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[REVIEW]

Ecdysteroids during Early Embryonic Development in Silkworm *Bombyx mori*: Metabolism and Functions

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ABSTRACT—It has been well established that eggs of insects, including those of the silkworm *Bombyx mori*, contain various molecular species of ecdysteroids in free and conjugated forms. In *B. mori* eggs, 20-hydroxyecdysone (20E) is a physiologically active molecule. In nondiapause eggs, 20E is produced by the conversion of maternal conjugated ecdysteroids (ecdysteroid-phosphates) and by *de novo* biosynthesis. In contrast, in diapause eggs, neither of these metabolic processes occurs. In *de novo* biosynthesis of 20E in *B. mori* eggs, hydroxylation at the C-20 position of ecdysone, which is catalyzed by ecdysone 20-hydroxylase, is a rate-limiting step. Furthermore, we found that a novel enzyme, called ecdysteroid-phosphate phosphatase (EPPase), specifically catalyzes the conversion of ecdysteroid-phosphates to free ecdysteroids. The developmental changes in the expression pattern of EPPase mRNA correspond closely to changes in the enzyme activity and in the amounts of free ecdysteroids in eggs. EPPase is localized in the cytosol of yolk cells, and the bulk of maternal ecdysteroid-phosphates is bound to vitellin and stored in yolk granules. The vitellin-bound ecdysteroid-phosphates are scarcely hydrolyzed by EPPase. Therefore, to examine how ecdysteroid-phosphates are hydrolyzed by EPPase during embryonic development further investigations were focused on yolk granules. Recent data indicate that acidification in yolk granules, induced by vacuolar H^+-ATPase, triggers the dissociation of ecdysteroid-phosphates from the vitellin-ecdysteroid-phosphates complex and the dissociated ecdysteroid-phosphates are released from yolk granules to the cytosol. To explain the process of the increase in the level of 20E during embryonic development in *B. mori* eggs, a possible model is proposed.

Key words: ecdysteroids, phosphatase, diapause, yolk granule acidification, silkworm

INTRODUCTION

In 1940, Fukuda (1940a, b) demonstrated in the silkworm *Bombyx mori* that the prothoracic glands are the organ producing the molting hormone that induces molting and metamorphosis in insects. His epoch-making discovery set a solid foundation for the search of the endocrine mechanism of molting in insects, establishing the theory now called the “classical theory” or “central dogma”.

In 1971, Ohnishi and co-workers observed a high activity of the molting hormone in *B. mori* eggs before embryos form the prothoracic glands, and subsequently his groups suggested that the molting hormone in eggs originates from ovaries (Hanaoka and Ohnishi, 1974; Mizuno and Ohnishi, 1975; Ohnishi and Chatani, 1977; Watanabe and Ohnishi, 1984). Thus, they predicted that the molting hormone may participate in controlling embryonic development and advanced their theory beyond the “classical theory” (reviewed by Ohnishi, 1986, 1990). Since then, their findings prompted intense research of the involvement of ecdysteroids[#1] in reproduction and/or embryonic development in various insects (reviewed by Hoffmann and Lagueux, 1985;

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#1: Ecdysteroids are used as a generic term, in accordance with Lafont and Horn (1989) and Karlson (1995), where the steroid nucleus bears a *cis*-fused A/B ring junction, a 7-en-6-one chromophore and a 14α-OH, irrespective of a molting hormone activity.

Lanot *et al*., 1989; Thompson *et al*., 1990).

This review mainly focuses on studies on ecdysteroids in *B. mori* eggs for the following reasons: (1) Studies on this topic have not been reviewed recently. (2) Recent available information on biochemical studies of ecdysteroids in early-stage embryos has been accumulated probably most systematically using *B. mori*. (3) The silkworm has long been utilized for endocrinological studies as a model system with which we can compare data obtained in other species.

We will focus on the following questions:

(1) What kinds of ecdysteroid are found in ovaries and eggs?

(2) What is the function of ecdysteroids in eggs?

(3) How is the level of 20-hydroxyecdysone (20E)[#2], the physiologically active molecule in *B. mori* eggs, regulated during early embryonic development?

Characterization of ecdysteroids in ovaries and eggs

In *B. mori* eggs, Ohnishi *et al*. (1971) first found a high molting hormone activity during development and a low activity during diapause. Subsequently, the occurrence of ecdysteroids, such as ecdysone (E), 20E, 26-hydroxyecdysone and makisterone A, was also demonstrated in ovaries and eggs of various insects, *e.g.*, the tobacco hornworm *Manduca sexta* (Kaplanis *et al*., 1973), the milkweed bug *Oncopeltus faciatus* (Kaplanis *et al*., 1975), the mosquito *Aedes aegypti* (Hagedorn *et al*., 1975), the migratory locust *Locusta migratoria* (Hetru *et al*., 1978) and the desert locust *Schistocerca gregaria* (Gande and Morgan, 1979). In *B. mori* ovaries, Ohnishi's group isolated and identified six free ecdysteroids, namely, 20E, E, 2-deoxy-20-hydroxyecdysone (2d20E), 2-deoxyecdysone (2dE), 2,22-dideoxy-20-hydroxyecdysone (2,22d20E) and Bombycosterol[#3], and their phosphoric esters (conjugated forms of ecdysteroids) (Ohnishi *et al*., 1977; Ikekawa *et al*., 1980; Ohnishi *et al*., 1981; Fujimoto *et al*., 1985; Hiramoto *et al*., 1988; Ohnishi *et al*., 1989).

Ohnishi's group detected these ecdysteroids mainly by their ultraviolet absorbance after high-performance liquid chromatography (HPLC). We further detected seven more free ecdysteroids and their conjugates in ovaries and eggs of *B. mori* by radioimmunoassay using two types of antiserum with different specificities (S-3 and H-22) after reverse-phase HPLC, and purified mainly them by thin-layer chromatography and HPLC. These ecdysteroids are 22-deoxy-20-hydroxyecdysone (22d20E), 2,22-dideoxy-23(*S*)-hydroxyecdysone (2,22d23(*S*)E), 3-epiecdysone (E'), 3-epi-2-deoxyecdysone (2dE'), 3-epi-22-deoxy-20-hydroxyecdysone (22d20E'), 3-epi-22-deoxy-20,26-dihydroxyecdysone (22d20,26E') and 3-epi-22-deoxy-16β,20-dihydroxyecdysone (22d16(β)20E'), and their 2-, 3- or 22-phosphoric esters (Fig. 1) (Kamba *et al*., 1994, 1995, 2000a, b; Mamiya *et al*., 1995). Of the twelve free ecdysteroids and their conjugated forms isolated from *B. mori* ovaries and eggs by Ohnishi's group and our group, six free ecdysteroids (E, 20E, 2dE, 2d20E, E' and 2dE') and five conjugated forms (E22P, 20E22P, 2dE22P, 2d20E22P and E'22P) have also been identified in some other species (Lafont and Wilson, 1996), but the other six free ecdysteroids (2,22d20E, 22d20E, 22d20E', 22d20,26E', 22d16(β)20E' and 2,22d23(*S*)E) and seven conjugated forms (2dE'22P, 2,22d20E3P, 22d20E3P, 22d20E'2P, 22d20,26E2P, 22d16(β)20E'2P and 2,22d23(*S*)E3P) have not been found in other insects (Fig. 1). Furthermore, by tracer experiments using ^{3}H-ketodiol (2,22,25-trideoxyecdysone), which has been demonstrated to be derived from cholesterol, labeled 2,22-dideoxyecdysone (2,22dE), 3-epi-2,22-dideoxyecdysone (2,22E'), 22,25-dideoxyecdysone (22,25dE), 2-deoxyecdysone (2dE) and 3-epi-22-deoxyecdysone (22dE') were detected in *B. mori* eggs (Sonobe *et al*., 1999). The possible metabolic pathways of these ecdysteroids have been described by Sonobe (1995).

