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Ecdysone Metabolism in Diapause Eggs and Non-Diapause Eggs of the Silkworm, *Bombyx mori*

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ABSTRACT—In order to compare ecdysone metabolism between diapause eggs and non-diapause eggs of the silkworm, *Bombyx mori*, ^{3}H-ecdysone and its derivatives (^{3}H-3-epiecdysone and ^{3}H-ecdysone 22-phosphate) were injected into the eggs at various stages during early embryogenesis, and the resultant labelled metabolites were analyzed by high-performance liquid chromatography. From the quantitative and qualitative changes in the labelled metabolites between diapause eggs and non-diapause eggs, it was demonstrated that epimerization of ecdysone occurred during early embryogenesis irrespective of the embryonic stage in both diapause eggs and non-diapause eggs, and that phosphorylation of ecdysone was a major metabolic step in diapause eggs, whereas dephosphorylation of ecdysone 22-phosphate and its subsequent hydroxylation at the C-20 and C-26 positions were characteristic in non-diapause eggs.

INTRODUCTION

In various insects, it has been suggested that embryonic (egg) ecdysteroids probably control early events during embryogenesis, including cuticle formation (Hoffmann and Lagueux, 1985), gastrulation (Lanot *et al*., 1989), and embryonic diapause (Gharib *et al*., 1981, a, b; Ohnishi, 1990).

In the eggs of the silkworm, *Bombyx mori*, thirteen free ecdysteroids and their corresponding phosphorylated conjugates have been isolated and identified by Ohnishi's group and our laboratory (Ohnishi *et al*., 1989; Ohnishi, 1990; Kamba *et al*., 1994, 1995; Mamiya *et al*., 1995). Using a combination of high-performance liquid chromatography (HPLC) with radioimmunoassay, the amounts of the nine major ecdysteroids were demonstrated to fluctuate during embryogenesis in both diapause eggs and non-diapause eggs (Sonobe *et al*., 1997). Furthermore, tracer experiments using ^{14}C-cholesterol and ^{3}H-5β-ketodiol, radioactive precursors of ecdysteroids, revealed that the mode of synthesis of ecdysteroids differed between these two types of eggs (Sonobe *et al*., 1999). In these experiments, of interest was the observation that 20-hydroxyecdysone, which is considered to be an active hormone in silkworm eggs (Gharib *et al*., 1981, a), was formed in non-diapause eggs, whereas it was scarcely formed in diapause eggs.

The present study was designed to define the differences in ecdysone metabolism between diapause eggs and non-diapause eggs of the silkworm. Radiotracer experiments with ^{3}H-ecdysone, which is an immediate precusor of 20-hydroxyecdysone, (or when necessary, ^{3}H-3-epiecdysone and ^{3}H-ecdysone 22-phosphate) were carried out at various stages during early embryogenesis in diapause eggs and non-diapause eggs. Results obtained are discussed in relation to the metabolic differences in the formation of 20-hydroxyecdysone between diapause eggs and non-diapause eggs.

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MATERIALS AND METHODS

The bivoltin race (kinshu × showa) of the silkworm, *Bombyx mori*, which was destined to lay diapause eggs, was used to obtain eggs. Some of the diapause eggs obtained were treated with hydrochloric acid (HCl) prior to the onset of diapause (20 hr after oviposition) in order to obtain non-diapause eggs (Sonobe and Odake, 1986). In diapause eggs, embryos ceased to develop at the late gastrula stage (approximately 72 hr after oviposition), but in non-diapause eggs, larvae hatched 11 days after oviposition.

[23,24-^{3}H]Ecdysone (2,112.7 GBq/mmol) was obtained from NEN Life Science Products (USA). ^{3}H-3-Epiecdysone and ^{3}H-ecdysone 22-phosphate were extracted and purified from diapause eggs injected with ^{3}H-ecdysone. The procedure used for the extraction and purification of these radioactive ecdysteroids was performed according to the method of our previous paper (Kamba *et al*., 1995). Three nanoliters of physiological saline containing 2,000 dpm of ^{3}H-ecdysone, 1,200 dpm of ^{3}H-3-epiecdysone or 600 dpm of ^{3}H-ecdysone 22-phosphate 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 ecdysteroids were injected into 300 eggs at five stages during early embryogenesis: 3, 24, 48, 72 and 96 hr after oviposition. Details of the morphological and physiological characterics of the developing embryos were described in previous papers (Sonobe *et al*., 1986; Sonobe and Odake, 1986). After injection, the eggs were incubated in a wet chamber at 25°C for 3 hr and stored at –80°C until the extraction of metabolites.

