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3.3 Ecdysteroid Chemistry and Biochemistry

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3.3.1. Introduction

It is not the aim here, owing to the limitations of space, to review all the literature concerning the chemistry and biochemistry of ecdysteroids, and the reader should consult older articles in several excellent reviews by Denis Horn (Horn, 1971, Horn and Bergamasco, 1985) and the book edited by Jan Koolman (Koolman, 1989). After a short historical introduction, we will focus on recent data concerning (1) the techniques used for ecdysteroid analysis and (2) the biosynthesis and metabolism of these hormones. However, we will not give details about chemical synthesis procedures, and we will limit this topic to a few chemical reactions that can be performed by (trained) biologists and do not require an experienced chemist.

The endocrine control of insect molting was established in part by the pioneering work of Stefan Kopec (1922), who demonstrated the need for a diffusible factor originating from the brain (see **Chapter 3.2**) in order for metamorphosis to take place in the gypsy moth, *Lymantria dispar*. This role of the brain was further substantiated by Wigglesworth (1934) with experiments using the blood-sucking bug *Rhodnius prolixus* to define "critical periods" for the necessity of the brain to produce a molting factor (see **Chapter 3.2**). The presence in the hemolymph of a molting/metamorphosis hormone was also established by other approaches: Frew (1928) cultivated imaginal discs *in vitro* in hemolymph from either larvae or pupae and he observed their evagination in the second medium only; Koller (1929) and Von Buddenbrock (1931) observed that the injection of hemolymph of molting animals into younger ones was able to accelerate molting of the latter. Fraenkel (1935) ligated larvae of *Calliphora erythrocephala* and observed pupation in the anterior part only; this provided a basis for the establishment of a bioassay for molting hormone activity, later used for the isolation of ecdysone (E) by Butenandt and Karlson (1954). The use of double ligation experiments with *Bombyx mori* larvae allowed Fukuda (1940) to demonstrate that a factor originating in the brain (i.e., the "brain hormone") (see **Chapter 3.2**) was relayed to an endocrine gland situated in the prothorax that produced the true "molting hormone." This

prothoracic gland was first described by Ke (1930); it is termed "ventral gland" in locusts, and in Diptera it is part of a complex endocrine structure, the "ring gland." In fact, the direct demonstration that prothoracic glands indeed produce E came much later (Chino *et al.*, 1974; King *et al.*, 1974; Borst and Engelmann, 1974), by the analysis of the secretory products of prothoracic glands cultivated *in vitro*. One year later, Hagedorn *et al.* (1975) demonstrated that the ovary of adult mosquitoes was another source of E, and this was the basis for establishing the role of ecdysteroids in the control of reproduction.

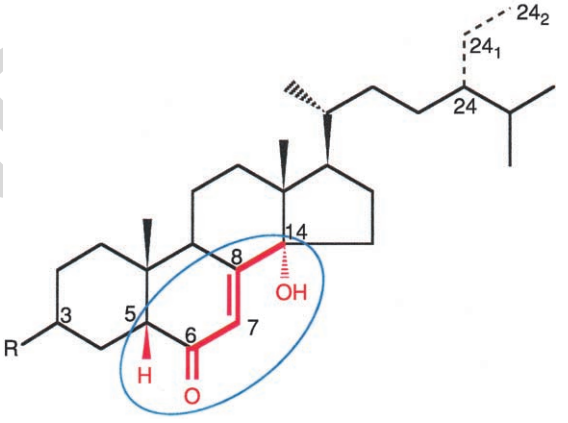
p0015 The first attempts to purify the molting hormone were performed by Becker and Plagge (1939) using the *Calliphora* bioassay derived from Fraenkel's work. α-Ecdysone was isolated in 1954 by Butenandt and Karlson after 10 years of attempts to do so. These authors used *B. mori* pupae because this material was available in large amounts. Ten more years of work was necessary to elucidate E's structure, which was finally achieved using X-ray crystallography analysis (Huber and Hoppe, 1965). A detailed description of this story can be found in Karlson and Sekeris (1966), and it is of great interest to follow how E was progressively characterized and to compare this work with what can be accomplished with presently available tools.

p0020 A second molecule, 20-hydroxyecdysone (20E) (initially termed β-ecdysone) was described soon thereafter, and this molecule was also isolated from a crayfish (Hampshire and Horn, 1966). It was rapidly established that 20E was the major molting hormone of all arthropods. Amazingly, E, which was the first of these compounds to be isolated, is at best a minor compound in most insect species when detectable, with the exception of young lepidopteran pupae, i.e., the material used by Butenandt and Karlson. Other closely related molecules were then isolated and the generic term of "ecdysteroids" was proposed for this new steroid family (Goodwin *et al.*, 1978). Meanwhile, ecdysteroids had also been isolated from plants, hence the terms "zooecdysteroids" and "phytoecdysteroids."

s0010 3.3.2. Definition, Occurrence, and Diversity

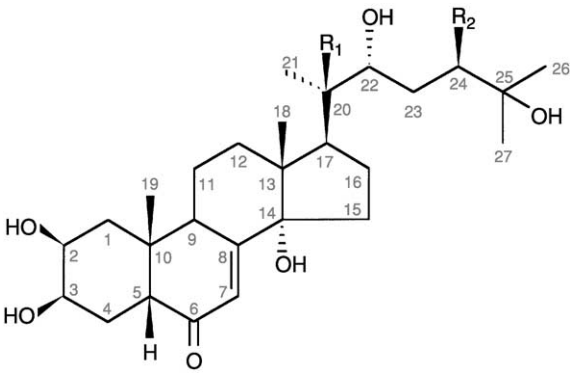
p0025 Ecdysteroids could be defined either by their biological activity (molting hormones) or their chemical structure. They represent a specific family of sterol derivatives comprising more than 300 members that bear common structural features: a *cis* (5β-H) junction of rings A and B, a 7-ene-6-one chromophore, and a *trans* (14α-OH or −H) junction of rings C and D (**Figure 1**). Most of them also bear the 3β-OH already present in their sterol precursor, and a set of other hydroxyl groups located both on the steroid nucleus and the side chain (**Figure 2**) that renders them rather water soluble. The major insect ecdysteroid is 20E, a 27-carbon (27C) molecule derived from cholesterol (C), but some insect species contain its 28C or 29C homologs, makisterone A and makisterone C, respectively (**Figure 3**).

p0030 The huge chemical diversity of ecdysteroids results from variations in the number and position of −OH groups, which can be either free or conjugated to various polar or apolar moieties, giving rise to the 70 different molecules presently described in insects listed in **Table 1** (more information about these molecules can be found on the Ecdybase website). The greatest diversity has been found in the eggs/embryos of Orthoptera and Lepidoptera, probably because they contain very large amounts of these molecules (up to 40 $\mu g\, g^{-1}$ fresh weight, i.e., 10 to 100 times the concentrations found in larvae or pupae). The nomenclature of insect ecdysteroids is generally derived from the ecdysone name, but



f0005 **Figure 1** Common structural features of ecdysteroids (major characteristics are indicated in red/blue; R = 3β-OH in most cases).

f0010 **Figure 2** Space-filling model of the 20-hydroxyecdysone molecule. Blue: carbon atoms; red: oxygen atoms; white: hydrogen atoms. (Courtesy of Pr J.-P. Girault.)



f0015 **Figure 3** Structures of the major insect ecdysteroids. $R_1 = R_2 = R_3 = H$: ecdysone (E); $R_1 = OH$, $R_2 = H$: 20-hydroxyecdysone (20E); $R_1 = OH$, $R_2 =$ methyl: makisterone A; $R_1 = OH$, $R_2 =$ ethyl: makisterone C.

this is always the case, and the trivial name of many ecdysteroids (especially phytoecdysteroids) is related to the name of the species from which they have been isolated; standardized abbreviations have been proposed for insect ecdysteroids (Lafont[bib1465] *et al.*, 1993a) and are used throughout this chapter.

s0015 ## 3.3.3. Methods of Analysis

s0020 ### 3.3.3.1. Methods of Identification

s0025 **3.3.3.1.1. Physicochemical properties**

s0030 p0035 *3.3.3.1.1.1. UV spectroscopy* The 7-en-6-one group of ecdysteroids has a relatively strong absorption at $\lambda_{max} \sim 243$ nm with an ε of ~ 10–$16\,000$: for instance E, has a λ_{max} at 242 nm and an ε of 12 400 in EtOH. This maximum of absorbance is shifted to 247 nm in water.