Detailed analyses of ecdysteroids in ovaries and eggs of *B. mori* and comparison with those found in other insect species revealed the following three distinct characteristics of ecdysteroids in ovaries and eggs of *B. mori*:

(1) Previously, it had been postulated that the possible sequence of hydroxylation of ecdysteroids at post-embryonic stages is mainly in the order of C-25 → C-22 → C-2 → C-20 (reviewed by Rees, 1989; Grieneisen, 1994). However, when free ecdysteroids in ovaries and eggs of *B. mori* are arranged in the order of the possible sequence of hydroxylation in 20E biosynthetic pathways, a unique sequence of hydroxylation, that is, hydroxylation at C-20 can precede hydroxylation at C-22 and C-2, is suggested (Fig. 2).

(2) Conjugated ecdysteroids in *B. mori* ovaries and eggs can be classified into three groups by the position of the phosphate group: (i) C-22 phosphoric esters include E22P, 20E22P, 2d20E22P, 2dE22P, E22P, and 2dE'22P; (ii) C-3 phosphoric esters, 2,22d20E3P, 22d20E3P and 2,22d23(*S*)E3P; and (iii) C-2 phosphoric esters, 22d20E'2P, 22d20,26E'2P and 22d16(β)20E'2P (Fig. 1). It should be noted that ecdysteroids with a hydroxyl group at C-22 give rise to a 22-phosphoric ester, whereas those lacking the group at a C-22 form C-3 phosphoric ester. However, in 3-epiecdysteroids lacking the hydroxyl group at C-22, the phosphate group is conjugated to the C-2 position instead of the C-3 position. Ecdysteroids are phosphorylated by ATP: ecdysteroid-phosphotransferase (ecdysteroid kinase) (Kabbouh and Rees, 1991; Takahashi *et al*., 1992). However, it is not clear at present whether a single enzyme can catalyze such phosphorylation at the three positions, or different enzymes are involved in these reactions.

(3) Among ecdysteroid-phosphates, C-22 and C-3 phosphoric esters are predominantly detected in the ovaries, whereas C-2 phosphoric esters are detected in eggs but not in ovaries. This suggests that the physiological significance of ecdysteroid 22- and 3-phosphates is different from that of ecdysteroid 2-phosphates: C-22 and C-3 phos-

#2: The ecdysteroid abbreviations used are those of Lafont *et al*. (1993).

#3: Bombycosterol has a peculiar structure that is almost outside the category of ecdysteroids.

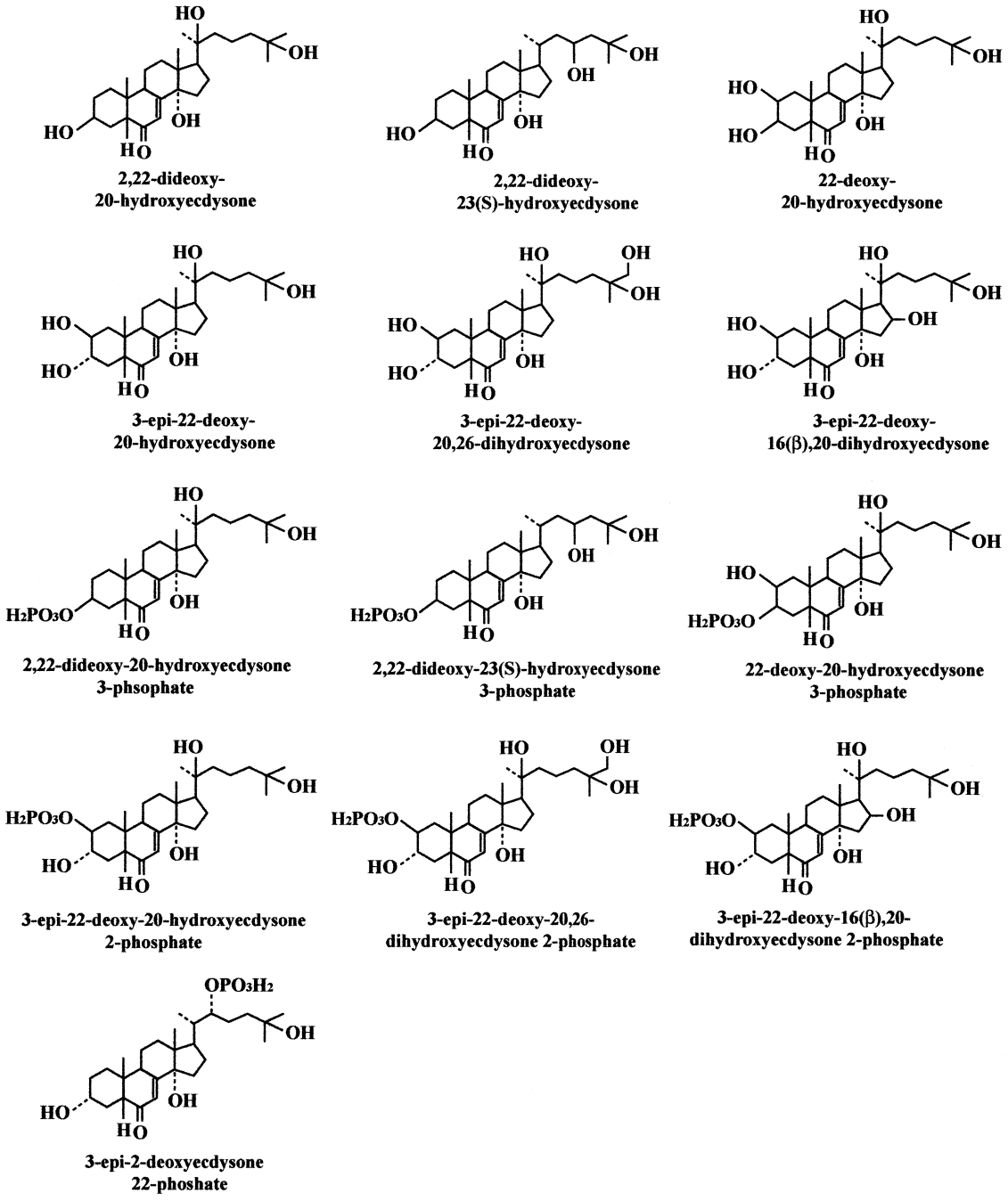

Fig. 1. Ecdysteroids found only in *B. mori* ovaries and eggs.

phoric esters in ovaries may serve as a storage for free ecdysteroids that can be used as the sources of 20E during embryonic development (Fig. 2), and C-2 phosphoric esters in eggs may be the end-products of metabolisms of ecdysteroids in *B. mori* eggs. This issue will be discussed later.

Biosynthesis and metabolism of ecdysteroids in diapause and nondiapause eggs

In *B. mori*, there are two types of egg that are developmentally different: diapause and nondiapause eggs. Diapause eggs are characterized by a cessation of embryonic development at the late gastrula stage, whereas nondiapause eggs develop continuously to the larval stage and larvae hatch 10–11 days after oviposition.

In diapause eggs, the bulk of ecdysteroids exist as conjugated forms (phosphoric esters), but in nondiapause eggs, free forms coexist with conjugated forms (Ohnishi *et al.*, 1977; Mizuno *et al.*, 1981). By a quantitative analysis of egg ecdysteroids carried out during the early embryonic development of *B. mori*, we obtained the following results (Sonobe *et al.*, 1997): In nondiapause eggs, (1) E and 20E