The procedure used for the extraction of ecdysteroids from eggs was essentially the same as described in our previous paper (Sonobe *et al*., 1999). The free ecdysteroid fraction was analyzed by HPLC equipped with a reverse phase (RP) column (Wakosil 5C18, 4.6×250 mm, Wako, Japan). The conjugated (phosphoric) ecdysteroids were

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hydrolyzed to their free forms using *Helix* hydrolases (a crude enzyme preparation from *Helix pomatia*, IBF, France) prior to HPLC analysis. The column was eluted (40 min) with a linear gradient of acetonitrile from 15 to 30% in 0.4% acetic acid (Somme-Martin *et al*., 1988), at a flow rate of 1 ml/min at 40°C.

Ecdysteroids (ecdysone, 3-epiecdysone, 20-hydroxyecdysone, and 3-epi-20-hydroxyecdysone) were identified by comparing their retention times with those of authentic compounds. 3-Epi-20,26-dihydroxyecdysone, which could not be obtained for use as a reference, was identified by the method described in our previous paper (Sonobe *et al*., 1999).

RESULTS AND DISCUSSION

In order to compare the developmental changes in the metabolism of ^{3}H-ecdysone between diapause eggs and non-diapause eggs, ^{3}H-ecdysone was injected into the two types of eggs at various developmental stages. Aliquots of the radioactive metabolites (5,000 dpm of the free ecdysteroid fraction and 10,000 dpm of the conjugated ecdysteroid fraction) were applied to RP-HPLC.

Figure 1 shows radiochromatograms of the free ecdysteroid fraction. In diapause eggs, only 3-epiecdysone was detected as a metabolite in 3-hr-old eggs. However, from 24 hr after oviposition, a small amount of 3-epi-20-hydroxyecdysone was detected in addition to 3-epiecdysone. In non-diapause eggs, in addition to 3-epiecdysone which was detected at every stage, 20-hydroxyecdysone and 3-epi-20,26-dihydroxyecdysone were also detected from 48 hr after oviposition. Only a negligible amount of unmetabolized ^{3}H-ecdysone was detected in both diapause eggs and non-diapause eggs, suggesting that ^{3}H-ecdysone is almost completely metabolized within 3 hr after its injection.

Figure 2 shows radiochromatograms of metabolites hydrolyzed by *Helix* hydrolases. In diapause eggs, only ecdysone and 3-epiecdysone were detected at each of the stages tested. In non-diapause eggs, in addition to ecdysone and 3-epiecdysone, 20-hydroxyecdysone and 3-epi-20,26-dihydroxyecdysone appeared at 72 and 96 hr after oviposition. In silkworm eggs, it has been demonstrated that 20-hydroxyecdysone, ecdysone and 3-epiecdysone bind a phosphoric group at the C-22 position (Ohnishi *et al*., 1989; Kamba *et al*., 1995). However, conjugated 3-epi-20,26-dihydroxyecdysone has not been reported yet. Thus, in order to characterize the conjugated form of 3-epi-20,26-dihydroxyecdysone, the conjugated ecdysteroid fraction obtained from the 96-hr-old non-diapause eggs was incubated with calf intestine alkaline phosphatase (Grade II, Boehringer Mannheim, Germany), and the hydrolyzed products were analyzed using RP-HPLC. 3-Epi-20,26-dihydroxyecdysone was detected in the hydrolyzed products of calf intestine alkaline phosphatase (data not shown). This result, therefore, suggests that 3-epi-20,26-dihydroxyecdysone is contained as a phosphoric ester in the eggs.

Figure 3 summarizes the quantitative changes in the metabolites of ^{3}H-ecdysone during early embryogenesis. The amounts of individual ecdysteroids were calculated from the HPLC profiles shown in Figs. 1 and 2, and expressed in dpm per 50 eggs. The yield of radioactive metabolites was 75–86% at each stage of development.

