysteroids. However, 24 hr after oviposition, ^{3}H-5β-ketodiol was tranformed into various polar ecdysteroids, such as 22, 25dE, 2, 22dE', 22dE', 22dE, 2, 22d20E and E (and/or E') in diapause eggs, and these components were almost identical following this stage. In contrast, in non-diapause eggs, from 48 hr after oviposition, more polar ecdysteroids such as 20E (and/or 20E') and 20,26E (and/or 20,26E') appeared in addition to ecdysteroids detected in diapause eggs.

As shown in Figs. 1 and 2, 3α-epimers of polar ecdysteroids were not well separated from 3β-ecdysteroids with the solvent system 1. Thus, components with retention times between 8 min and 16 min were pooled and then re-chromatographed using solvent system 2 (see Materials and Methods). Fig. 3 (A, B) shows the HPLC patterns of the free ecdysteroid fractions and the hydrolyzed conjugated ecdysteroid fractions in 96-hr-old non-diapause eggs, respectively. As shown in these figures, seven ^{3}H-ecdysteroids (20, 26E', 20E, 20E', E, E', 22d20E and 22d20E') were throughly separated. The same analyses were performed at each developmental stage in diapause eggs and non-diapause eggs (radiochromatograms are not illustrated, but results are summarized in Fig. 4). On the basis of these analyses, it was evident that 3α-epimerization and subsequent conjugation of ecdysteroids occurred in both diapause eggs and non-diapause eggs.

The putative 2, 22d20E peak from RP-HPLC (the peak with the retention time of 17 min in chromatogram of D96 in Fig. 2) was collected and its identity further ascertained by NP-HPLC analysis. The compound was eluted as a single sharp peak at 30.1 min using the solvent system 3, and 16.4 min using the solvent system 4. In both cases, this compound behaved just as an authentic 2,22d20E sample, thus establishing the identity of this metabolite.

Among the ecdysteroids shown in Figs. 1, 2 and 3, 22, 25dE, 2, 22dE', 2, 22dE, 22dE', 22dE, and their conjugated forms have not been identified so far in silkworm eggs. In order to characterize the conjugated forms of 22, 25dE, 2, 22dE', 2, 22dE, 22dE', 22dE and ketodiol, the conjugated fraction obtained from 96-hr-old non-diapause eggs was incubated with calf intestine alkaline phosphatase (Grade II), and the hydrolyzed products were analyzed using HPLC. The HPLC profiles scarcely differed from the HPLC profiles of the *Helix* enzyme-hydrolized products shown in Figs. 2 and 3(B) (data no shown). This result, therefore, suggests that the six conjugated ecdysteroids mentioned above may be phosphoric esters. However, the position of conjugation was not further analyzed.

Fig. 4 summarizes quantitative changes in metabolites of ^{3}H-5β-ketodiol during embryogenesis. The amounts of individual ecdysteroids were calculated from HPLC profiles shown in Fig. 1, 2 and 3, and were expressed in dpm per egg. The yield of radioactive metabolites was 70–80 % in each stage. In diapause eggs, ^{3}H-5β-ketodiol remained mostly unmetabolized in 3-hr-old eggs, but was metabolized to various radioactive ecdysteroids from 24 hr after oviposition. Among these ecdysteroids, 22, 25dE, 2, 22dE', 2, 22dE, 22dE', 22dE, 2dE, E' and their conjugated forms did not show appreciable quantitative changes throughout embryogenesis until 96 hr after oviposition. The amout of conjugated E (E 22-phosphate, Ohnishi *et al.*, 1989), which appeared from 48 hr after oviposition, was also almost constant until 96 hr after oviposition. The amount of the conjugated 2, 22d20E (2, 22d20E 3-phosphate, Ohnishi *et al.*, 1989) gradually increased from 24 hr to 72 hr, and levelled off at 96 hr after oviposition.