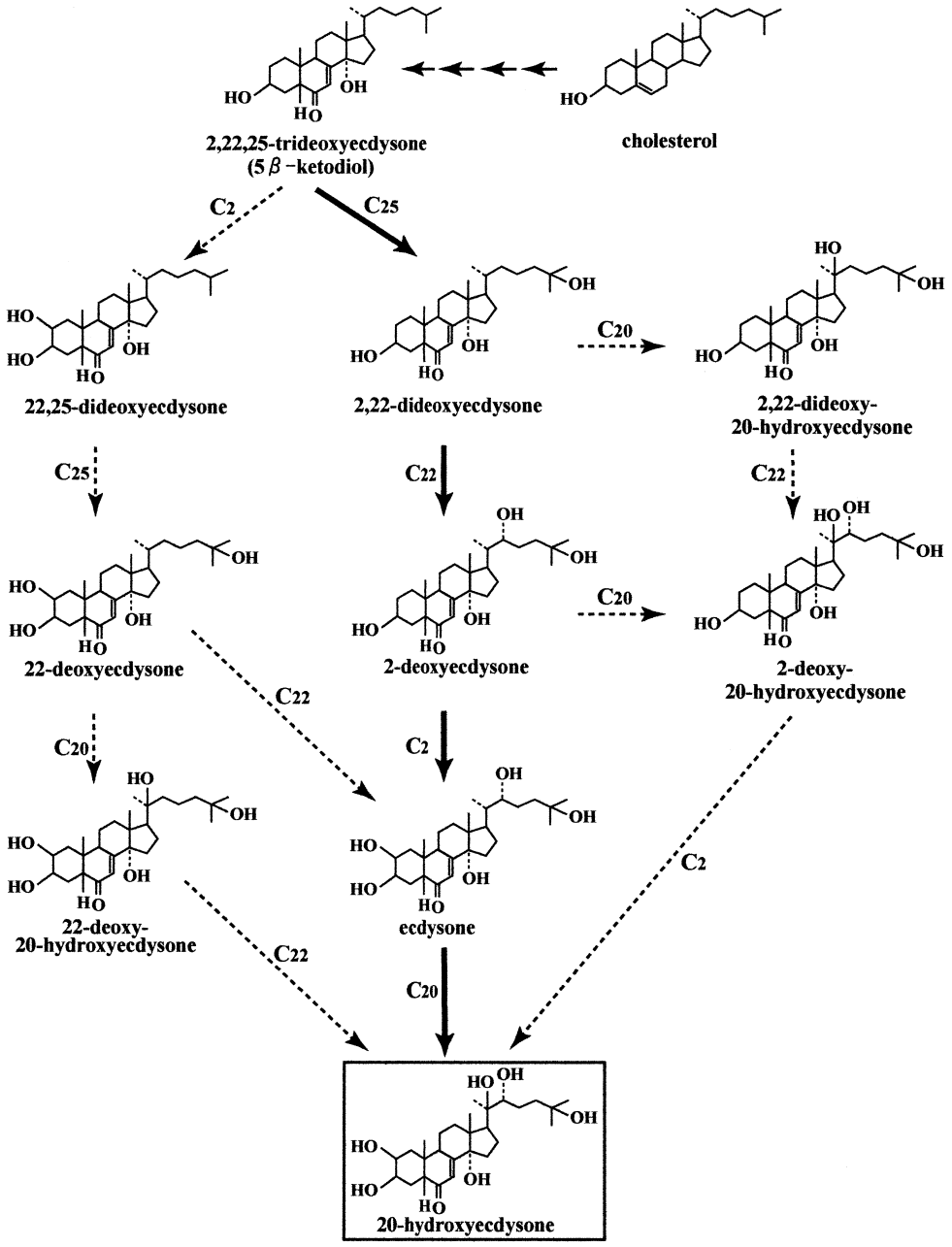

Fig. 2. Pathways of 20E biosynthesis. Steps that are common to most insects, including *B. mori*, are designated by solid lines, while possible diverging steps in *B. mori* ovaries and eggs are represented by dashed lines. The possibility that 22d20E is derived from 2,22d20E has also been suggested in *B. mori* ovaries (Kamba *et al.*, 1994).

constitute minor components, while intermediate ecdysteroids in 20E biosynthesis, such as 2d20E, 2dE and 2,22d20E, constitute major components. However, (2) in nondiapause eggs, among the egg ecdysteroids, E and 20E sharply increase in level from the second day (late gastrula stage) to the fourth day (organogenesis stage) (Fig. 3A), whereas (3) in diapause eggs, the levels of free ecdysteroids, including E and 20E, hardly increase during early embryonic development, and the low level is maintained during diapause (Fig. 3A). (4) The levels of almost all the conjugated ecdysteroids so far analyzed in diapause eggs, including E22P and 20E22P, continuously increase from the second day to the fourth day (late gastrula stage) on which embryonic development ceases. However, 20E22P as well as E22P remains almost unchanged in non-diapause eggs (Fig. 3B). These results strongly suggest that egg ecdysteroids are metabolized in different ways in diapause and nondiapause eggs.

As will be discussed in the next section, we are convinced that 20E is a physiologically active molecule in *B. mori* eggs on the basis of the results of *in vitro* binding-assay using the ecdysteroid receptor of *B. mori* (BmEcR/

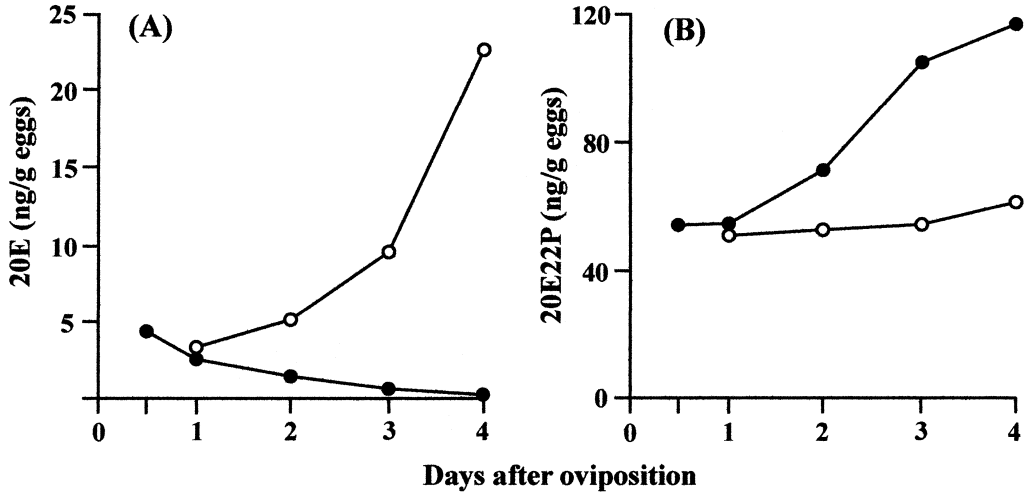

Fig. 3. Changes in the levels of 20E and 20E22P during early embryonic development. A, 20E; B, 20E22P. The closed circles and open circles indicate diapause eggs and nondiapause eggs, respectively. Based on Sonobe *et al.* (1986), the stages of embryogenesis in nondiapause eggs are as follows: 12 hr, cellular blastoderm; 1 day, early gastrula; 2 days, late gastrula; 3 days, early organogenesis; and 4 days, late organogenesis. Diapause eggs cease to develop at the late gastrula stage. Ecdysteroid abbreviations are as in the text. Modified from Sonobe *et al.* (1997).

BmUSP) and those of microinjection experiments (Makka *et al.*, 2002). Thus, to obtain metabolic information that explains the quantitative difference in 20E level between diapause and nondiapause eggs, we next carried out tracer experiments using radioactive precursors of 20E. Two possible metabolic pathways responsible for the increase in 20E level during early embryonic development may be considered: the *de novo* synthesis of 20E and dephosphorylation of ecdysteroid-phosphates of maternal origin. To examine the former possibility, we injected ^{14}C-cholesterol, ^{3}H-ketodiol or ^{3}H-ecdysone into *B. mori* eggs (Sonobe *et al.*, 1999; Makka and Sonobe, 2000), and to examine the latter possibility, we injected ^{3}H-E22P or ^{3}H-20E22P (Makka and Sonobe, 1998, 2000). In both experiments, radioactive metabolites were analyzed by HPLC.