s0035 p0040 *3.3.3.1.1.2. IR spectroscopy* The presence of a number of hydroxyl groups on most ecdysteroids ensures a strong absorption in the infrared (IR) spectrum in the region of 3340–3500 cm^{-1}. The $\alpha\beta$-unsaturated ketone results in a characteristic cyclohexenone absorption at 1640–1670 cm^{-1}, with a weaker alkene stretch at approximately 1612 cm^{-1}. An additional carbonyl absorption is seen with 3-dehydroecdysteroids: thus 3-dehydroecdysone (3DE) and 3-dehydro-20-hydroxyecdysone (3D20E) show a carbonyl absorption at 1712 cm^{-1} and 1700 cm^{-1}, respectively, in addition to the usual 7-ene-6-one signal. Among conjugates, the absorption of phosphates ranges from 1100 to 1000 cm^{-1}. Acetates and acyl esters can be characterized by additional bands corresponding to the carbonyl and to the ester group. Ecdysteroids are not soluble in the usual infrared solvents (CS_2, CCl_4, $CHCl_3$) so IR spectra are done on KBr discs or mineral oil (Nujol) mulls. The development of Fourier transform spectrometers (FT-IR) allows the solubility problem to be overcome, as dilute solutions can be analyzed. Thus, IR spectra can even be recorded in-line during HPLC analysis (Louden[bib1565] *et al.*, 2001, 2002).

s0040 p0045 *3.3.3.1.1.3. Fluorescence* In the presence of sulfuric acid or aqueous ammonia, ecdysteroids are induced to fluoresce (Horn[bib0895], 1971). Typical values for excitation and emission wavelengths are in the region of 380 and 430 nm, respectively. Some variations in excitation and emission wavelength and fluorescence intensity are observed with ecdysteroids (Koolman[bib1270], 1980).

s0045 p0050 **3.3.3.1.2. Mass spectrometry** Mass spectrometry (MS) has been invaluable in the elucidation of ecdysteroid structures and has been particularly useful for insect samples containing low levels of ecdysteroids. Most of the early work on ecdysteroids has been performed using relatively unsophisticated direct introduction techniques (electron impact, EI), but new ionization techniques are now increasingly employed.

s0050 *3.3.3.1.2.1. Modes of ionization*

s0055 p0055 3.3.3.1.2.1.1. Electron impact (EI-MS) In EI-MS, an electron beam is used for the ionization and the collision will break the molecule into several characteristic fragments. With nonvolatile substances like ecdysteroids, the use of a 70 eV electron beam results in extensive fragmentation. The abundant fragments correspond to the steroid nucleus or to the side chain and are highly informative for structural elucidation.

s0060 p0060 3.3.3.1.2.1.2. Chemical ionization (CI-MS) This method uses a reagent gas that is ionized by the electron beam and gives rise to a set of ions that in turn can react with ecdysteroids (Lafont[bib1420] *et al.*, 1981). The ionization reaction proceeds as follows:

$$M + HR^+ \rightarrow MH^+ \text{(pseudo-molecular ion)} + R$$

$$M + HR^+ \rightarrow MHR^+ \text{(adduct ion)}$$

where R = the reagent gas, e.g., ammonia. The relative intensity of these ions is rather high. Among the various reagent gases, ammonia is most widely used, giving both MH^+ and $(M+NH_4)^+$ ions of high intensity. This method is of interest for the determination of the molecular mass (M) of ecdysteroids. Both positive and negative ionization can be used. The CI/D techniques were analyzed in depth by Lusby[bib1595] *et al.* (1987) who considered the role of

t0005 **Table 1** Ecdysteroids identified (and isolated in most cases) from insects (see also Rees, 1989a; for a complete list of ecdysteroids and for structures, see Ecdybase)

Ecdysteroid	*Species*	*Reference*
Low polarity ecdysteroids (≤5-OH groups)		
2,14,22,25-Tetradeoxyecdysone (5β-ketol)	*Locusta migratoria* ovaries	Hetru *et al.* (1978)
2,22,25-Trideoxyecdysone (5β-ketodiol)	*Locusta migratoria* ovaries	Hetru *et al.* (1978)
2,22-Dideoxyecdysone	*Locusta migratoria* ovaries	Hetru *et al.* (1978)
2,22-Dideoxy-20-hydroxyecdysone	*Bombyx mori* ovaries	bib0945 Ikekawa *et al.* (1980)
2,22-Dideoxy-23-hydroxyecdysone	*Bombyx mori*	bib1060 Kamba *et al.* (2000a)
22-Deoxy-20-hydroxyecdysone	*Bombyx mori*	bib1050 Kamba *et al.* (1994)
Ecdysone	*Bombyx mori* pupae	bib0240 Butenandt and Karlson (1954)
2-Deoxyecdysone	*Bombyx mori* ovaries/eggs	bib1815 Ohnishi *et al.* (1977)
2-Deoxy-20-hydroxyecdysone	*Schistocerca gregaria*	bib0980 Isaac *et al.* (1981a)
20-Deoxy-makisterone A (= 24-methyl-ecdysone)	*Drosophila* ring glands	bib1965 Redfern (1984)
14-Deoxy-20-hydroxyecdysone	*Gryllus bimaculatus*	bib0860 Hoffmann *et al.* (1990)
Phase I metabolites		
+ 20-OH		
20-Hydroxyecdysone	*Bombyx mori* pupae	bib0840 Hocks and Wiechert (1966)
Makisterone A	*Oncopeltus fasciatus*	bib1075 Kaplanis *et al.* (1975)
24-Epi-makisterone A	*Acromyrmex octospinosus*	bib1655 Maurer *et al.* (1993)
Makisterone C	*Dysdercus fasciatus*	bib0550 Feldlaufer *et al.* (1991)
+ 26-OH (and the subsequent oxidation products)		
20,26-Dihydroxyecdysone	*Manduca sexta* pupae	bib2410 Thompson *et al.* (1967)
26-Hydroxyecdysone	*Manduca sexta* eggs	bib1080 Kaplanis *et al.* (1973)
Ecdysonoic acid	*Schistocerca gregaria* eggs	bib0965 Isaac *et al.* (1983)
20-Hydroxyecdysonoic acid	*S. gregaria* eggs; *Pieris brassicae* pupae	bib0965 Isaac *et al.* (1983); bib1430 Lafont *et al.* (1983)
20-Hydroxyecdysone 26-aldehyde (2 hemiacetal forms)	*Chironomus tentans cells*	bib1160 Kayser *et al.* (2002)
+ 3-oxo/3α-		
3-Dehydroecdysone	*Calliphora erythrocephala*	bib1120 Karlson *et al.* (1972)
3-Dehydro-20-hydroxyecdysone	*Calliphora vicina*	bib1310 Koolman and Spindler (1977)
3-Dehydro-2-deoxyecdysone	*Locusta migratoria*	bib2475 Tsoupras *et al.* (1983a)
3-Epi-20-hydroxyecdysone	*Manduca sexta*	bib2405 Thompson *et al.* (1974)
3-Epiecdysone	*Manduca sexta*	bib1090 Kaplanis *et al.* (1979)
3-Epi-2-deoxyecdysone	*Schistocerca gregaria*	bib0980 Isaac *et al.* (1981a)
3-Epi-26-hydroxyecdysone	*Manduca sexta*	bib1095 Kaplanis *et al.* (1980)
3-Epi-20,26-dihydroxyecdysone	*Manduca sexta*	bib1090 Kaplanis *et al.* (1979)
3-Epi-22-deoxy-20-hydroxyecdysone	*Bombyx mori*	bib1615 Mamiya *et al.* (1995)
3-Epi-22-deoxy-20,26-dihydroxyecdysone	*Bombyx mori*	bib1065 Kamba *et al.* (2000b)
3-Epi-22-deoxy-16β,20-dihydroxyecdysone	*Bombyx mori*	bib1065 Kamba *et al.* (2000b)
Phase II metabolites: apolar conjugates		
Acetates: 3-acetylation		
Ecdysone 3-acetate	*Schistocerca gregaria*	bib0985 Isaac *et al.* (1981b)
20-Hydroxyecdysone 3-acetate	*Locusta migratoria*	bib1735 Modde *et al.* (1984)
20-Hydroxyecdysone 22-acetate	*Drosophila melanogaster*	bib1635 Maroy *et al.* (1988)
Acyl esters: 22-acylation		
Ecdysone 22-palmitate	*Gryllus bimaculatus*	bib0855 Hoffmann *et al.* (1985)
Ecdysone 22-palmitoleate	*Gryllus bimaculatus*	bib0855 Hoffmann *et al.* (1985)
Ecdysone 22-oleate	*Gryllus bimaculatus*	bib0855 Hoffmann *et al.* (1985)
Ecdysone 22-linoleate	*Gryllus bimaculatus*	bib0855 Hoffmann *et al.* (1985)
Ecdysone 22-stearate	*Gryllus bimaculatus*	bib0855 Hoffmann *et al.* (1985)
20-Hydroxyecdysone 22-palmitate	*Heliothis virescens*	bib1350 Kubo *et al.* (1987)
20-Hydroxyecdysone 22-oleate	*Heliothis virescens*	bib1350 Kubo *et al.* (1987)
20-Hydroxyecdysone 22-linoleate	*Heliothis virescens*	bib1350 Kubo *et al.* (1987)
20-Hydroxyecdysone 22-stearate	*Heliothis virescens*	bib1350 Kubo *et al.* (1987)
Phase II metabolites: polar conjugates		
Glucosides: 22-glucosylation		
26-Hydroxyecdysone 22-glucoside	*Manduca sexta*	bib2400 Thompson *et al.* (1987a)
Phosphates		
2-Phosphates		
Ecdysone 2-phosphate	*Schistocerca gregaria*	bib0960 Isaac *et al.* (1984)