In diapause eggs (Fig. 3, a), approximately 58%, 28% and 14% of the ^{3}H-ecdysone injected into 3-hr-old eggs was converted into ecdysone 22-phosphate, 3-epiecdysone and 3-epiecdysone 22-phosphate, respectively. Then, the amounts of ecdysone 22-phosphate and 3-epiecdysone decreased sharply as embryogenesis proceeded. On the other hand, the amount of 3-epiecdysone 22-phosphate increased sharply as embryogenesis proceeded and reached a plateau 72 hr after oviposition. The amount of 3-epiecdysone 22-phosphate accounted for approximately 80% of the total radioactive metabolites in 72-hr-old diapause eggs.

In non-diapause eggs (Fig. 3, b), the amounts of ecdysone 22-phosphate and 3-epiecdysone decreased with embryonic development, as in the case of diapause eggs. The amount of 3-epiecdysone 22-phosphate increased sharply until 48 hr after oviposition, which was comparable to the case of diapause eggs, after which it decreased rapidly as embryogenesis proceeded. The metabolic differences between diapause eggs and non-diapause eggs in regard to the formation of 3-epiecdysone 22-phosphate will be described later. 20-Hydroxyecdysone and 3-epi-20,26-dihydroxyecdysone, which were not detected in diapause eggs, appeared from 48 hr after oviposition. The amount of 20-hydroxyecdysone was relatively constant throughout early embryogenesis, but the amount of 3-epi-20,26-dihydroxyecdysone increased markedly as embryogenesis proceeded. In addition to these ecdysteroids, 20-hydroxyecdysone 22-phosphate and 3-epi-20,26-dihydroxyecdysone phosphate were also formed. These results suggest that hydroxylation reactions of ecdysone at the C-20 and C-26 positions increased rapidly as embryogenesis proceeded in non-diapause eggs. This suggestion is consistent with our previous results showing that ecdysone 20-monooxygenase activity increased as embryogenesis proceeded in non-diapause eggs; however, little or no activity of the enzyme was detected in diapause eggs (Horike and Sonobe, 1999).

In order to elucidate the metabolic differences between diapause eggs and non-diapause eggs in the formation of 3-epiecdysone 22-phosphate, putative immediate precursors of 3-epiecdysone 22-phosphate (Fig. 4; Rees and Isaac, 1985; Thompson *et al*., 1989), namely, ^{3}H-3-epiecdysone and ^{3}H-ecdysone 22-phosphate, were injected into 96-hr-old diapause eggs and non-diapause eggs, and the resultant metabolites were analyzed by RP-HPLC (Fig. 5 and 6).

When ^{3}H-3-epiecdysone was injected into diapause eggs, about 66% was converted into 3-epiecdysone 22-phosphate and about 25% remained unmetabolized (Fig. 5, a). On the other hand, when ^{3}H-3-epiecdysone was injected into non-diapause eggs, only about 12% was converted into 3-epiecdysone 22-phosphate, and most of the ^{3}H-3-epiecdysone was metabolized to 3-epi-20-hydroxyecdysone and 3-epi-20,26-hydroxyecdysone (Fig. 5, b). The yield of radioactive metabolites in the ecdysteroid fraction after the injection of ^{3}H-3-epiecdysone was approximately 91%. These results sug-