Thus, we obtained several pieces of interesting information about 20E production: (1) In *B. mori*, the eggs are capable of synthesizing 20E from cholesterol via ketodiol. To our knowledge, we are the first to show the direct evidence that the insect egg is a site of ecdysteroid synthesis. Furthermore, by tracer experiments using ^{3}H-ketodiol and ^{3}H-ecdysone, 20E was demonstrated to be synthesized in yolk cells (Fig. 4). (2) Whereas in nondiapause eggs ketodiol was metabolized to 20E, in diapause eggs radioactive 20E

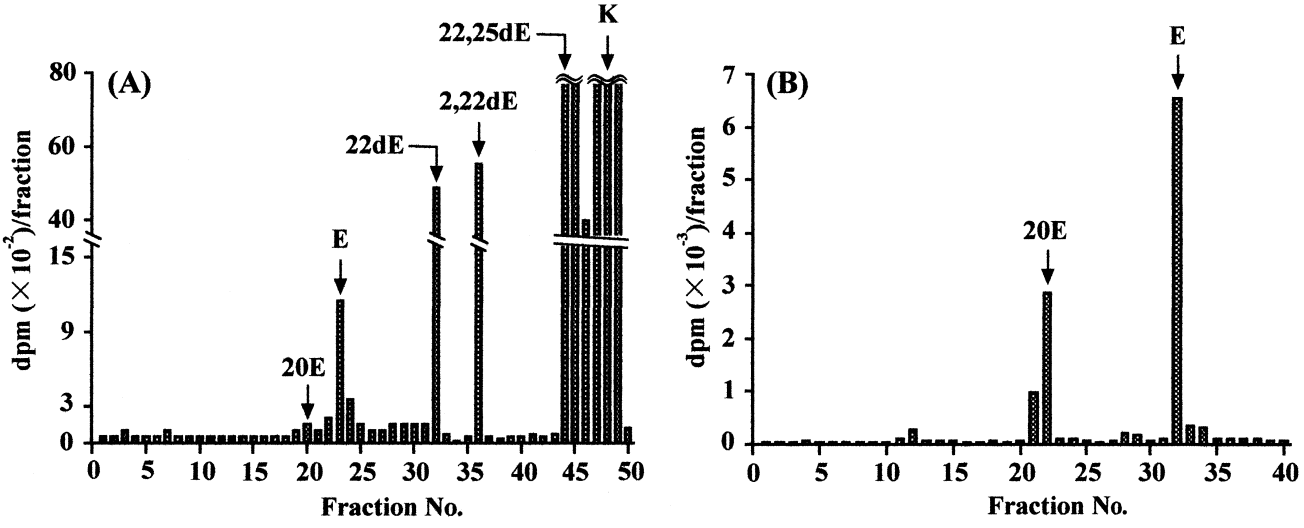

Fig. 4. Metabolism of ^{3}H-ketodiol and ^{3}H-ecdysone in yolk cells *in vitro*. Yolk cells were dissected out from 3-day-old nondiapause eggs, transferred into a small volume of Grace's insect cell culture medium containing ^{3}H-ketodiol (A) or ^{3}H-ecdysone (B), and incubated at 25°C for 12 hr. Ecdysteroids were extracted from yolk cell suspension with 85% methanol, and the free ecdysteroid fraction was analyzed by reverse-phase HPLC according to the procedures described previously (Sonobe *et al.*, 1999; Makka and Sonobe, 2000). Retention times of ecdysteroid standards are indicated by arrows. Ketodiol is abbreviated to "K". Other abbreviations of ecdysteroids are as in the text.

was not formed, although various radioactive precursors of 20E, such as 2,22dE, 22,25dE, 22dE and E, were detectable. These results suggest that hydroxylation at C-20 of E, which is catalyzed by ecdysone 20-hydroxylase (E20OHase), may be a rate-limiting step in the formation of 20E from ketodiol in *B. mori* eggs. (3) The epimerization of ecdysteroids occurred during embryonic development irrespective of the embryonic stage, in both diapause and nondiapause eggs. (4) The phosphorylation of E and 20E was the major metabolic step in diapause eggs, whereas the dephosphorylation of E22P and 20E22P was characteristic of nondiapause eggs. In conclusion, the increase in 20E level in nondiapause eggs is due to an increase in the activities of both E20OHase and ecdysteroid-phosphate phosphatase (EPPase), the latter catalyzes the dephosphorylation of ecdysteroid-phosphates. The biochemical characterization of these enzymes will be discussed later.

Biological functions of egg ecdysteroids

Up to the mid-1960s, it had generally been accepted that embryonic molting occurs independent of the formation of the brain and prothoracic glands (reviewed by Hoffmann and Lagueux, 1985). Meanwhile, Ohnishi and co-workers (1971) discovered the occurrence of molting hormone activity in *B. mori* eggs before the prothoracic glands differentiate, and predicted that egg ecdysteroids play an essential role in embryonic development. To elucidate the function of egg ecdysteroids, changes in the levels of free ecdysteroids such as E and 20E have been investigated during embryonic development in various insect species. It was demonstrated that embryonic molting coincides with a surge in the level of free ecdysteroids in various species, *e.g. L. migratoria* (Lagueux *et al*., 1979), the cockroach *Blaberus craniifer* (Bullière *et al*., 1979), the phasmid *Clitumnus extradentatus* (Cavallin and Fournier, 1981), *S. gregaria* (Gande and Morgan, 1979), and the cockroach *Nauphoeta cinerae* (Imboden and Lanzrein, 1982). Although evidence of the involvement of egg ecdysteroids in embryonic molting is based mainly on a temporal correlation rather than direct evidence, such as the induction of embryonic molting following the application of 20E into eggs, one of the functions of free ecdysteroids in eggs has been postulated to be the control of embryonic molting.

Besides the embryonic molting, it has also been demonstrated that the application of 20E inhibits the induction of embryonic diapause in the cochineal insect *Lepidosophes ulmi* (Gharib *et al*., 1981a), and promotes the elongation and segmentation of the germ band of *M. sexta* cultured *in vitro* (Lanot *et al*., 1989).

Recently, some *Drosophila* lethal mutations affecting cuticle formation have been studied from the viewpoint of molecular biology and developmental genetics. According to Chávez *et al*. (2000), *disembodied* and *spook* mutants have very low levels of E and 20E in eggs and fail to express the 20E-inducible genes *IMP-E1* and *L1* in the embryonic epidermis. These mutations produce little or no cuticle during the second half of embryogenesis and exhibit severe defects in late morphogenetic processes. Thus, Chávez *et al*. (2000) concluded that embryonic free ecdysteroids, which have occurred before their prothoracic glands function, regulate morphogenetic processes such as cell movements and cuticle deposition in embryos.

In *B. mori* eggs, the deposition of the serosal cuticle occurs within 24 hr after oviposition, and then, in nondiapause eggs, the first and second layers of embryonic cuticles are formed when the labral lobe differentiates (approximately 72-hr embryos) and the head and thorax appear (approximately 96-hr embryos), respectively (Takei and Nagashima, 1975; Ohtsuki *et al*., 1976). According to Mizuno *et al*. (1981), no marked increase in free ecdysteroid level was detected during cuticlogenesis. In contrast, Gharib's group reported that three distinct peaks are observed at stages when the serosal cuticle and the first and second layers of embryonic cuticles are deposited (Gharib and De Reggi, 1983; Gharib *et al*., 1983). Recently, we have demonstrated that the first and second layers of embryonic cuticles are formed during a marked upsurge in the levels of free ecdysteroids (Fig. 5A), including 20E, which begins to increase at the gastrula stage and peaks at the balstokinesis (Yamada and Sonobe, 2003), although no two conspicuous peaks coinciding with the formation of the first and second layers of embryonic cuticles were observed. Therefore, results obtained so far are contradictory with regards to the relationship between cuticle formation and the pattern of ecdysteroid fluctuation during embryonic development. However, results are consistent in that the levels of egg ecdysteroids are related to the embryonic diapause (Ohnishi *et al*., 1971; Gharib *et al*., 1981b; Sonobe *et al*., 1997).

Thus, we focused our study on demonstrating how egg ecdysteroids participate in the progress and the cessation of embryonic development of *B. mori*. We adopted two experimental approaches (Makka *et al*., 2002). First, interactions between the ecdysteroid receptor and various ecdysteroids found in *B. mori* eggs were analyzed by the ligand-binding assay using the ecdysteroid receptor, B1 isoform (BmEcR-B1) (Kamimura *et al*., 1996) and its heterodimeric partner ultraspiracle (BmUSP) (Tzertzinis *et al*., 1994), expressed *in vitro*. Next, several ecdysteroids found in *B. mori* eggs were injected into diapause-type eggs (eggs before the onset of embryonic diapause: prospective diapause eggs) to directly examine the effect of ecdysteroids on the induction of embryonic diapause.