Continued

Table 1 Continued

Ecdysteroid	*Species*	*Reference*
26-Hydroxyecdysone 2-phosphate	*Manduca sexta*	bib2440 Thompson *et al.* (1987b)
3-Epi-22-deoxy-20-hydroxyecdysone 2-phosphate	*Bombyx mori*	bib1615 Mamiya *et al.* (1995)
3-Epi-22-deoxy-20,26-dihydroxyecdysone 2-phosphate	*Bombyx mori*	bib1065 Kamba *et al.* (2000b)
3-Epi-22-deoxy-16β,20-dihydroxyecdysone 2-phosphate	*Bombyx mori*	bib1065 Kamba *et al.* (2000b)
3α-Phosphates		
3-Epi-2-deoxyecdysone 3-phosphate	*Locusta migratoria*	bib2480 Tsoupras *et al.* (1982b)
3-Epi-20-hydroxyecdysone 3-phosphate	*Pieris brassicae*	bib0100 Beydon *et al.* (1987)
3β-phosphates		
Ecdysone 3-phosphate	*Locusta migratoria*	bib1495 Lagueux *et al.* (1984)
22-Deoxy-20-hydroxyecdysone 3-phosphate	*Bombyx mori*	bib1050 Kamba *et al.* (1994)
2,22-Dideoxy-20-hydroxyecdysone 3-phosphate	*Bombyx mori*	bib0830 Hiramoto *et al.* (1988)
2,22-Dideoxy-23-hydroxyecdysone 3-phosphate	*Bombyx mori*	bib1060 Kamba *et al.* (2000a)
22-phosphates		
2-Deoxyecdysone 22-phosphate	*Schistocerca gregaria*	bib0990 Isaac *et al.* (1982)
2-Deoxy-20-hydroxyecdysone 22-phosphate	*Locusta migratoria*	bib2490 Tsoupras *et al.* (1982a)
Ecdysone 22-phosphate	*Schistocerca gregaria*	bib0990 Isaac *et al.* (1982)
20-Hydroxyecdysone 22-phosphate	*Locusta migratoria*	bib2490 Tsoupras *et al.* (1982a)
3-Epi-2-deoxyecdysone 22-phosphate	*Bombyx mori*	bib1055 Kamba *et al.* (1995)
3-Epi-ecdysone 22-phosphate	*Bombyx mori*	bib1055 Kamba *et al.* (1995)
26-phosphates		
26-Hydroxyecdysone 26-phosphate	*Manduca sexta*	bib2445 Thompson *et al.* (1985b)
Phase II metabolites/complex conjugates		
Acetyl-phosphates		
3-Acetylecdysone 2-phosphate	*Schistocerca gregaria*	bib0960 Isaac *et al.* (1984)
3/2-Acetylecdysone 22-phosphate	*Schistocerca gregaria*	bib0975 Isaac and Rees (1984)
3-Acetyl-20-hydroxyecdysone 2-phosphate	*Locusta migratoria; Schistocerca gregaria*	bib1735 Modde *et al.* (1984); bib0975 Isaac and Rees (1984)
3/2-Acetyl-20-hydroxyecdysone 22-phosphate	*Locusta migratoria*	bib2485 Tsoupras *et al.* (1983b)
Ecdysone 2,3-diacetate 22-phosphate	*Locusta migratoria*	bib1495 Lagueux *et al.* (1984)
Nucleotides		
2-Deoxyecdysone 22-AMP	*Locusta migratoria*	bib2475 Tsoupras *et al.* (1983a)
Ecdysone 22-AMP	*Locusta migratoria*	bib0800 Hétru *et al.* (1984)
Ecdysone 22-isopentenyl-AMP	*Locusta migratoria*	bib2495 Tsoupras *et al.* (1983b)
Ecdysteroid-related steroids		
Bombycosterol	*Bombyx mori*	bib0605 Fujimoto *et al.* (1985a)
Bombycosterol 3-phosphate	*Bombyx mori*	bib0830 Hiramoto *et al.* (1988)

parameters such as source pressure and source temperature on fragmentation.

s0065 p0065 3.3.3.1.2.1.3. Field desorption (FD-MS) With FD-MS, ionization proceeds in a high electrostatic field; such conditions allow ecdysteroid molecules to lose one electron ($\rightarrow M^+$) without excessive heating. In such cases, the organic molecules have not received any large amount of energy and, therefore, almost no fragmentation is observed (bib1310 Koolman and Spindler, 1977).

s0070 p0070 3.3.3.1.2.1.4. Fast-atom bombardment (FAB-MS) FAB-MS uses a beam of neutral atoms (argon or xenon) bearing a high kinetic energy for the ionization of involatile ionic or nonionic compounds present either in the solid state or as a solution in a glycerol matrix. FAB-MS is particularly suitable for polar ecdysteroids – phosphate conjugates (see e.g., bib0990 Isaac *et al.*, 1982b; bib1735 Modde *et al.*, 1984), ecdysonoic acids (bib0960 Isaac *et al.*, 1984) – but of course it works also with nonionic ecdysteroids (bib0955 Rees and Isaac, 1985). Sodium or potassium salts of conjugates and ecdysonoic acids give, respectively, $[M+Na]^+$ and $[M+K]^+$ ions. FAB^+ has been used to characterize fatty acyl derivatives of ecdysteroids (bib0420 Dinan, 1988), but FAB^- can also be used (Wilson *et al.*, 1988a). When using a deuterated matrix, extensive deuterium exchange takes place and it allows the number of hydroxyl groups present in the molecule to be determined (bib1920 Pis and Vaisar, 1997).

s0075 p0075 3.3.3.1.2.1.5. Electrospray (ES-MS) ES-MS has been introduced more recently (Hellou *et al*., 1994). It allows a high intensity of molecular ions to be obtained and is recommended for fragile molecules. Moreover, it can be conveniently coupled with HPLC for in-line analysis.

s0080 p0080 *3.3.3.1.2.2. Low/high-resolution mass spectrometry (HR-MS)* All the above methods provide low-resolution mass spectra. Such data result, in fact, from an approximation, as the exact molecular mass of atoms (with the exception of the ^{12}C carbon atom, which by definition is equal to 12) is not a whole number (for example, the mass of ^{1}H is 1.00783 and that of ^{16}O is 15.99491). By using high-resolution instruments, the mass resolution is much increased and the masses of fragments can be obtained with a great accuracy, allowing the spectroscopist to unambiguously determine the elementary composition of ions and of their parent molecules. This technique has fully replaced the former elementary analysis.

s0085 p0085 *3.3.3.1.2.3. Tandem mass spectrometry (MS-MS)* The determination of fragment structure and filiation is by no means easy. The MS-MS techniques correspond to the sequential operation of several chambers (quadrupoles) where fragment separation takes place. Thus, the first quadrupole is used to select one ion, and following the passage of that ion through a "fragmentation cell," the second one is used to analyze the further fragmentation of this selected ion. An extensive analysis of this type allows the establishment of genetic relationships between all the observed fragments and is of considerable help in the understanding of fragmentation mechanisms. The same method can also be used to search for the presence of a specific compound in a crude mixture (Mauchamp *et al*., 1993).

s0090 p0090 *3.3.3.1.2.4. General rules for mass spectrometry* The fragmentation patterns of ecdysteroids are dominated by ions resulting from the ecdysteroid nucleus and those due to the side chain (Nakanishi, 1971; **Figure 4**). Because of the ready elimination of water from these polyhydroxy compounds, molecular ions, if present, are usually weak (<1%) when using EI-MS, although $[M-18]^+$ ions are often readily apparent. Indeed, the high mass region of the spectra of ecdysteroids tends to be characterized by ions 18 mass units apart resulting from the sequential losses of water (Horn, 1971). The presence of a 20,22-vicinal diol allows the cleavage of the side chain (2), giving rise to a prominent fragment at m/z 99 for 20E (otherwise dependent on the structure of the side chain), frequently forming the base peak of the EI spectrum. An ion at m/z 81 is also frequently observed, which probably results from the elimination of water from the side chain fragment (Horn, 1971; Nakanishi, 1971). In the absence of a 20,22-diol, fragmentation (1) between C_{17} and C_{20} is observed. This cleavage is less dominant than the 20,22-scission, and the result is a more complex pattern. Where the side chain has been modified to include methyl or ethyl substituents at C_{24} (e.g., the makisterones A and C), the fragments at m/z 99 and 81 are replaced by ions 14 and 28 mass units higher (Nakanishi, 1971).