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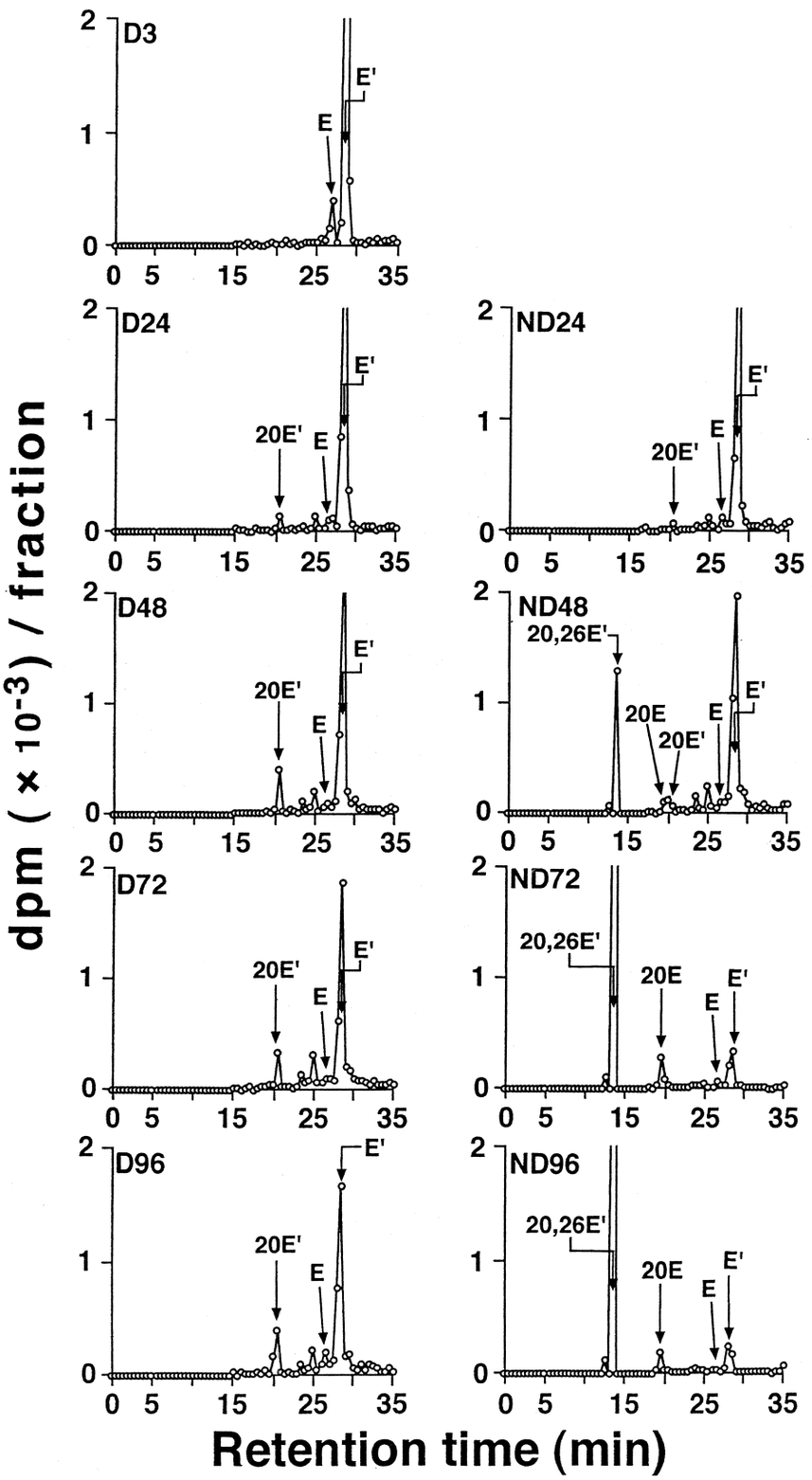

Fig. 1. RP-HPLC analysis of metabolites occurring in the free ecdysteroid fraction after injection of ^{3}H-ecdysone into diapause eggs and non-diapause eggs. Diapause (D) eggs and non-diapause (ND) eggs were injected with ^{3}H-ecdysone at 3, 24, 48, 72 and 96 hr after oviposition, and incubated for 3 hr at 25°C. The free ecdysteroid fraction was prepared, and analyzed using RP-HPLC as described in the text. The retention times of ecdysteroids, determined as described in the text, are indicated by arrows: 20,26E', 3-epi-20,26-dihydroxyecdysone; 20E, 20-hydroxyecdysone; 20E', 3-epi-20-hydroxyecdysone; E, ecdysone; E', 3-epiecdysone.