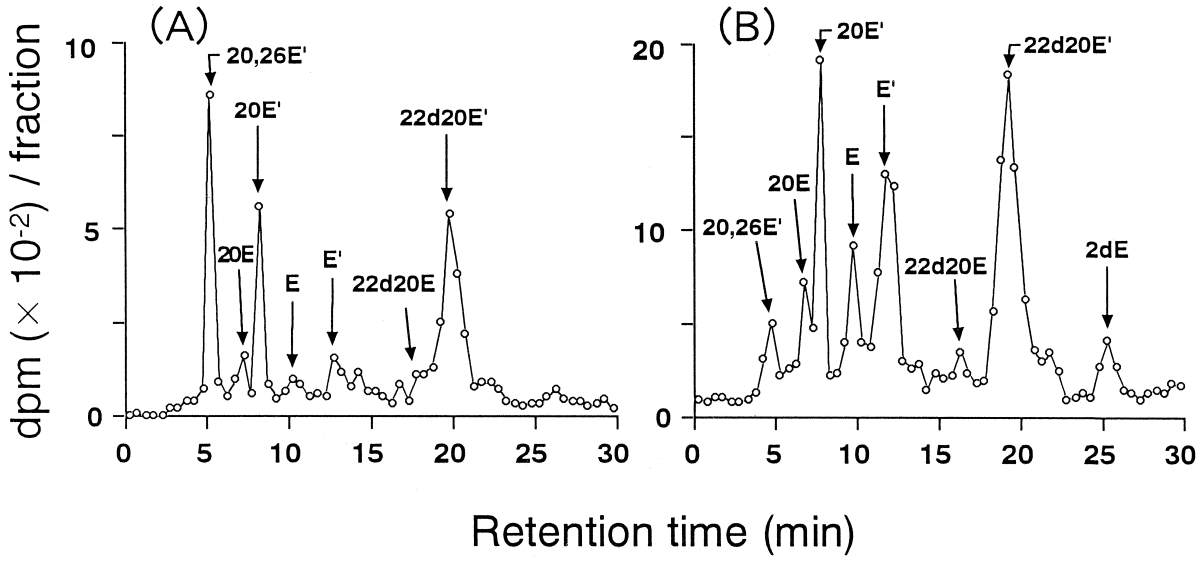

Fig. 3. Examples of chromatograms separating 3α-epimers from 3β-ecdysteroids. Fractions with retention times of 8–16 min in chromatograms of ND96 in Fig. 1 or Fig. 2 were pooled and analyzed by RP-HPLC using solvent system 2. (A) and (B) show chromatograms of ND96 in Fig. 1 and ND96 in Fig. 2, respectively. Retention times of ecdysteroid standards are indicated by arrows (see text for ecdysteroid abbreviations).