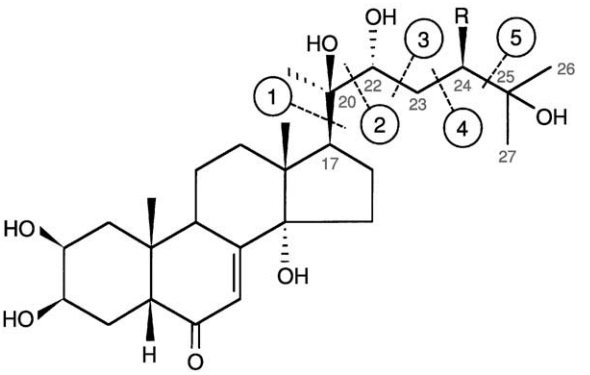


Figure 4 Major and minor fragmentations of ecdysteroid side chain. 1: C_{17}–C_{20} cleavage; 2: C_{20}–C_{22} cleavage (major if 20,22-diol); 3: C_{22}–C_{23} cleavage (minor); 4: C_{23}–C_{24} cleavage (if branching at C_{24}); 5: C_{24}–C_{25} cleavage (for TMS ethers). f0020

s0095 p0095 **3.3.3.1.3. Nuclear magnetic resonance (NMR)** NMR spectroscopy is the method of choice for determining the structure of ecdysteroids. Proton ^{1}H-NMR and carbon ^{13}C-NMR are of general use, whereas the use of ^{31}P-NMR is restricted to phosphate conjugates (Hétru *et al*., 1985; Rees and Isaac, 1985) and will not be discussed here.

p0100 Obtaining a high-quality ^{1}H-NMR spectrum using modern equipment requires about 50 µg of a pure compound and this is generally sufficient to determine elementary changes like epimerization, appearance of an extra-OH group, or the position involved in a conjugation process. More important changes require ^{13}C-NMR studies, which until recently required amounts in the milligrams. Complete NMR spectra of E and 20E have been published (Kubo *et al*., 1985b; Girault and Lafont, 1988, Lee and Nakanishi, 1989), and general tables are given in several reviews (e.g., Hoffmann and Hétru, 1983; Horn and Bergamasco, 1985; Rees and Isaac, 1985; see also Ecdybase website). Most NMR data have been obtained using C_5D_5N as solvent, except for very apolar compounds (apolar conjugates or acetate derivatives analyzed in $CDCl_3$) and polar conjugates (analyzed in D_2O). These

solvents may be of interest for use with specific compounds in connection with either solubility problems or the presence of signals, which would be masked by signals due to the classical solvents. For ^{1}H-NMR studies using modern machines, the water solubility of many common ecdysteroids is high enough to obtain excellent spectra (Girault and Lafont, 1988).

3.3.3.1.3.1. Proton NMR There have been spectacular improvements in proton NMR with the introduction of high-field machines and sophisticated software, and this has resulted not only in the possibility of using smaller amounts of ecdysteroids but also of obtaining more information from the spectra. Classical proton NMR spectroscopy (in C_5D_5N) allows the easy determination of methyl groups (usually C_{18}, C_{19}, C_{21}, C_{26}, and C_{27}, δ 0.8–1.4 ppm) and of a few additional signals corresponding to 7-H (vinylic proton, δ *ca.* 6.2 ppm) and to primary or secondary alcoholic functions (2-H, 3-H and 22-H δ 4.0–4.2 ppm). Other signals (CH or CH_2 groups) overlap in the same area of the spectrum (δ 1.5–2.3 ppm), with the noticeable exception of 9-H (δ *ca.* 3.5 ppm) and 5-H (δ *ca.* 3.0 ppm) (Horn, 1971). Furthermore, two-dimensional techniques such as 2D-COSY and relayed correlated spectroscopy (COSY) have allowed the complete assignment of all protons of the ecdysone molecule (Kubo *et al.*, 1985a; Girault and Lafont, 1988). The proton NMR spectrum strongly depends upon the solvent and, to a lesser extent, on the temperature used. In most cases the solvent used was C_5D_5N or $CDCl_3$ (for acetate derivatives essentially), but for some compounds such data are lacking and the available spectra were obtained in D_2O, CD_3OD, ..., so they are more difficult to compare. Coupling constants also provide useful information concerning the stereochemistry of the ecdysteroid molecule, and, for example, they allow 3α- and 3β-OH compounds to be distinguished.

3.3.3.1.3.2. Carbon-NMR The introduction of ^{13}C NMR (Lukacs and Bennett, 1972) represented a major advance in the determination of ecdysteroid structures. The signals of the carbon atoms are spread over 200 ppm. Thus, ^{13}C NMR spectra are better resolved than ^{1}H spectra. Due to the low natural abundance of ^{13}C, relatively large samples (≥10 mg) are required. Off-resonance decoupled spectra show the coupling of carbon atoms to protons, allowing the carbons to be distinguished from the number (0–3) of protons they bear and allowing an easier interpretation of spectra.

New methods have recently become available for collecting ^{13}C information thanks to inverse correlations with proton signals: the heteronuclear multiple-quantum correlation (HMQC) and the heteronuclear multiple-bond correlation (HMBC), which make use of the direct ($^{1}J^{13}$C-H) and long range ($^{2}J^{13}$C-H and $^{3}J^{13}$C-H) couplings, respectively (**Figure 5**; Girault, 1998); they allow ^{13}C data to be obtained with less than 500 µg of ecdysteroid. The production of higher field instruments (up to 800 MHz) will continue to reduce sample requirements (to 100 µg or even less, if completely pure).

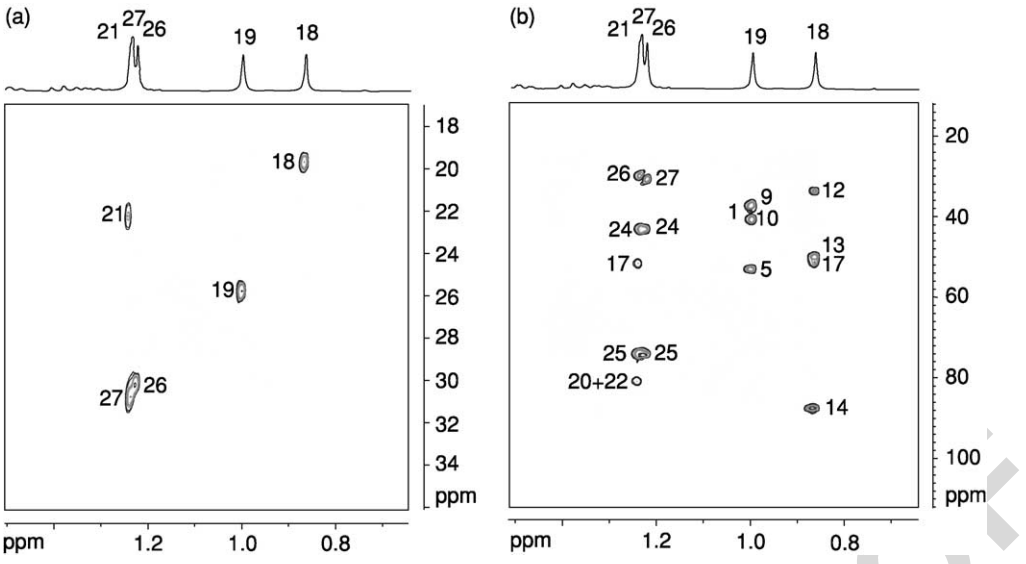
3.3.3.2. Methods of Purification

Ecdysteroids form a group of rather polar compounds and, as a consequence, their extraction is usually performed using a polar solvent such as MeOH. The next step usually involves one or more solvent partitions with the aim of removing the bulk of polar and nonpolar contaminants prior to chromatography (**Figure 6**).

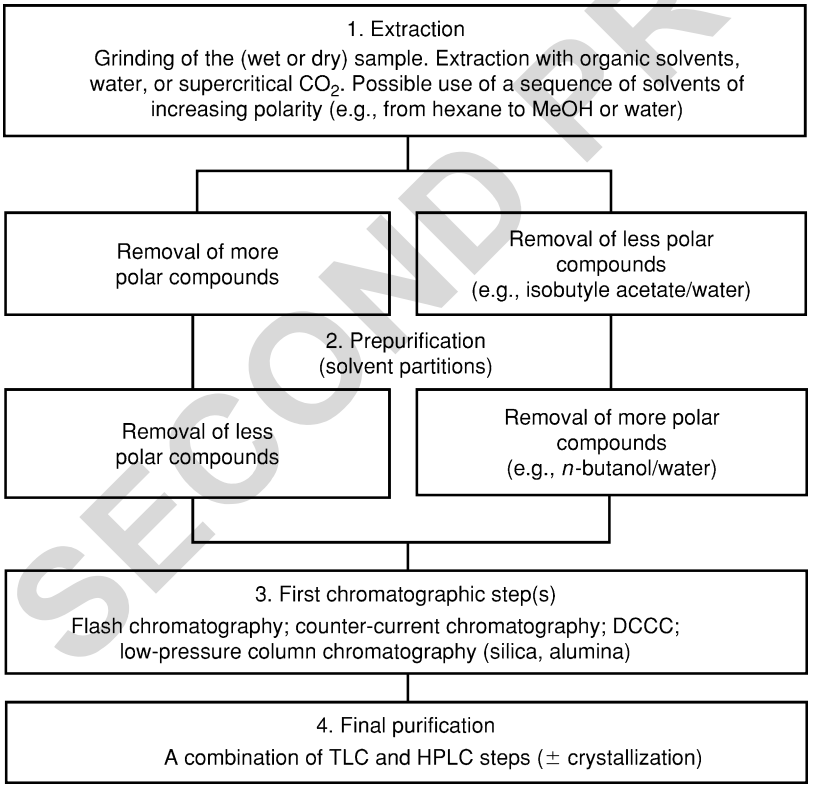
3.3.3.2.1. Extraction procedures Usually, a polar solvent (MeOH, at least 10× sample weight v/w) is used. Two or three repeats are necessary to extract ecdysteroids quantitatively. Alternative solvents include the less toxic EtOH, or Me_2CO, MeCN, MeOH-H_2O or H_2O. Extraction can be performed at room temperature, but slight heating (<60 °C) or even refluxing conditions (Soxhlet apparatus) may significantly improve its efficiency.