Our results indicate that the relative binding affinities of egg ecdysteroids to the BmEcR-B1/BmUSP heterodimer decrease in the order of 20E > 2d20E > 22d20E > E > 2dE > E22P (Table 1). This data represents the first study on the binding affinity of egg ecdysteroids to the EcR/USP heterodimer, indicating that hydroxylation at the C-2, C-20 and C-22 positions increases the binding affinity and that the modifications of the C-22 hydroxyl group, as shown in

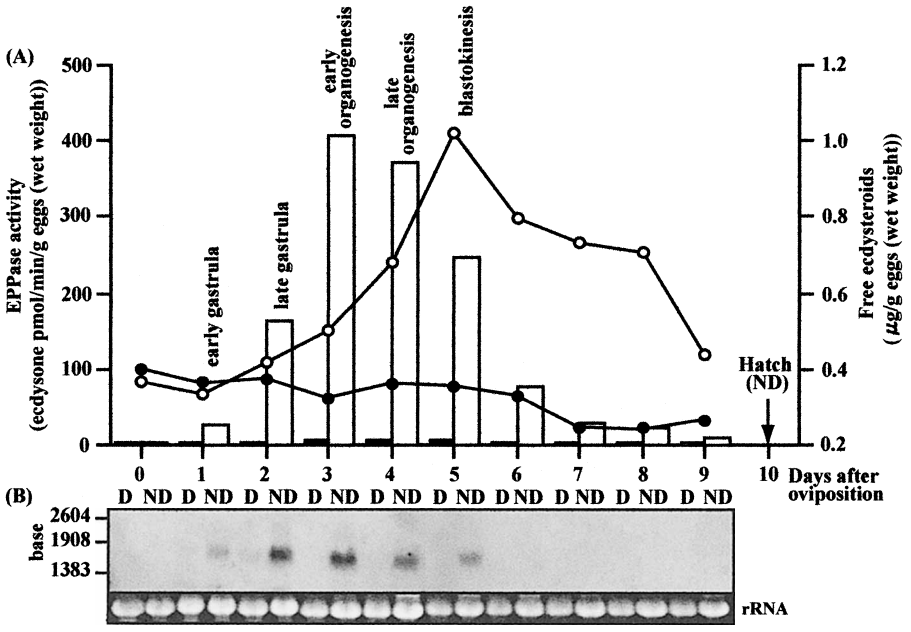

Fig. 5. Expression pattern of EPPase during embryonic development. A, profiles of EPPase activity and level of free ecdysteroids. The closed circles and open circles indicate the levels of free ecdysteroids in diapause eggs and nondiapause eggs, respectively. The solid bars and open bars indicate EPPase activities in diapause eggs and nondiapause eggs, respectively. B, expression pattern of the EPPase mRNA. Total RNA (20 μg) from eggs at various developmental stages was used for Northern blot analysis. The blot was hybridized with an alkaline phosphatase-labeled probe, which corresponds to the whole open reading frame of the EPPase. Ethidium bromide-stained ribosomal RNA is shown to indicate equal loading of total RNA. Positions of RNA markers are shown on the left. D, diapause eggs; ND, nondiapause eggs. From Yamada and Sonobe (2003).

Table 1. Competitive inhibition of ^{3}H-ponasterone A binding to ecdysteroid receptor by various ecdysteroids.

Ecdysteroid	IC_{50}	Ratio
Ponasterone A	8.9×10^{-8} M	0.09
20E	9.5×10^{-7} M	1.0
2d20E	5.6×10^{-5} M	58.9
22d20E	8.1×10^{-5} M	85.3
20E22Ac	1.1×10^{-4} M	115.8
E	1.8×10^{-4} M	189.5
2dE	5.1×10^{-3} M	5368.4
E22P	5.9×10^{-3} M	6210.5

The ecdysteroid receptor (BmEcR-B1/BmUSP heterodimer) was incubated with 5nM ^{3}H-ponasterone A with various unlabeled competitors. The concentration required to give a 50% inhibition, IC_{50}, was calculated. Ecdysteroid abbreviations are as in the text. From Makka *et al.* (2002).

22d20E, 20-hydroxyecdysone 22-acetate (20E22Ac) and E22P, decrease the binding affinity. These findings are consistent with the structure-physiological activity relationship found in the morphological response in *Drosophila* Kc-H cells (Cherbas *et al.*, 1980), and also with qualitative assignments by an electrophoretic mobility shift assay using the mosquito ecdysteroid receptor (Wang *et al.*, 2000), although 22d20E, 2dE and E22P were not included in their experiments.

Next, several egg ecdysteroids of *B. mori* were injected into prospective diapause eggs (18–21-hr eggs), and their effects on embryonic development were examined (Makka *et al.*, 2002). Approximately 7% of the eggs injected with 20E ($p<0.002$, χ^2-test) developed beyond the gastrula stage without entering diapause. In contrast, the injection of ecdysteroids other than 20E into prospective diapause eggs was not effective in inducing embryonic development. These results indicate that the absence or presence of 20E correlates with the developmental difference between diapause and nondiapause in *B. mori* embryos. However, in this experiment, not all the eggs treated with 20E developed without entering diapause. This rather low efficiency of injected 20E to induce embryonic development may be due to the prompt inactivation of 20E injected into diapause-type eggs, as previously demonstrated that exogenous E and 20E are indeed promptly inactivated due to epimerization and phosphorylation in diapause-type eggs (Makka and Sonobe, 1998, 2000; Sonobe *et al.*, 1999). Therefore, the continuous supply of 20E may be required for the embryonic development of *B. mori*; in other words, the deficiency in the continuous supply of 20E may result in embryonic diapause. This suggestion is consistent with the results of the study of Gharib *et al.* (1981b) using *B. mori* eggs. They showed that most of the diapause eggs resumed development after soaking in an isotonic solution of 20E for 24 hr.

In *B. mori*, according to Yaginuma of Nagoya University (personal communication), BmEcR-B1 and BmUSP were constantly expressed throughout early embryogenesis in

both diapause and nondipause eggs, but little or no BmEcR-A, the ecdysteroid receptor isoform of *B. mori* (Kamimura *et al*., 1997), was detected throughout early embryogenesis. These results strongly suggest that the level of 20E production, but not the expression level of ecdysteroid receptors, may be the rate-limiting step in controlling the early embryogenesis of *B. mori*. Therefore, in order to understand the hormonal regulation of embryonic diapause of *B. mori*, it is urgent to analyze the regulatory mechanisms of enzymes related to the increase in the level of 20E, namely, EPPase and E20OHase.

Enzyme systems involved in 20E production

(1) Dephosphorylation of ecdysteroid-phosphates

In many insect species, ecdysteroids are synthesized in developing ovaries, accumulated in mature ovaries and are transferred to eggs. Several lines of evidence indicate that ecdysteroid-phosphates in newly laid eggs are physiologically inactive storage conjugates that are used as the source of free hormones in embryonic development. (Warren *et al*., 1986; Makka and Sonobe 1998, 2000; reviewed by Hoffmann and Lagueux, 1985; Thompson *et al*., 1990). However, little attention has so far been paid to the enzyme, EPPase, which is responsible for the dephosphorylation of ecdysteroid-phosphates. We isolated EPPase from *B. mori* eggs, which is involved in the conversion of E22P to E and is distinct from nonspecific phosphatases. The following are the major characteristics of EPPase (Yamada *et al*., 2002; Yamada and Sonobe, 2003).

EPPase in nondiapause eggs of *B. mori* exists in the cytosol, and is most active at pH 7.5. The kinetic analysis of purified EPPase showed that although the affinity and specificity of EPPase for *p*-nitrophenylphosphate (*p*NPP), which is generally used as the substrate of phosphatases, are much lower than those for E22P, both E22P and *p*NPP are hydrolyzed at the same active site of EPPase. However, the enzyme activity was not affected by L-tartrate and fluoride, which are the strong inhibitors of acid phosphatase. Therefore, there is no doubt that EPPase is a kind of phosphatase, but is different from acid phosphatase. Furthermore, lysosomal acid phosphatase prepared from *B. mori* eggs scarcely hydrolyzed E22P (Yamada and Sonobe, unpublished data), and alkaline phosphatase could not be detected in the early stage of nondiapause eggs (Chino, 1961). These results indicate that E22P in *B. mori* eggs is hydrolyzed exclusively by EPPase.