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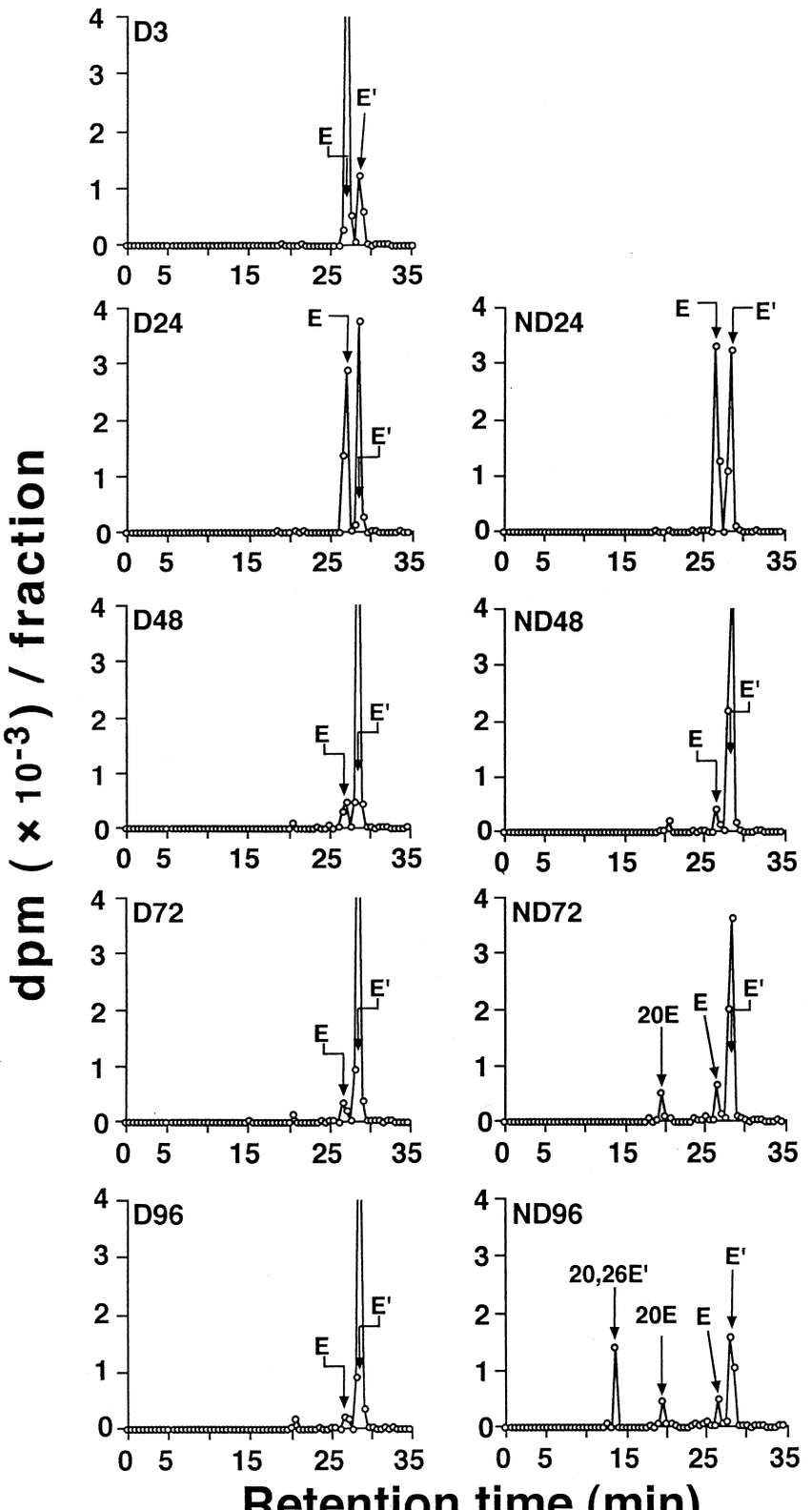

Fig. 2. RP-HPLC analysis of metabolites of ^{3}H-ecdysone occurring in the free ecdysteroid fraction released after hydrolysis of conjugated ecdysteroids by *Helix* hydrolases. Conjugated ecdysteroids were prepared from the same eggs which were used to prepare the free ecdysteroid fraction in Fig. 1. The free ecdysteroid fraction obtained after treatment with *Helix* hydrolases was analyzed using RP-HPLC as described in the text. The retention times of ecdysteroids are indicated by arrows: the abbreviations used for ecdysteroids are the same as those in Fig. 1.