3.3.3.2.2. Partition techniques The crude extract is concentrated under reduced pressure to give an oily residue. The rather peculiar polarity of ecdysteroids allows their purification by solvent partitioning. This of course applies to compounds having a polarity in the same range as E and 20E. In the cases where polar or apolar ecdysteroids are present (e.g., phosphate or acyl esters), it is clear that those derivatives will partition in a very different way according to their respective polarity, and they may be lost.

3.3.3.2.2.1. Solvent partitioning In early studies (see Horn, 1971), the initial partition was made between an aqueous concentrate and Bu-1-OH. The Bu-1-OH residue, into which the ecdysteroids were extracted, was then partitioned between aqueous MeOH and hexane to remove nonpolar material such as lipids. However, reversing this order of operations was found to be beneficial and led to reduced emulsion formation during the partition step and less problems with frothing during evaporation. Other suitable solvents for removing lipids include hexane-MeOH (7:3, v/v), light petroleum (b.p. 40–60 °C), or Pr-1-OH-hexane (3:1, v/v), the



f0025 **Figure 5** Inverse correlation NMR spectra of 20-hydroxyecdysone in D_2O. (a) Heteronuclear multiple-quantum correlation allows the observation of the direct $^{1}J^{13}C$-H couplings (e.g., between the 18-Me protons and C-18); (b) Heteronuclear multiple-bond correlation allows the observation of $^{2}J^{13}C$-H (e.g., between 18-Me protons and C-13) and $^{3}J^{13}C$-H (e.g., between 18-Me protons and C-12, C-14, and C-17) long-range couplings. (Courtesy of Dr J.-P. Girault.)



f0030 **Figure 6** Example of an extraction and purification chart for the large-scale isolation of ecdysteroids (Reprinted with permission from Lafont, R., Morgan, E.D., Wilson, I.D., **1994b**. Chromatogrpahic procedures for phytoecdysteroids. *J. Chromatogr. 658*, 31–53; © Elsevier.)

ecdysteroids remaining in the aqueous phase. The addition of $(NH_4)_2SO_4$ improves phase separation. The separation of polar impurities from the ecdysteroids can be achieved by partition between H_2O and Bu-1-OH (ecdysteroids partition into the organic phase) and H_2O-EtOAc (ecdysteroids remain in the aqueous phase). The major factors governing the choice of solvent partition system are the

type of contaminants to be removed (i.e., mainly lipids or mainly polar compounds, etc.) and the nature of the ecdysteroids to be isolated. Thus, the addition or removal of one-OH group, or conjugation to polar (e.g., sulfate) or nonpolar (e.g., acetate or fatty acyl) groups can significantly affect partition ratios. A combination of two successive partitions allows elimination of both polar and apolar contaminants. The use of two systems having one solvent in common avoids the need for an evaporation step between the two partitions. It is thus possible to combine first $CHCl_3/H_2O$ followed by H_2O/Bu-1-OH. $CHCl_3$ can be replaced by a more polar organic solvent, such as isobutyl acetate that, allows ecdysteroids to remain in the water phase (Matsumoto and Kubo, 1989).

3.3.3.2.2.2. Counter-current distribution (CCD) Counter-current distribution between Bu-1-OH and H_2O is effective for removing polar contaminants and several other systems have been successfully used in the past (Horn and Bergamasco, 1985). For instance, Butenandt and Karlson (1954) used this method to purify ecdysone from *Bombyx* pupae by means of Craig countercurrent distribution with butanol-cyclohexane-water (6:4:10, v/v/v). This method allowed an efficient separation of two different fractions containing E and 20E, respectively. The same two ecdysteroids were also isolated from an extract of 15 kg *Calliphora* pupae (Karlson, 1956).

3.3.3.2.2.3. Droplet counter-current chromatography (DCCC) DCCC provides an efficient means for purifying samples up to the gram range. It belongs to the family of liquid–liquid partition chromatographic methods. In favorable cases, DCCC can enable the preparation of pure compounds (Kubo *et al.*, 1985a). Generally, a subsequent HPLC step is required to get pure ecdysteroids. In a related procedure, high-speed countercurrent chromatography (HSCCC), the column is a multilayer coil and efficient mixing is achieved through planetary rotation (Ito, 1986). The centrifugal forces allow separations to be achieved within hours instead of days with DCCC.

3.3.3.2.3. Column chromatography (low pressure)

3.3.3.2.3.1. Preparative columns This technique can be easily scaled-up according to sample size; it just suffices to keep a low sample to sorbent ratio. It can be used with various stationary phases (NP: silica, alumina; RP: hydrophobic resins, polyamide, or C_{18}-bonded silica, ion-exchange phases, etc.). Elution usually proceeds with a step-gradient of the mobile phase with increasing eluting power; fractions are collected and assayed, for example, by TLC or HPLC.

3.3.3.2.3.2. Small columns and/or disposable cartridges

3.3.3.2.3.2.1. Normal phase Low-pressure chromatography on a small column was used in very early methods for the fractionation of crude extracts: silica or alumina columns (normal phase systems) were generally eluted with binary mixtures, e.g., a step-gradient of alcohol in chloroform or benzene.

3.3.3.2.3.2.2. Reversed phase The availability of hydrophobic phases (resins like Amberlite® or hydrocarbon-bonded silica) has led to a complete renewal of the early procedures. Small cartridges or syringes containing 0.2–1 g of HPLC phase are ideally suited for a rapid clean up of small samples (Lafont *et al.*, 1982; Watson and Spaziani, 1982; Pimprikar *et al.*, 1984; Rees and Isaac, 1985; Lozano *et al.*, 1988b; Wilson *et al.*, 1990b). They can also be used for desalting purposes, e.g., direct adsorption of ecdysteroids from culture media, from buffers used for enzymatic hydrolysis of conjugates, or from reversed phase HPLC fractions when an involatile buffer is used.

3.3.3.3. Methods of Separation

The general characteristics of separation are summarized in **Table 2**.

3.3.3.3.1. Thin-layer chromatography (TLC, HPTLC, OPLC)

3.3.3.3.1.1. Chromatographic procedures Normal-phase (absorption) chromatography on silica gel has been used extensively for the isolation of ecdysteroids and for metabolic studies (Horn, 1971; Morgan and Poole, 1976; Morgan and Wilson, 1989).

3.3.3.3.1.1.1. Normal-phase and reversed-phase systems A general normal phase system consists of 95% EtOH-$CHCl_3$ (1:4, v/v). For reproducible results, plates should be heated for 1 h at 120 °C, and then deactivated to constant activity over saturated saline, and TLC tanks should be saturated with the vapor of the solvent used for the chromatography before the plates are developed. Reversed-phase TLC on either bonded or paraffin coated plates has been widely used (Wilson *et al.*, 1981, 1982b; Wilson, 1985). Bonded silicas with various alkyl chain lengths of C_2 to C_{18} (or alternatively, various degrees of coating) are available and all seem

t0010 **Table 2** Methods of analysis and of characterization

Method	*Subtypes*	*Detection methods*	*Comments*	*Selected references*
TLC	1D/2D, AMD	UV/fluorescence quenching		Mayer and Svoboda (1978); Wilson (1985); Wilson and Lafont (1986); Wilson and Lewis (1987); Wilson (1988); Wilson *et al.* (1988b); Báthori *et al.* (2000); Read *et al.* (1990); Wilson *et al.* (1990c); Lafont *et al.* (1993b);
	NP/RP	Color reactions		
	HPTLC	Radioactivity/ autoradiography		
	OPLC	Off-line MS Immuno- or bioassay		
LC	Low-pressure/high-pressure (HPLC)	UV Fluorescent derivatives MS (+ IR, NMR)	Analytical or preparative	Lafont and Wilson (1990); Kubo and Komatsu (1986, 1987); Marco *et al.* (1993); Evershed *et al.* (1993); Wainwright *et al.* (1997); Louden *et al.* (2002)
	NP, RP, ion-exchange, affinity	Radioactivity Immuno- or bioassay		Lozano *et al.* (1988a, 1988b) Warren *et al.* (1986)
SFC		UV MS	Very fast and efficient	Raynor *et al.* (1988, 1989); Morgan *et al.* (1988); Morgan and Huang, 1990
DCCC	Ascending, descending	UV	Preparative	Kubo *et al.* (1985a); Bathori (1998)
CE, MCE		UV	Analytical only	Large *et al.* (1992); Davis *et al.* (1993); Yasuda *et al.* (1993)
GLC	Filled or capillary columns	FID ECD MS	Requires derivatization	Poole *et al.* (1975); Bielby *et al.* (1986); Evershed *et al.* (1987)