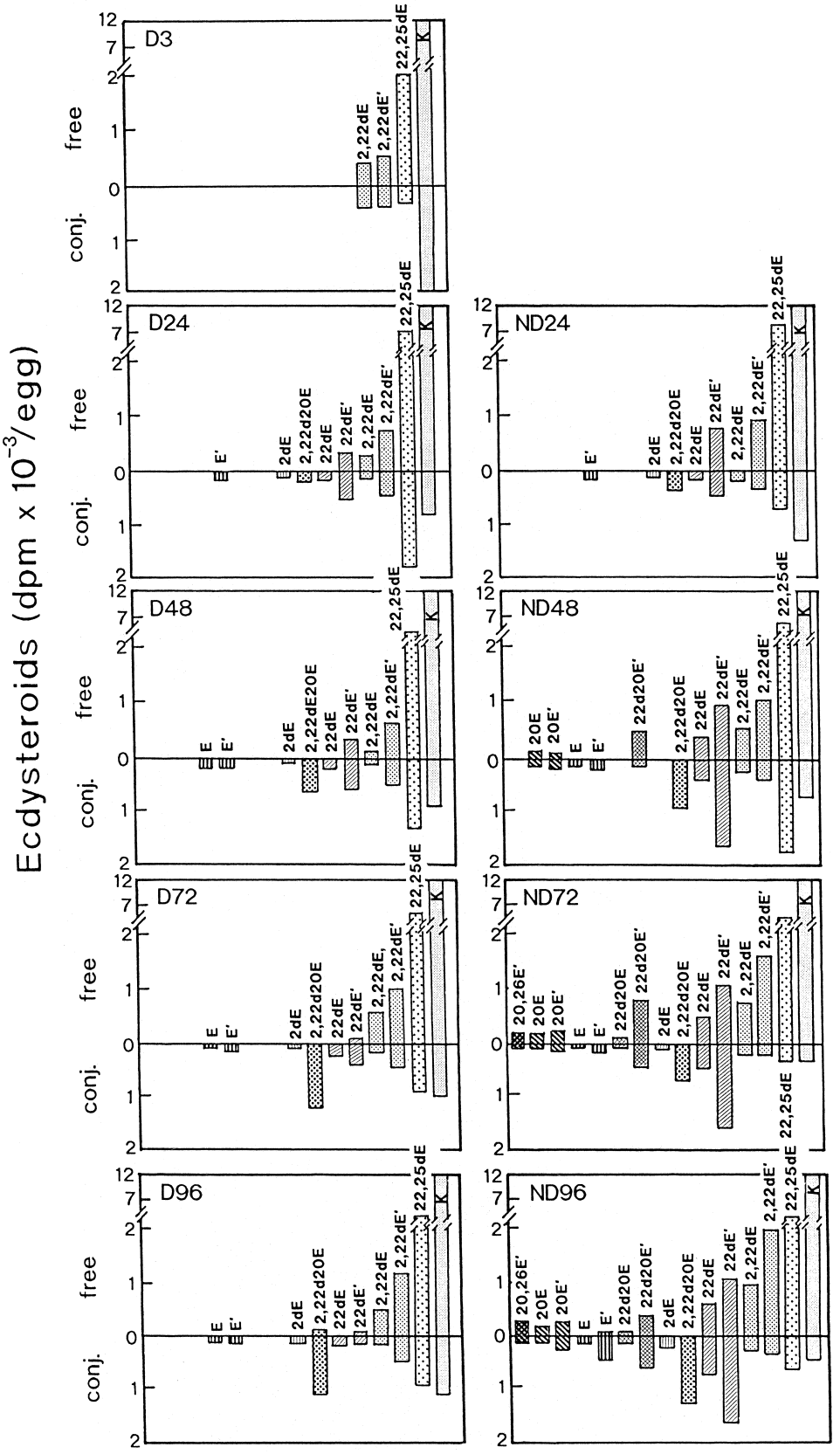

Fig. 4. Quantitative changes in metabolites of ^{3}H-ketodiol in diapause eggs and non-diapause eggs during early embryogenesis. The amounts of individual ecdysteroids were calculated from the profiles shown in Figs 1, 2 and 3. The abscissa shows ecdysteroids arranged in order of their retention time. The ordinate shows ecdysteroid content in the free form (upper column) and in corresponding conjugated forms (lower column). Abbreviations of ecdysteroids are as in the text.