Interestingly, EPPase hydrolyzed 20E22P and 2dE22P as well as E22P, but the enzyme scarcely hydrolyzed 22d20E3P. Since 2,22d20E and 22d20E have been suggested to be converted to 20E during embryonic development (Fig. 2), their C-3 phosphoric esters may be regarded as storage forms of ecdysteroids from which free 20E is generated. If so, it is conceivable that another phosphatase that has a greater specificity for C-3 phosphoric esters of ecdysteroids may exist in *B. mori* eggs. According to the possibility, it seems that phosphatase involved in the dephosphorylation of C-2 phosphoric ester does not exist in *B. mori* eggs, because 3-epiecdysteroids, which give rise to C-2 phosphoric esters as described earlier (Fig. 1), are ecdysteroid inactivation products formed irreversibly (reviewed by Rees and Isaac, 1985; Weirich, 1989; Thompson *et al*., 1990). Further experiments are necessary to confirm this possibility.

We then attempted to purify EPPase. EPPase was purified by about 3,000-fold to homogeneity by seven steps of column chromatography. The cDNA clone encoding EPPase was isolated by reverse transcription polymerase chain reaction (RT-PCR) using degenerate primers on the basis of the partial amino acid sequence obtained from purified EPPase and by the subsequent 3'- and 5'-rapid amplifications of cDNA ends (RACE). The full-length cDNA of EPPase was composed of 1620 bp with an open reading frame encoding a protein of 331 amino acid residues. In the database Pfam (protein family of alignments), phosphatases have been classified into many groups according to their consensus patterns of amino acid sequences, such as acid phosphatase, alkaline phosphatase, protein phosphatase, and fructose-1-6-bisphosphatase. We could not find phosphatases that show amino acid sequences similarity to EPPase in Pfam. This result indicates that EPPase is a novel phosphatase distinct from previously known phosphatases.

However, the tBLASTn analysis of SilkBase shows that the C-terminal region of EPPase (amino acids 170–259) shares 93% similarity with fbs2026 cloned from the fat body of *B. mori*. The FASTA analysis of the database Swiss-Prot also shows that EPPase (amino acids 64–329) has 38% similarity with the C-terminal region of the UBASH3A protein homolog in *D. melanogaster* (amino acids 489–748). Although the functions of both proteins have not yet been clarified at present, it is predicted that these proteins are involved in ecdysteroid metabolism. Thus, the detailed analysis of the biochemical roles of these proteins is necessary.

The pattern of changes in EPPase activity in diapause and nondiapause eggs approximately coincided with that of the changes in levels of free ecdysteroids (Fig. 5A). This result is consistent with those of our previous tracer experiments using ^{3}H-E22P and ^{3}H-20E22P (Makka and Sonobe, 1998, 2000), in which it has been demonstrated that the increase in free ecdysteroid levels results from the hydrolysis of ecdysteroid-phosphates. Furthermore, the pattern of changes in EPPase activity coincided with the expression pattern of EPPase mRNA (Fig. 5A, B), indicating that gene transcription is required for eliciting an increase in EPPase activity.

Anti-EPPase IgG was used to immunocytochemically locate EPPase in the nondiapause eggs. As shown in Fig. 6, EPPase is located in the cytoplasm around the nuclei of yolk cells, which are formed about 24 hr after oviposition, but not in yolk granules and embryonic cells.

(2) Biosynthesis of 20E from cholesterol

As described previously, *B. mori* eggs are capable of synthesizing 20E from cholesterol via ketodiol, and the hydroxylation at C-20 position of E may be a rate-limiting step. However, little is known about the biochemical characteristics and changes in E20OHase activity during embryonic development. Thus, we analyzed the hydroxylation reaction at the C-20 position during embryonic development (Horike and Sonobe, 1999; Horike *et al*., 2000). We demonstrated that E20OHase activity in *B. mori* eggs is associated with microsomes, and the patterns of changes in activity of this enzyme in diapause and nondiapause eggs approximately coincide with changes in 20E level (Fig. 3A, 7). It has been generally known that all the microsomal P450-mediated hydroxylation reactions are dependent on the presence of NADPH-cytochrome P450 oxidoreductase (P450 reductase). Although P450 reductase genes have been reported in several insects (Mayer and Durrant, 1979; Zhang *et al*., 1998), little is known about their physiological functions. We attempted the molecular cloning of P450 reductase and the production of its antibody to better understand the mechanism of 20E biosynthesis in *B. mori* eggs (Horike *et al*., 2000).

Using RT-PCR, a cDNA fragment of P450 reductase from *B. mori* was cloned from 3-day-old nondiapause eggs. RACE was used to isolate the ends of the cDNA. The full-length cDNA obtained was composed of 3471 bp with an open reading frame encoding a protein of 687 amino acid residues with a relative molecular mass of 77,700. The deduced amino acid sequence of *B. mori* P450 reductase shows several domains that are highly conserved among the enzymes of various species (Horike *et al*., 2000). When

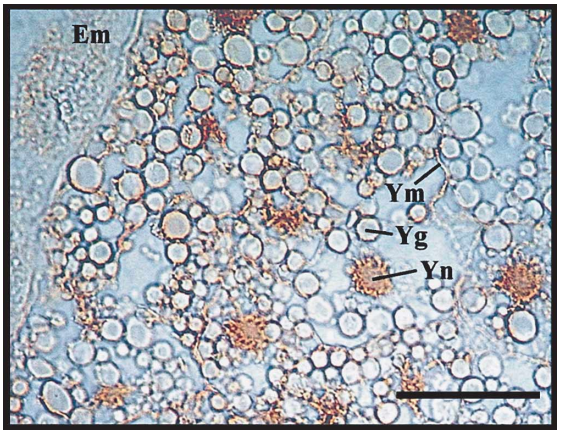

Fig. 6. Immunolocalization of EPPase in *B. mori* eggs. Sections of 72-hr nondiapause eggs were treated with EPPase antiserum. Note that EPPase is localized in the cytoplasm around nuclei of yolk cells. Em, embryo; Ym, yolk cell membrane; Yn, yolk cell nucleus; Yg, yolk granule. Scale bar represents 40 μm.

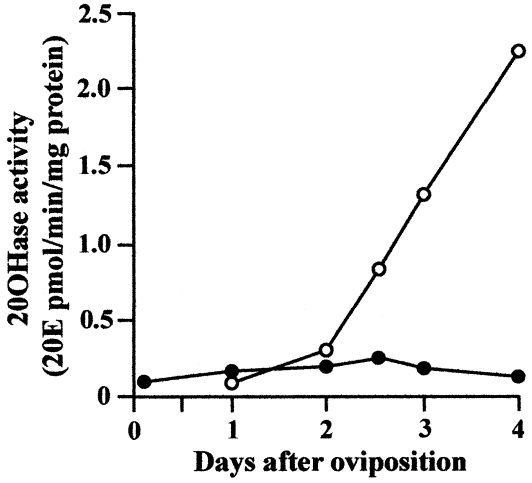

Fig. 7. Changes in microsomal E20OHase activity during embryonic development. The closed circles and open circles indicate E20OHase activities in diapause eggs and nondiapause eggs, respectively. From Horike and Sonobe (1999).