TLC, thin-layer chromatography; AMD, automated multiple development; NP, normal-phase; RP, reversed-phase; HPTLC, high-performance thin-layer chromatography; OPLC, over-pressure thin-layer chromatography; SFC, supercritical fluid chromatography; DCCC, droplet counter-current chromatography; CE, capillary electrophoresis; MCE, micellar CE; GLC, gas-liquid chromatography; MS, mass spectrometry; IR, infrared; NMR, nuclear magnetic resonance; FIR, flame ionization detector; ECD, electron capture detector.

suitable for ecdysteroids. MeOH-H_2O, Pr-2-OH-H_2O, EtOH-H_2O, MeCN-H_2O, and Me_2CO-H_2O solvent systems have been used for chromatography; MeOH-H_2O mixtures are most commonly used (Wilson *et al.*, 1981).

s0185 p0180 3.3.3.3.1.1.2. Two-dimensional TLC A given biological sample can contain a very complex mixture of ecdysteroids. In such cases, two-dimensional techniques have proved to be very efficient: e.g., silica plates developed with (1) toluene-Me_2CO-EtOH-25% aqueous ammonia (100:140:32:9 v/v), then (2) $CHCl_3$-MeOH-benzene (25:5:3 v/v) (Báthori *et al.*, 2000).

s0190 p0185 3.3.3.3.1.1.3. Visualization techniques Detection of ecdysteroids on the TLC plate can be accomplished using a variety of techniques of varying specificities. Non-specific techniques include iodine vapors or heating in the presence of ammonium carbonate, which produces fluorescent spots or fluorescence quenching when a fluor (ZnS) is incorporated into the silica. More specific reagents, such as the vanillin-sulfuric acid spray, can be used to give spots of characteristic color (Horn, 1971). A slightly more specific fluorescence reaction than that obtained using ammonia detection can be achieved by spraying the plate with sulfuric acid.

s0195 p0190 3.3.3.3.1.1.4. Scanners Plate analysis can benefit from the wide array of available detectors (scanners) such as UV, UV-Vis, fluorescence, FT-IR, radioactivity, or mass spectrometry (Wilson *et al.*, 1988a, 1990a,c).

s0200 p0195 *3.3.3.3.1.2. General rules for TLC* Ecdysteroid behavior on TLC is directly related to the number and position of free hydroxyl groups and the presence of various substituents. The migration of a given compound is given by its R_f (= reference front), defined as [migration of the substance/migration of the solvent front]. From the R_f value, another parameter can be calculated, the R_m, defined as [$R_m = \log(1/R_f - 1)$]. A single modification of an ecdysteroid results in a change in its R_m, expressed as ΔR_m which is to some extent characteristic of the modification (Koolman *et al.*, 1979).

s0205 p0200 *3.3.3.3.1.3. New TLC techniques* Several methods providing improved resolution when compared with conventional TLC are available.

s0210 p0205 3.3.3.3.1.3.1. Automated or Programmed multiple development (AMD and PMD) In this system, the plate is developed repeatedly with the same solvent, which is allowed to migrate further and further with each development. This method allows a reconcentration of ecdysteroids at each run, in particular by suppressing tailing, and this finally results in sharper bands and improved resolution (Wilson and Lewis, 1987).

s0215 p0210 3.3.3.3.1.3.2. Overpressure thin-layer chromatography (OPLC) In OPLC, the plate is held under an inert membrane under hydrostatic pressure (*ca.* 25 bar) and the solvent is forced through the layer by an HPLC pump. Therefore, the flow of the solvent is controlled and is no longer due to capillarity, and the plate can be developed within minutes. To be efficient, this technique requires the use of HPTLC plates. OPLC has been used with ecdysteroids in only a few instances (Read *et al.*, 1990).

s0220 p0215 **3.3.3.3.2. High-performance liquid chromatography (HPLC)** HPLC is the most popular technique for ecdysteroid separations, both for analytical and preparative purposes. It offers a wide choice of techniques that can be optimized for polar or apolar metabolites. The identification of any ecdysteroid by comigration with a reference compound must rely on the simultaneous use of several (at least two) different HPLC systems, generally one normal-phase (NP) and one reversed-phase (RP) system (reviews: Lafont *et al.*, 1980, 1981; Touchstone, 1986; Lafont, 1988; Thompson *et al.*, 1989; Lafont and Wilson, 1990).

s0225 *3.3.3.3.2.1. Chromatographic procedures*

s0230 p0220 3.3.3.3.2.1.1. Normal phase (NP) systems Silica columns are usually run using mixtures of a chlorinated solvent (e.g., CH_2Cl_2, dichloroethane) and an alcohol (e.g., MeOH, EtOH, Pr-2-OH). Water added to just below saturation partially deactivates silica and this results in more symmetrical peaks; a classical mixture is CH_2Cl_2–Pr-2-OH–H_2O (Lafont *et al.*, 1979). The respective proportions of the three components can be adapted to suit the sample polarity. Thus, specific mixtures can be prepared for nonpolar compounds, e.g., acetates or E precursors (125:15:1 v/v/v), for medium-polarity compounds (125:25:2 or 125:30:2 for E and 20E), or for more polar ecdysteroids (125:40:3 for 26-hydroxyecdysteroids) including glycoside conjugates (100:40:3).

p0225 Polar bonded columns (-diol or -polyol,-APS = aminopropylsilane,) can also be used instead of silica (Dinan *et al.*, 1981). Diol-bonded columns have been used with the phasmid *Carausius morosus* for the separation of a wide array of metabolites (Fournier and Radallah, 1988), whereas the APS phase proved particularly efficient for the separation of mixtures of 3α-OH, 3β-OH, and 3-oxo ecdysteroids (Dinan *et al.*, 1981). In addition, these columns allow the use of gradients without the problems linked with the long re-equilibration times encountered with silica. CH_2Cl_2-based solvents, although very efficient for chromatographic separations, suffer from a high UV-cutoff and the quenching properties of this compound, which preclude diode-array detection or efficient in-line radioactivity monitoring (see below). This problem may be overcome with cyclohexane based mixtures (Lafont *et al.*, 1994a), which also display a different selectivity.

s0235 p0230 3.3.3.3.2.1.2. Reversed-phase systems C_{18}-bonded silicas are the most widely used phases, with elution performed with MeOH-H_2O. MeCN-H_2O or MeCN-buffer mixtures, and are more efficient, especially when polar conjugates and/or ecdysonoic acids are present. The use of ion suppression, e.g., using an acidic buffer, is absolutely essential in the case of polar conjugates or 26-acids. Otherwise, the ionized molecules do not partition well, resulting in variable retention times. Systems have been designed for polar or apolar metabolites, both of which may exist within the same animal. The use of various pHs (Pis *et al.*, 1995a) can modify selectively the retention time of polar (ionizable) metabolites and give access to the pK value of ionizable groups, which can help to characterize conjugates.

s0240 p0235 3.3.3.3.2.1.3. Detection procedures Several types of detectors can be used for the monitoring of ecdysteroids in the eluates.

p0240 *UV detector.* Ecdysteroids possess a chromophore that is strongly absorbing at ~245 nm, which allows the easy detection of 10 pmol amounts.

p0245 *Fluorescence detector.* There have been several attempts to prepare fluorescent derivatives of ecdysteroids. The potential interest is great, as this would enhance the sensitivity of detection by at least 100 times and possibly also the selectivity as compared to UV. Precolumn or postcolumn derivatization can be used. There are several prerequisites for precolumn reactions: (1) they must be simple and give a single derivative for each ecdysteroid; (2) they must be quantitative, even at very low concentrations of

ecdysteroids present in a crude sample; (3) they must be specific; and (4) the excess reagent (if fluorescent itself) must be removed prior to HPLC analysis. Two approaches have been described: the first used phenanthrene boronic acid (Poole *et al.*, 1978), which is specific for diols and would therefore react also with sugars; the second used 1-anthroyl nitrile (Kubo and Komatsu, 1986), which reacts with alcohols, in this case the 2-OH of ecdysteroids. Interest in the second case would essentially be increased sensitivity, but it is not a specific reaction in that the authors use the same reagent for the determination of prostaglandins! These methods have not received further applications, and we must emphasize that the use of precolumn derivatization would negate one major advantage of HPLC over GLC. Postcolumn derivatization could make use of the sulfuric acid-induced fluorescence (see above), but this has not been investigated.