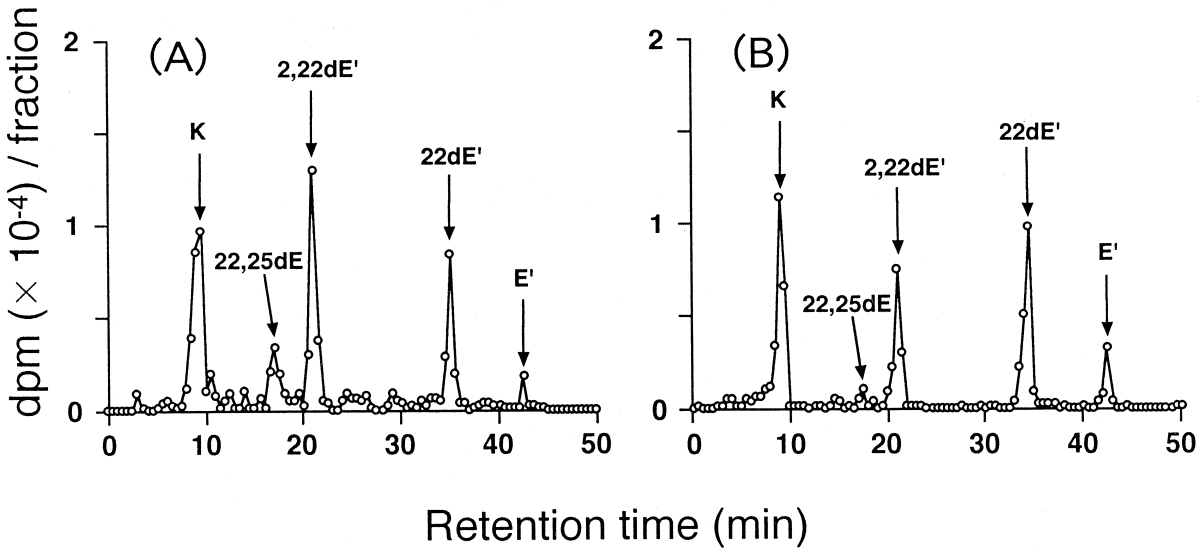

Fig. 5. NP-HPLC analysis of metabolites of ^{14}C-cholesterol in 24-hr-old non-diapause eggs. (A) shows the free ecdysteroid fraction, (B) shows the free ecdysteroids released after *Helix* enzyme hydrolysis of the conjugated ecdysteroid fraction. Ecdysteroids were analyzed using solvent system 3. Retention times of ecdysteroid standards are indicated by arrows (see text for ecdysteroid abbreviations).

As shown in Fig. 4, there was no remarkable difference in the amounts of radioactive ecdysteroids between diapause eggs and non-diapause eggs 24 hr after oviposition. However, in non-diapause eggs, the amounts of radioactive ecdysteroids in both free and conjugated forms gradually increased as embryogenesis proceeded. It is especially noticeable that 20E and 22d20E (and their epimers and conjugated forms), which were not detected in diapause eggs, appeared from 48 hr after oviposition, and the amounts of these ecdysteroids were approximately constant throughout embryogenesis. The amount of conjugated 2, 22d20E (2, 22d20E 3-phospate) seemed to gradually increase as embryogenesis proceeded. The hydroxylation at the C-20 position of ecdysteroids in diapause eggs and non-diapause eggs will be discussed later.

HPLC pattern of labelled metabolites formed from ^{14}C-cholesterol

In order to examine whether ketodiol is a true endogenous molecule in silkworm eggs, we investigated ecdysteroid biosynthesis using ^{14}C-cholesterol as a precursor. ^{14}C-Cholesterol was injected into 24-hr-old non-diapause eggs, and ecdysteroids (*Helix* enzyme-hydrolyzable ecdysteroids in the case of the conjugated fraction) were analyzed using NP-HPLC (solvent 3). An apparent peak which has the same retention time as reference ketodiol was observed in both free (Fig. 5A) and conjugated fractions (Fig. 5B). In addition to ketodiol, four distinkt peaks of radioactivity corresponding to the retention times of 22, 25dE, 2, 22dE', 22dE' and E' were detectable (Fig. 5A, 5B). These peaks were collected separately, and confirmed to be the predicted ecdysteroids by RP-HPLC using solvent system 1: E', 22dE', 2, 22dE', 22, 25dE and ketodiol eluted at 13.5, 22.5, 26.0, 31.5 and 33.5 min, respectively. Approximately 0.05% and 0.07% of the injected cholesterol were transformed into free ecdysteroids and conjugated ecdysteroids, respectively, during the 12-hr-incubation of injected eggs.

DISCUSSION

Recently, we demonstrated in silkworm eggs that the amount of 20E sharply increased in non-diapause eggs as embryogenesis proceeded, but remained at a low level in diapause eggs (Sonobe *et al*., 1997). However, the mechanism controlling the 20E formation in silkworm eggs is still not elucidated. Thus, we investigated the 20E formation using ^{3}H-5β-ketodiol. The transformation of ketodiol into ecdysteroids has been substantiated in *Locusta* ovaries (Hetru *et al*., 1982), especially in the ovarian follicles (Kappler *et al*., 1986). Furthermore, it has also been demonstrated that labelled cholesterol was incorporated into ketodiol by *Locusta* ovaries incubated *in vitro* (Hetru *et al*., 1982). These results, taken together, corroborated the idea that the follicle cells of the developing ovary may be a ecdysiosynthetic tissue (Hoffmann and Lagueux, 1985). This finding has been used to explain the presence of ecdysteroids in the ovaries of many insect species (Hoffmann and Lagueux, 1985; Rees, 1989; Delbeque *et al*., 1990; Ohnishi, 1990; Grieneisen, 1994). As concerns the developing eggs, it has been indicated that free ecdysteroids may be derived from hydrolysis of maternal conjugated ecdysteroids (Warren *et al*., 1986; Isaac and Rees, 1985; Hoffmann and Lagueux, 1985). In addition, using maternal radioactive cholesterol, Thompson and co-workers (1988) suggested that there is little or no biosynthesis of ecdysteroids during embryogenesis. However, in the present experiments using radioactive ketodiol and cholesterol microinjected into the silkworm eggs, we demonstrated that ketodiol was formed from cholesterol and the former was efficiently