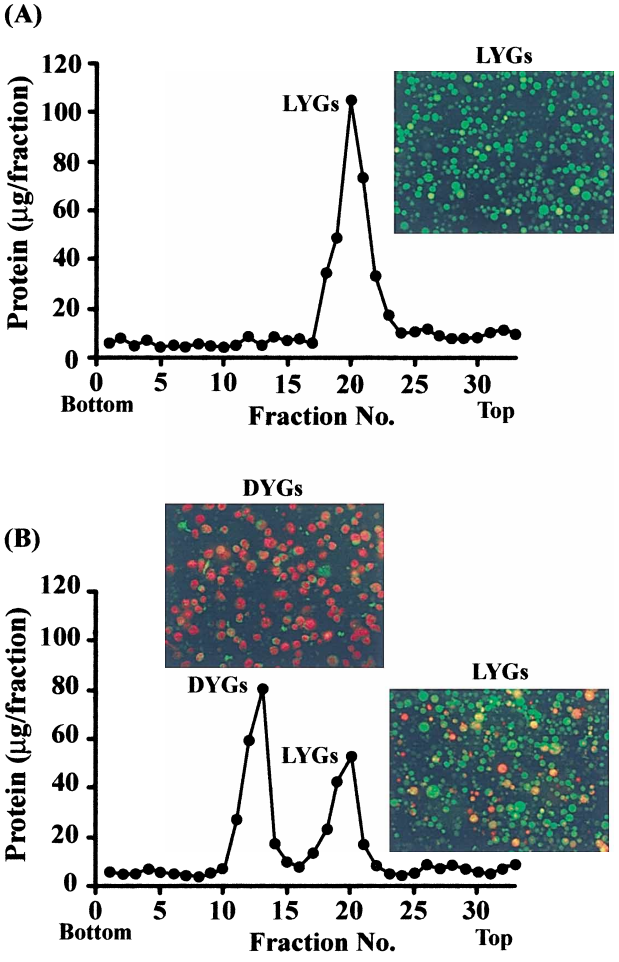

Fig. 8. Percoll density gradient patterns of yolk granules, and acridine orange fluorescence of the yolk granules. Yolk granules prepared from mature oocytes (A) and 36-hr nondiapause eggs (B) were centrifuged by Percoll gradients for 30 min at 1,000x*g*. The distribution of yolk granules was determined by measuring protein concentration in each fraction using the method of Bradford (1976). The yolk granules, stained with 100 μM acridine orange, were observed by fluorescence microscopy. LYGs, light yolk granules; DYGs, dense yolk granules.

the microsomes prepared from the nondiapause eggs were incubated with an antibody raised against the P450 reductase, which was expressed in *Escherichia coli* and purified to homogeneity, E20OHase activity was inhibited in a dose-dependent manner. The immunoblot analyses of egg microsomes in various developmental stages indicated that the P450 reductase protein was scarcely detected in diapause eggs, but the P450 reductase protein content in nondiapause eggs gradually increased parallel to the increase in 20E level from the early gastrula stage to the organogenesis stage (Horike *et al*., 2000). Actinomycin D and α-amanitin prevented the 20-hydroxylation of E in *B. mori* eggs, indicating that gene transcription for both E20OHase and P450 reductase is required for 20E biosynthesis.

Recently, it has been demonstrated that the wild-type genes of three members of Halloween family of embryonic lethals, namely *disembodied*, *shadow* and *shade*, code for cytochrome P450s that mediate the last three hydroxylation reactions in the ecdysteroidogenic pathway in *D. melanogaster*, namely the 22-, 2- and 20-hydroxylases (Warren *et al*., 2002; Petryk *et al*., 2003). These studies indicate the importance of ecdysteroids in the embryonic development of insects in general.

Release of ecdysteroid-phosphates from yolk granules

Vitellin stored in yolk granules of oocytes is a nutritional source for subsequent embryonic development. Lagueux *et al*. (1981) were the first to report in *L. migratoria* that most ecdysteroid-phosphates extracted with a buffer solution are bound to vitellin, and the complex is decomposed when treated with protease or ethanol. According to Bownes *et al*. (1988), in *Drosophila* eggs, fatty acid ecdysteroid conjugates were also tightly bound to yolk proteins, and free ecdysteroids could be obtained when yolk proteins were incubated with a combination of protease and esterase. From these results, it is predicted that the degradation of yolk proteins or vitellin *in vivo* leads to the release of conjugated ecdysteroids, followed by the increase in free ecdysteroid levels that trigger key events in embryonic development.

In newly laid eggs of *B. mori*, the bulk of ecdysteroid-phosphates forms a complex with vitellin in yolk granules, and the ecdysteroid-phosphates bound to vitellin are scarcely hydrolyzed by EPPase (Yamada *et al*., in preparation). The fact that EPPase is found in the cytosol but not in yolk granules (Fig. 6) suggests that ecdysteroid-phosphates are dissociated from vitellin and released into the cytosol before they are hydrolyzed by EPPase. Thus, we presumed that in *B. mori* eggs, the dissociation of ecdysteroid-phosphates from vitellin may be caused by the degradation of vitellin. Indeed, in the cockroach *Blattella germanica* (Nordin *et al*., 1991), the African soft tick *Ornithododos moubata* (Fagotto, 1991), the stick insect *Carausius morosus* (Fausto *et al*., 2001) as well as in *B. mori* (Yamahama *et al*., 2003), vitellin has been reported to be degraded by protease that is activated when yolk granules are acidified. However, according to Zhu *et al*. (1986), the level of vitellin in *B. mori* eggs remains almost unchanged from the early to middle stages of embryonic development, but abruptly begins to decrease after the middle stage when larval differentiation is in progress. Thus, we wondered how ecdysteroid-phosphates dissociate from vitellin from the early to middle stages of embryonic development when vitellin is hardly degraded.

Our recent findings on the dissociation of ecdysteroid-phosphates from vitellin are summarized as follows (Yamada *et al*., in preparation): (1) In nondiapause eggs, the level of unbound ecdysteroid-phosphates increased as embryonic development proceeds from the cellular blastoderm stage to the gastrula stage. This suggests that ecdysteroid-phosphates are released from vitellin without vitellin degradation during embryonic development. In contrast, in diapause eggs, the level of unbound ecdysteroid-phosphates did not increase. (2) Ecdysteroid-phosphates dissociated from vitellin prepared from mature ovaries or newly laid eggs, when incubated at acidic pH *in vitro*. (3) Ecdysteroid-phosphates were released from yolk granules when yolk granules, prepared from mature ovaries or newly laid eggs, were incubated with ionophore monensin at acidic pH

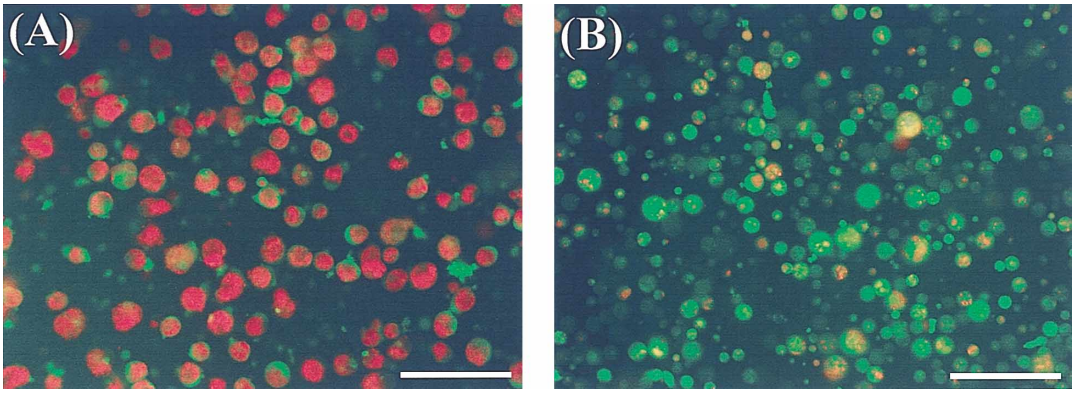

Fig. 9. Effect of bafilomycin on acidification of DYGs. DYGs from 36-hr nondiapause eggs were incubated for 30 min in the absence (A) or presence (B) of 5 μM bafilomycin, and stained with 100 μM acridine orange. Scale bars represent 40 μm.

in vitro. (4) Mature oocytes and newly laid eggs were found to contain a single population of yolk granules, tentatively named light yolk granules (LYGs), that could be isolated by Percoll density gradient centrifugation (Fig. 8A). However, after the cellular blastoderm stage (12-hr eggs), another population of yolk granules appeared in the higher-density fraction, tentatively named dense yolk granules (DYGs) (Fig. 8B). When the pH of yolk granules was estimated using fluorescent dye acridine orange, all LYGs in mature oocytes and newly laid eggs were found to be neutral (Fig. 8A), whereas in developing eggs all DYGs were acidic, but most LYGs remained neutral (Fig. 8B). These results indicate that ecdysteroid-phosphates may be released mainly from DYGs at the stages of cellular blastoderm and gastrulation. (5) As shown in Fig. 9, the acidification of DYGs was inhibited by bafilomycin, a specific inhibitor of vacuolar H^+-ATPase (V-ATPase). Indeed, (6) the presence of V-ATPase in the membrane fraction of yolk granules was also verified by