p0250 *Diode-array detectors*. Such detectors represent valuable tools since they provide the absorbance spectrum of all eluted peaks. Thus, in the case of ecdysteroids, whether or not a compound that comigrates with a reference ecdysteroid has a UV absorbance with a maximum at 242 nm can be directly checked. This is an additional criterion for assessing the identity of peaks and it works equally well with very small amounts of ecdysteroids (less than 100 ng).

p0255 *Radioactivity monitors*. On-line radioactivity measurement in ecdysteroid metabolic or biosynthesis studies is of considerable interest because: (1) it provides an immediate result; (2) it saves time and avoids the need for collecting many fractions, filling scintillation vials, and waiting for their measurement; (3) it provides direct comparison with the signal of another detector, e.g., UV monitor, and allows an easy check for the coelution of radioactive peaks with reference compounds; and (4) it does not significantly decrease the resolution when compared with the UV signal: this means that at a flow rate of 1 ml min^{-1} for the column effluent and 3 ml min^{-1} for the scintillation cocktail, with a detector cell size of 0.5 ml, the result is equivalent or even better than collecting 0.2 ml fractions (i.e., 200 tubes for a 40 min analysis).

p0260 *Mass spectrometry (MS)*. On-line HPLC-MS provides useful information on the structure of ecdysteroids eluting from the column, and has become a routine technique thanks to the thermospray and electrospray techniques. Two groups (Evershed *et al.*, 1993; Marco *et al.*, 1993) reported the successful use of HPLC-MS with ecdysteroids. These techniques work in the nanogram range with single ion monitoring (SIM) detection (Evershed *et al.*, 1993). Their use with acetonide derivatives results in a stabilization of the molecules and the production of abundant molecular ions (Marco *et al.*, 1993). A further improvement was obtained with atmospheric pressure chemical ionization (APCI-MS) resulting in a much lower limit of detection – 10 pg with E and 20E (Wainwright *et al.*, 1996, 1997).

p0265 *Nuclear magnetic resonance (NMR)*. Using superheated D_2O as eluent, it has been possible to get on-line proton NMR spectra, but this requires high (100–400 μg) amounts of ecdysteroids to be injected on the column (Louden *et al.*, 2002).

s0275 *3.3.3.3.2.2. Different aims of HPLC*

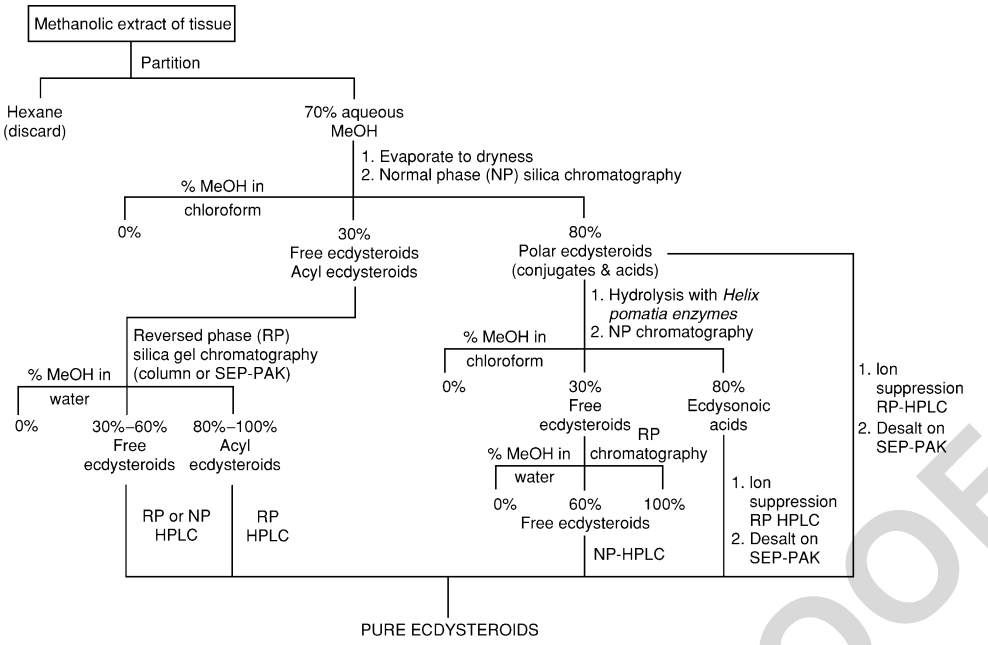
s0280 p0270 3.3.3.3.2.2.1. Analytical and preparative HPLC Columns of different sizes exist with the same packings, and it is therefore very easy to scale up any chromatographic method. Analytical (i.d. 4.6 mm), semi-preparative (i.d. 9.4 mm), and preparative (i.d. *ca.* 20 mm or more) columns are available. By increasing the flow rate in proportion to the cross-section, one obtains similar separations with increased maximal load.

s0285 p0275 3.3.3.3.2.2.2. Microbore HPLC Microbore HPLC has been designed essentially for coupling with mass spectrometry. It uses columns of small internal diameter (e.g., 1 mm) run with a reduced solvent flow rate (*ca.* 50 μl min^{-1}), which is compatible with a direct coupling with a mass spectrometer.

s0290 p0280 3.3.3.3.2.2.3. Quantitative analyses HPLC has been used for the direct quantification of individual ecdysteroids in biological samples. This requires of course high sensitivity because of the low concentrations encountered and adequate sample clean up. Quantification is best obtained if an internal standard is added to the sample either before HPLC analysis, or better, before sample purification (Lafont *et al.*, 1982). Many phytoecdysteroids can be used as internal standards (Wilson *et al.*, 1982a).

p0285 The fundamental place of HPLC in ecdysteroid analysis will be exemplified by a detailed analytical protocol applied to locust eggs (**Figure 7**). A combination of HPLC at various pHs and of enzymatic hydrolyses may indeed allow a rational diagnosis of ecdysteroid types present in a given biological extract (**Table 3**).

s0295 p0290 **3.3.3.3.3. Gas-liquid chromatography (GLC)** This method was first introduced for ecdysteroids by Katz and Lensky (1970), and developed during the following years (Morgan and Woodbridge,



f0035 **Figure 7** A general procedure for the purification of ecdysteroids combining several chromatographic steps. (From Russel, G.B., Greenwood, D.R., **1989**. Methods of isolation of ecdysteroids. In: Koolman, J. (ED.), Ecodyssome. From Chemistry to Mode of Action. George Thieme Verlag, Stuttgart, p. 102. Reprinted by permission.)

t0015 **Table 3** Strategy for a rationale diagnosis of ecdysteroids

Polarity	*Electrically charged (Yes/No)*	*pK_a (if applicable)*	*Susceptibility to hydrolysis (Yes/No)*	*Conclusion*
High		6.5–7.0	Y	Phosphate esters
	Y	4.5	N	26-Oic acids
		2.0	Y	Sulfate esters[a]
			Y	Glucosides
Medium			N	26-OH derivatives
	N	–	N	E, 20E and related
Low			N	Precursors
			Y	Acyl esters

[a]Sulfate esters have not yet been found in insects.

1971; bib0950 Ikekawa *et al*., 1972; bib1725 Miyazaki *et al*., 1973). Despite the advantages possessed by gas-liquid chromatography (GLC) in terms of sensitivity and specificity as compared to other techniques, its use for ecdysteroids has been confined to only a few groups (bib0205 Borst and O'Connor, 1974; bib1755 Morgan and Poole, 1976; bib1325 Koreeda and Teicher, 1977; bib1425 Lafont *et al*., 1980; bib2630 Webster, 1985; bib0115 Bielby *et al*., 1986; bib0510 Evershed *et al*., 1987, 1990; bib0505). As all but a few biosynthetic intermediates of ecdysteroids are nonvolatile, it is necessary to convert them into volatile trimethylsilyl (TMS) ether derivatives. Detection can be carried out using either a flame ionization detector (FID, poor sensitivity), or better still, an electron capture detector (ECD) or a mass spectrometer (MS). In the 1970–1980 period, GC-MS represented a reference method for analyzing ecdysteroid mixtures (bib1425 Lafont *et al*., 1980).