converted into several ecdysteroids during early embryogenesis (Figs. 4, 5). To our knowledge, this is the first report to show that ketodiol may be an intermediate in the synthesis of ecdysteroids from cholesterol in insect eggs before the differentiation of their endocrine glands.

As shown in Fig. 4, it is evident that the phosphorylation and epimerization of ecdysteroids always occur in both diapause eggs and non-diapause eggs irrespective of their developmental stages, but the formation of 20E occurs exclusively after the gastrula stage in non-diapause eggs. Thus, we focused our discussion on the difference in hydroxylation steps between diapause eggs and non-diapause eggs. Current experimental evidence using radioactive tracers for the sequence of hydroxylation in the final steps of 20E biosynthesis from ketodiol supports three routes, comprising C-25→C-22→C-2→C-20 as a prime pathway and C-2→C-25→C-22→C-20 or C-25→C-20→C-22→C-2 as branched pathways (Rees, 1989; Ohnishi, 1990; Warren and Hetru, 1990; Grieneisen, 1994). In the silkworm eggs, although the amounts of labelled 22, 25dE, 2, 22dE, 22dE, 2dE, E and their conjugated forms were slightly lower in diapause eggs than in non-diapause eggs (Fig. 4), the fact that these ecdysteroids occurred in diapause eggs strongly suggests that the hydroxylation reactions at C-25, C-22 and C-2 positions function in diapause eggs. As shown in Fig. 4, 20E and its metabolites, including their epimers and phosphoric esters, were detected from 48 hr after oviposition in non-diapause eggs, but were not detected in diapause eggs. These results indicate a possibility that the hydroxylation reaction at the C-20 position of E, which is catalyzed by ecdysone 20-monooxygenase, may be a rate-limiting step in the formation of 20E from ketodiol in silkworm eggs. This hypothesis is supported by the following experiments. When ^{3}H-ecdysone was injected into silkworm eggs, it was converted efficiently into 20E, 20E' and 20, 26E' in non-diapause eggs, but was not appreciably converted into 20E and its metabolites in diapause eggs (Sonobe *et al.*, 1995; Sonobe *et al.*, 1997; Makka and Sonobe, 2000). Furthermore, the activity of ecdysone 20-monooxygenase in the diapause eggs remained at a low level, while that in the non-diapause eggs increased from the gastrula stage (Horike and Sonobe, 1999).

As shown in Fig. 4, 22d20E, 22d20E' and their phosphoric esters were detected as metabolites of ^{3}H-5β-ketodiol from 48 hr after oviposition in non-diapause eggs, but they were not detected in diapause eggs. These results suggest that 22d20E may be synthesized via 22dE, catalyzed by ecdysone 20-monooxygenase. However, it was unexpected that 2, 22d20E and its phosphoric ester would be detected in diapause eggs as well as in non-diapause eggs, because diapause eggs were not expected to have the ability to hydroxylate ecdysteroids at the C-20 position as discussed above. Although ecdysone 20-monooxygenase activity in diapause eggs was appreciably lower than that in non-diapause eggs, the reason why 2, 22dE was hydroxylated at the C-20 position in diapause eggs as well as in non-diapause eggs is not clear at present. However, one possible explanation is that ecdysone 20-monooxygenase may hydroxylate 2, 22dE more efficiently than E. This hypothesis is supported by the finding that 2, 25-dideoxyecdysone was a more effective substrate of ecdysone 20-monooxygenase than E (Smith, 1985), although whether or not ecdysone 20-monooxygenase efficiently hydroxylates 2, 22dE in comparison with E remains to be examined. Another possibility is that a specific enzyme which hydroxylates 2, 22dE at the C-20 position may exist in silkworm eggs. In any case, characterization of the hydroxylation reaction at the C-20 position of ecdysteroids is important for understanding the ecdysteroid metabolism in silkworm eggs.

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