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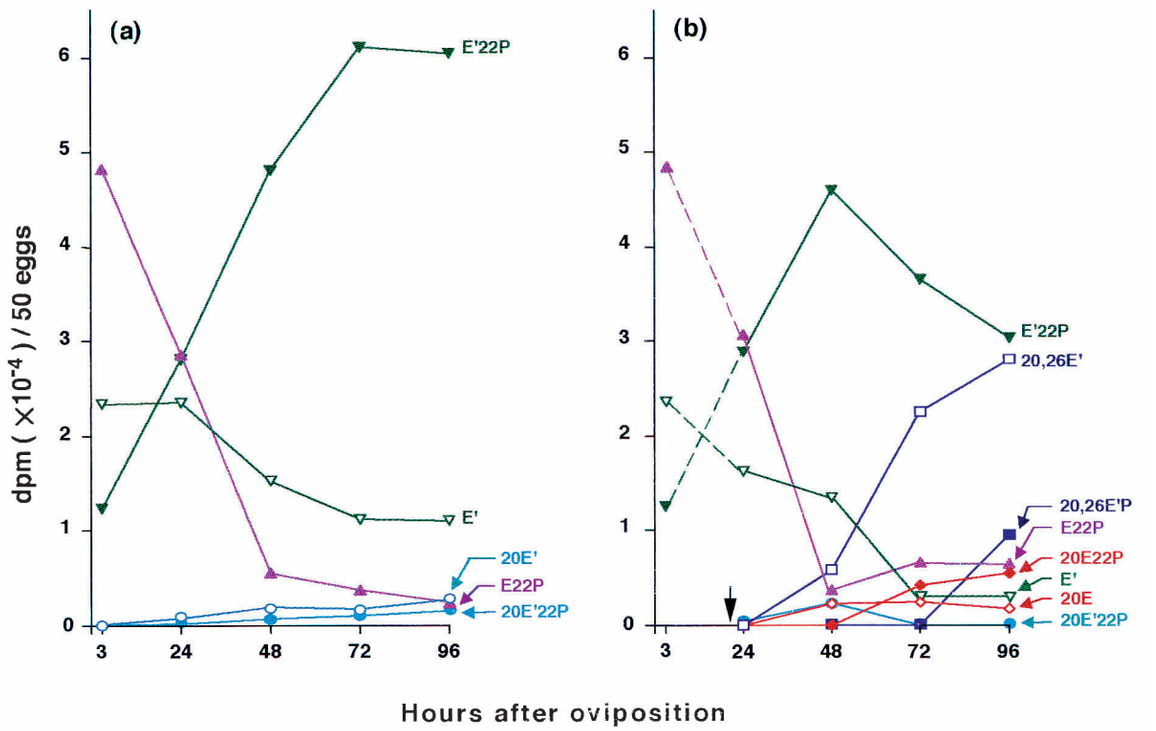

Fig. 3. Quantitative changes in metabolites of ^{3}H-ecdysone in diapause eggs and non-diapause eggs during early embryogenesis. The amounts of individual ecdysteroids were calculated from the profiles shown in Figs. 1 and 2. (a), diapause eggs; (b), non-diapause eggs. Symbols used for the ecdysteroids are: □, 3-epi-20,26-dihydroxyecdysone (20,26E'); ◇, 20-hydroxyecdysone (20E); ○, 3-epi-20-hydroxyecdysone (20E'); ▽, 3-epiecdysone (E'); ■, phosphoric ester of 3-epi-20,26-dihydroxyecdysone (20,26E'P); ◆, 20-hydroxyecdysone 22-phosphate (20E22P); ●, 3-epi-20-hydroxyecdysone 22-phosphate (20E'22P); ▲, ecdysone 22-phosphate (E22P); ▼, 3-epiecdysone 22-phosphate (E'22P). The arrow indicates the time at which eggs were treated with HCl in order to prevent diapause initiation.

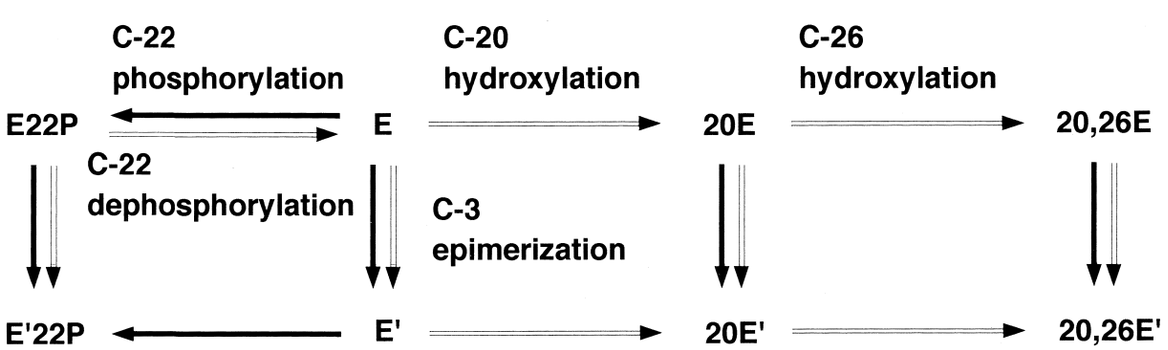

Fig. 4. Metabolic scheme of ecdysone in diapause eggs and non-diapause eggs of the silkworm. ➡, major pathways in diapause eggs; ⇨, major pathways in non-diapause eggs. 20,26E, 20,26-dihydroxyecdysone; E22P, ecdysone 22-phosphate; E'22P, 3-epiecdysone 22-phosphate. Other abbreviations used for the ecdysteroids are the same as those in figure 1.