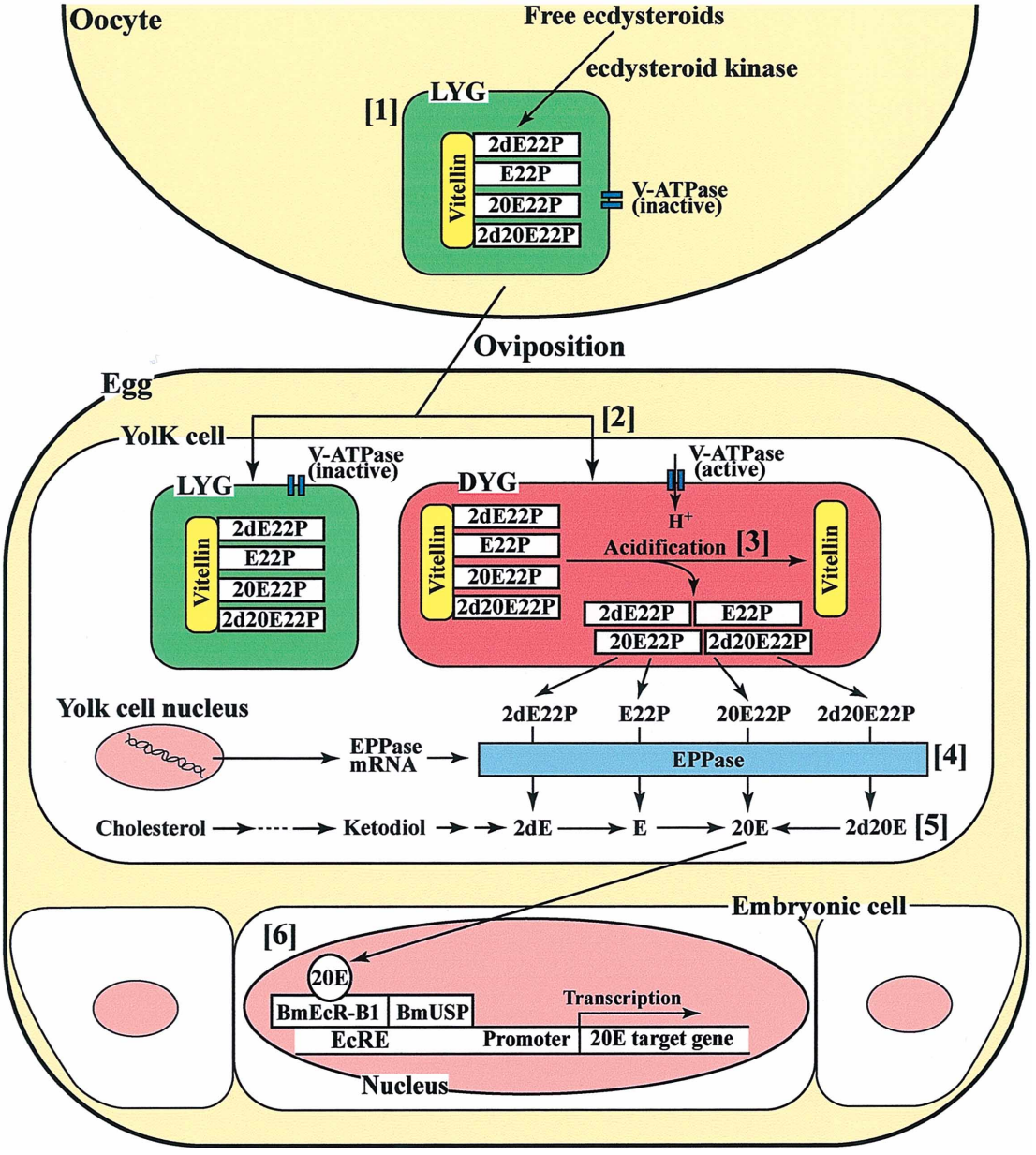

Fig. 10. Proposed pathways of 20E production from vitellin-ecdysteroid-phosphates complex stored in yolk granules of mature oocytes, and the role of 20E in embryonic development. [1] In mature oocytes, phosphoric esters of active hormone 20E and its precursors form a complex with vitellin, and the complex is stored in LYGs. [2] Some of the LYGs become DYGs after the cellular blastoderm stage (12-hr eggs); at the same time, acidification induced by V-ATPase occurs mainly in DYGs. [3] Ecdysteroid-phosphates dissociate from vitellin and are released from DYGs. [4] Ecdysteroid-phosphates are hydrolyzed by EPPase synthesized *de novo* in yolk cells from the gastrula stage to the organogenesis stage. [5] 20E is produced by the hydroxylation reactions of precursor ecdysteroids mainly in yolk cells. [6] 20E acts via the ecdysteroid receptor BmEcR-B1/BmUSP on particular target genes that are indispensable for embryonic development at the gastrula stage. Ecdysteroid abbreviations are as in the text.

Western blot analysis using an antiserum which cross-reacts with the *M. sexta* mid-gut V-ATPase A subunit.

Taken together, the process that leads to the production of free ecdysteroids from the vitellin-ecdysteroid-phosphates complex is summarized in Fig. 10. First, some of the LYGs become DYGs after the cellular blastoderm stage (12-hr eggs); at the same time, acidification, attributable to V-ATPase activity, occurs mainly in DYGs; Next, ecdysteroid-phosphates in the DYGs, such as 2dE22P, E22P, 20E22P and 2d20E22P, dissociate from vitellin and are released. Finally, these released ecdysteroid-phosphates are hydrolyzed by EPPase in the cytosol.

Hypothesis proposed and perspective

The silkworm enters diapause at the late gastrula stage of embryonic development. It has been proposed that two mechanisms are involved in the process determining embryonic diapause of *B. mori*: one is a process that is predetermined by the diapause hormone (DH) during oogenesis (Fukuda, 1951; Hasegawa, 1951; reviewed by Yamashita, 1996), and the other is a process that is determined by a genetic factor (*pnd*$^+$ gene) during embryonic development (Katsumata, 1968; Yoshitake and Hashiguchi, 1969). However, little is known how DH and the *pnd*$^+$ gene are involved in the biochemical mechanism controlling embryonic diapause.

Although our studies have not yet identified particular target genes on which 20E acts during embryonic development of *B. mori*, it is clear that 20E is responsible for the developmental differences between diapause eggs and non-diapause eggs. As shown in Fig. 10, in nondiapause eggs, 20E is produced by the dephosphorylation of ecdysteroid-phosphates released from the maternal vitellin-ecdysteroid-phosphates complex stored in yolk granules as well as by *de novo* biosynthesis. The release of ecdysteroid-phosphates is triggered by the acidification of yolk granules at the cellular blastoderm stage of nondiapause eggs. However, the release does not occur in the yolk granules at the cellular blastoderm stage of diapause eggs. The *pnd*$^+$ gene has been demonstrated not to be expressed at the cellular blastoderm stage (Sonobe and Odake, 1986). From these results, it is conceivable that the V-ATPase activity in yolk granules may be inhibited by mechanisms in which DH rather than the *pnd*$^+$ gene is involved. If so, the characterization of V-ATPase in yolk granules is indispensable for understanding mechanisms of DH action. On the other hand, since at the gastrula stage the *pnd*$^+$ gene has been expressed (Sonobe and Odake, 1986) as well as EPPase gene (Fig. 5), it is possible the *pnd*$^+$ gene may participate in the EPPase gene expression. The *pnd* mutant (Katsumata, 1968; Yoshitake and Hashiguchi, 1969; Sonobe, 1984; Sonobe and Okada, 1984; Sonobe *et al.*, 1986; Sonobe and Odake, 1986; Sonobe, 1989) will be useful for elucidating the molecular mechanism of the EPPase gene expression.

Our approaches involving biochemical, molecular, and morphological methods are beginning to reveal the metabolism and functions of ecdysteroids in embryonic development of *B. mori*. Although much remains to be clarified, the proposed pathways of 20E production in *B. mori* eggs (Fig. 10) open up a new field in the study of embryonic diapause. Furthermore, the new results of studies using *B. mori* eggs will facillitate the study of mechanisms of hormonal regulation in embryonic development of other insects.

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