gest that the phosphorylation reaction of ecdysteroids in diapause eggs is far more active than that in non-diapause eggs, by contrast, the hydroxylation reactions at the C-20 and C-26 positions in non-diapause eggs are far more prominent in comparison with those in diapause eggs. This suggestion is consistent with our previous results obtained from tracer experiments using ^{14}C-cholesterol and ^{3}H-5β-ketodiol (Sonobe *et al.*, 1999).

When ^{3}H-ecdysone 22-phosphate was injected into diapause eggs, about 36% remained unmetabolized, but about 54% was converted into 3-epiecdysone 22-phosphate (Fig. 6, a). On the other hand, in non-diapause eggs, ^{3}H-ecdysone 22-phosphate was not converted into 3-epiecdysone 22-phosphate to the same extent as in the case of diapasue eggs; only approximately 21% of the injected ^{3}H-ecdysone 22-phosphate was converted into 3-epiecdysone 22-phosphate (Fig. 6, b); by contrast, about 57% of ^{3}H-ecdysone 22-phosphate was converted into several free ecdysteroids such as ecdysone, 3-epiecdysone, 20-hydroxyecdysone, 3-epi-20-hydroxyecdysone and 3-epi-20,26-hydroxyecdysone (Fig. 6, b). The

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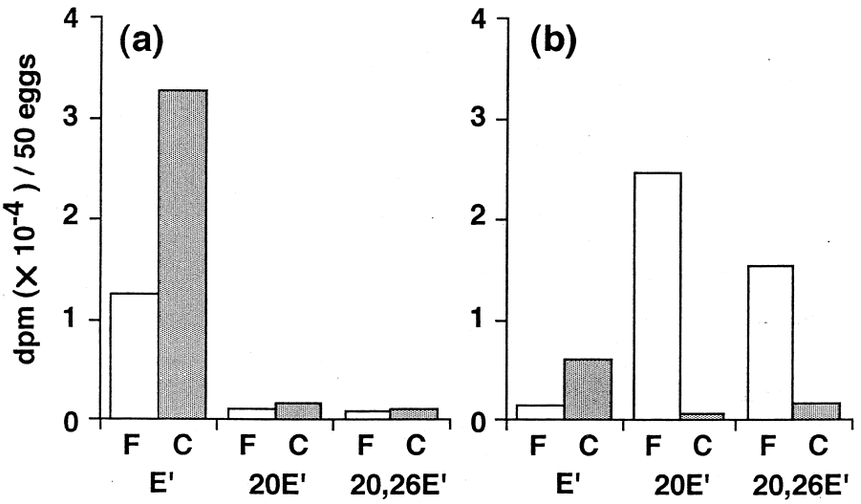

Fig. 5. RP-HPLC analysis of metabolites of ^{3}H-3-epiecdysone injected into diapause eggs and non-diapause eggs. Diapause eggs (a) and non-diapause eggs (b) were injected with ^{3}H-3-epiecdysone at 96 hr after oviposition, and incubated for 3 hr at 25°C. The free ecdysteroid fraction and conjugated ecdysteroid fraction (which was hydrolyzed to the free form using *Helix* hydrolases prior to HPLC analysis) were analyzed using RP-HPLC as described in the text. F, free form; C, conjugated form. Abbreviations used for the ecdysteroids are the same as those in figure 1.

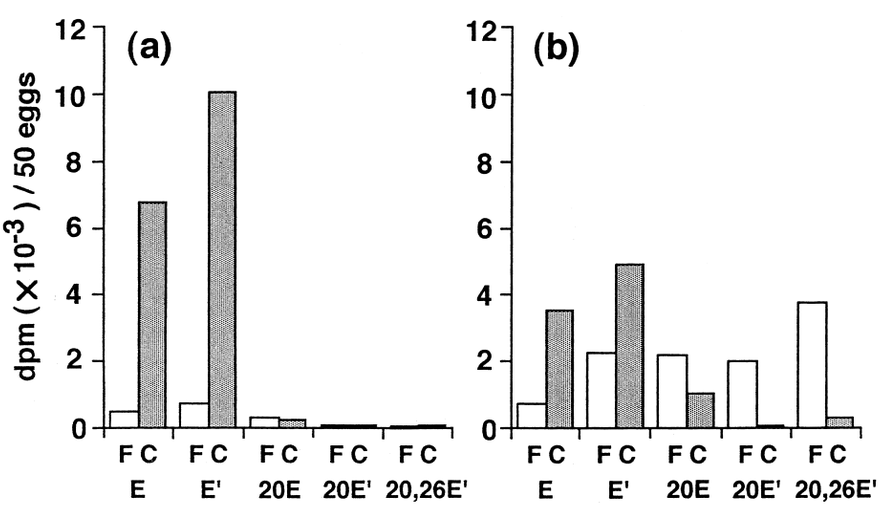

Fig. 6. RP-HPLC analysis of metabolites of ^{3}H-ecdysone 22-phosphate injected into diapause eggs and non-diapause eggs. Diapause eggs (a) and non-diapause eggs (b) were injected with ^{3}H-ecdysone 22-phosphate 96 hr after oviposition, and incubated for 3 hr at 25°C. The free ecdysteroid fraction and conjugated ecdysteroid fraction (which was hydrolyzed to the free form using *Helix* hydrolases prior to HPLC analysis) were analyzed using RP-HPLC as described in the text. F, free form; C, conjugated form. Abbreviations used for the ecdysteroids are the same as those in Fig. 1.

yield of radioactive metabolites in the ecdysteroid fraction after the injection of ^{3}H-ecdysone 22-phosphate was about 84%. These results suggest that dephosphorylation of conjugated ecdysteroids and subsequent hydroxylation are more active in non-diapause eggs than in diapause eggs.

Regarding the formation of 3-epiecdysone 22-phosphate, our present experiments demonstrated that 3-epiecdysone 22-phosphate is formed from ecdysone *via* both 3-epiecdysone and ecdysone 22-phosphate in silkworm eggs (Fig. 4), and the results suggested that the rapid decrease in the formation of 3-epiecdysone 22-phosphate in 72- and 96-hr-old non-diapause eggs (Fig. 3, b) might be caused by the increase in the hydroxylation of ecdysone at the C-20 and C-26 positions during embryonic development.

In some species of insects, *e.g.*, *Lepidosaphes ulmi* (Gharib *et al.*, 1981 b), *Chortoicetes terninifera* (Gregg *et al.*, 1987) and *Manduca sexta* (Lanot *et al.*, 1989), it has been suggested that 20-hydroxyecdysone may exert key roles in embryonic development. Similarly, in *Bombyx* eggs, there are three experiments demonstrating that 20-hydroxyecdysone is required for embryonic development to advance: (1) the application of exogenous 20-hydroxyecdysone caused dia-

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pause eggs to resume development (Gharib *et al*., 1981 a), (2) the 20-hydroxyecdysone titer increased sharply in non-diapause eggs as embryonic development proceeded, but remained at a low level in diapause eggs (Sonobe *et al*., 1997), (3) when 20-hydroxyecdysone was injected into the eggs prior to the onset of diapsuse (at 15 to 20 hr after oviposition), some of them changed their developmental fate by becoming non-diapause (Makka and Sonobe, unpublished data). Results of tracer experiments using ^{3}H-5β-ketodiol (Sonobe *et al*., 1999) and ^{3}H-ecdysone (Fig. 3) suggest that the hydroxylation at the C-20 position of ecdysone may be a rate-limiting step in the formation of 20-hydroxyecdysone in silkworm eggs. This suggestion is supported by enzymic experiments, in which the activity of ecdysone 20-monooxygenase was measured (Horike and Sonobe, 1999).

However, in our tracer experiments using ^{3}H-ecdysone 22-phosphate (Fig. 6) and ^{3}H-20-hydroxyecdysone 22-phosphate (Makka and Sonobe, 1998), another possibility, that the dephosphorylation reaction of phosphorylated ecdysteroids is also responsible for the increase in 20-hydroxyecdysone titer, was suggested. Therefore, characterization of the enzyme systems involved in the phosphorylation and dephosphorylation of ecdysteroids, that is, ATP:ecdysteroid phosphotransferase in diapause eggs and ecdysteroid-phosphate phosphohydrolase in non-diapause eggs, is also important for the elucidation of the ecdysteroid metabolism in silkworm eggs.

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