With filled columns (1–3 m long), chromatography is performed on nonpolar stationary phases such as OV-1 and OV-101 coated on Gas Chrom Q and Gas Chrom P. Typical operation temperatures for the columns are in the range of 280 °C, with carrier gas flow rates of 50 ml min^{-1} (Bielby *et al*., 1980, 1986; bib0115). Retention behavior on GLC is the result of both molecular mass and polarity, depending on the degree of silylation. Capillary GC using flexible fused silica columns provides improved resolution and sensitivity. Thus, 10 pg (20E) or 100 pg (E) could be detected by GC coupled with mass spectrometric (SIM) detection (bib0510 Evershed *et al*., 1987). p0295

s0300 p0300 **3.3.3.3.4. Supercritical fluid chromatography** Supercritical fluid chromatography (SFC) uses a supercritical fluid as mobile phase, which behaves like a compressible fluid of very low viscosity, where mass transfer operates very rapidly, allowing the use of high linear velocities and, therefore, very short analysis times without any loss of efficiency. SFC may use either conventional packed HPLC columns or capillary tubes, and the mobile phase is generally supercritical CO2 containing a small amount of an organic modifier, e.g., MeOH (Morgan *et al.*, bib1750 bib1950 bib1955 1988; Raynor *et al.*, 1988, 1989). Chromatographic parameters that can be monitored are the temperature, the pressure, and the percentage of the organic modifier. Supercritical CO_2 is a nonpolar fluid and, therefore, SFC is more or less equivalent to normal-phase HPLC; the eluting power is increased by adding MeOH. Various types of packed columns can be used, including silica or bonded silica; alternatively, fused silica capillary columns can be used (Raynor *et al.*, 1988). A major advantage of SFC bib1950 over HPLC is the shorter retention times; compounds elute as sharp peaks and the sensitivity of detection is accordingly increased. A second advantage concerns the high transparency of CO_2 in the infrared (IR), which allows the use of FT-IR detectors. Furthermore, SFC is compatible with both flame ionization detection (the universal detection used with GLC) and mass spectrometry. No derivatization is required, and this represents an important advantage over GLC.

s0305 p0305 **3.3.3.3.5. Capillary electrophoresis (CE)** Capillary electrophoresis is a recently introduced technique that allows extremely efficient separation of peptides, oligonucleotides, and also steroid hormones. The migration of substances is due to a combination of electroendosmotic flow of buffer towards the cathode and the (slower) migration of negatively charged solutes towards the anode. CE gives very efficient separations for a wide range of ionized and unionized compounds. As most of the ecdysteroids are not ionizable, CE has to be performed using some form of micellar CE (MCE, also known as MECK or micellar electrokinetic chromatography). In this mode, a surfactant such as sodium dodecyl sulfate (SDS) is added to the buffer at a concentration above its critical micellar concentration. The compounds of interest then partition between the aqueous phase and the micelles as these are drawn through the capillary by the electroosmotic flow. Such an approach has been shown to be well suited to the CE of ecdysteroids. Two types of methodology have been used in CE: with SDS and an organic modifier such as MeOH (Large *et al.*, 1992; bib1500 bib0390 Davis *et al.*, 1993); or with SDS and γ-cyclodextrin as modifiers (Yasuda *et al.*, 1993). CE has been bib2860 used for extracts from the eggs of *Schistocerca gregaria* (Davis *et al.*, 1993) and crayfish hemolymph bib0390 bib2860 (Yasuda *et al.*, 1993). The sensitivity of MCE for ecdysteroids was very good and the on-column detection of *ca.* 175 pg of ecdysone was readily achieved. In fact, due to the very small volume that can be injected on the column (5 nl), this corresponds in practice to a much larger sample containing 35 μg ml^{-1} (i.e., a 10 μl sample would have to contain 350 ng ecdysteroid).

3.3.3.4. Methods of Quantification

s0310

One can use the physicochemical techniques as discussed above (see **Table 4**) or, alternatively, bioassays and immunoassays (**Table 5**) performed either on crude extracts or on fractions collected after a chromatographic separation (TLC, HPLC).

p0310

s0315 p0315 **3.3.3.4.1. Bioassays** Various kinds of bioassays are used for testing ecdysteroids from animal or plant extracts, either *in vivo* or *in vitro*. Their purpose may be either the measurement of ecdysteroid concentrations in biological samples (they allowed the isolation of E by Butenandt and Karlson, 1954), bib0240 to analyze the relative biological activity of various ecdysteroids (structure–activity relationships), or to screen plant extracts for ecdysteroid content in a semiquantitative way.

p0320 We may distinguish between assays where disturbances or toxic effects are assessed (e.g., abnormal molting), and bioassays designed to measure a biological activity linked to a normal physiological process or even to quantify hormones. The naturally occurring ecdysteroids exhibit a wide range of molting hormone activities when measured in bioassay systems. Bioassay systems for molting hormones have tended to be based on the larval–pupal molt of a number of dipteran species (e.g., *Calliphora*, *Sarcophaga*, and *Musca*). In these assays, last instar larvae are ligated and those showing no further development of the abdomen are selected for use in the bioassay. Extracts of (pure) compounds are then injected into the abdomen and signs of development (tanning of the cuticle) are sought. The sensitivity of the assay varies depending on the dipteran species used and the assay conditions: in *Calliphora* it is of the order of 5–50 ng per abdomen for 20E versus 5–6 ng for *Musca*. A variation on the rather specialized larval–pupal molt used in the dipteran bioassays is the locust abdomens of fourth instar *S. gregaria* and is based on a larval-to-larval molt. Another useful bioassay, which does not require the injection of the test material, is based on the use of

t0020 **Table 4** Physicochemical methods of quantification: characteristics and limits of detection

Method	*Limits of detection*	*Specificity*	*References*
Direct or after a chromatographic separation			
UV absorbance (*ca.* 242 nm)	1 μg	Poor	bib2095 Sardini and Krepinsky (1974)
Chugaev's color reagent (380 nm)	<10 μg	Limited	bib1195 bib1200 bib0190 Kholodova (1977, 1981); Bondar *et al.* (1993)
Sulfuric acid induced fluorescence	10 pg	Limited	bib0680 bib1270 Gilgan and Zinck (1972); Koolman (1980)
Bioassay	*ca.* 5 ng	Good	bib0350 Cymborowski (1989)
Immunoassay	5–10 pg	(Very) good	bib1945 Porcheron *et al.* (1989)
TLC			
UV absorbance (scanner)	500 ng	Low	bib2790 bib0070 Wilson *et al.* (1990c); Báthori and Kalász (2001)
Fluorescence quenching (fluorescent TLC plates)	500 ng	Low	bib1665 Mayer and Svoboda (1978)
Mass spectrometer			
HPLC			
UV absorbance	<10 ng	Fair	bib1475 bib2845 Lafont *et al.* (1982); Wright and Thomas (1983)
Fluorescence (requires derivatization)	<10 pg	?	bib1930 bib1340 bib1345 Poole *et al.* (1978); Kubo and Komatsu (1986, 1987)
Mass spectrometer	50–100 pg	Very good	bib0520 bib1520 Evershed *et al.* (1993); Le Bizec *et al.* (2002)
GLC			
Flame ionization detector	50 ng	Poor	bib1150 Katz and Lensky (1970)
Electron capture detector	1–10 pg	Good	bib1925 bib1935 bib0115 Poole *et al.* (1975, 1980); Bielby *et al.* (1986)
Mass spectrometer	10–100 pg	Very good	bib0510 Evershed *et al.* (1987)

ligated larvae of *Chilo suppressalis* (bib2105 Sato *et al.*, 1968). These are simply dipped into methanolic solutions of the test compound of extract. An *in vitro* variation of this assay uses pieces of integument from ligated larvae of *Chilo* and has a reported sensitivity of *ca.* 15 ng per test (bib0030 Agui, 1973). In all *in vivo* test systems, it is difficult to assess whether the observed biological activity derives from the injected compound or an active metabolite. An obvious example of this is the conversion of 2dE and E into the more active metabolite, 20E (as shown by comparison with their activity in *in vitro* bioassays). The same considerations may apply to 3-oxo (3-dehydro) ecdysteroids.

p0325 *In vitro* systems, where it is possible to determine the biological activity of a compound in the quasi-absence of its metabolism, are therefore very useful for the determination of structure–activity relationships. Among them, a microplate bioassay using a *Drosophila* cell line (B II) was designed by bib0330 Clément and Dinan, 1991 and bib0325 Clément *et al.*, 1993 with good sensitivity and accuracy. This assay relies on a turbidimetric measurement of cell density in a system where ecdysteroids inhibit cell proliferation. More recently, new methods have been proposed, which use either *Drosophila* Kc cells transfected with a reporter plasmid containing the 5′ region of the *hsp27* promoter and the firefly luciferase gene (bib1680 Mikitani, 1995) or SL2 cells with the addition of an EcR expression plasmid (bib0050 Baker *et al.*, 2000). Cells were cultured for 24 h in the presence of ecdysteroids, and then cell lysates were used to measure luciferase activity.

p0330 A number of *in vitro* bioassay systems exist, including puffing of polytene chromosomes (reviews bib0085 Bergamasco and Horn, 1980; Cymborowski, bib0085 1989) and morphogenesis in imaginal discs. As Bergamasco and Horn (1980) pointed out, in bioassays where quantitative data are sought, the purity of the sample, especially the absence of other active ecdysteroids, is of paramount importance.

s0320 p0335 **3.3.3.4.2. Immunoassays** Immunoassay (IA) use antibodies obtained by immunization with ecdysteroids. As ecdysteroids are not directly immunogenic, they must be coupled to a suitable protein before immunization. Generally, they are derivatized to create a carboxylic function which can react with the amino groups of protein carriers (BSA, HSA or thyroglobulin). The protein–ecdysone complex is strongly immunogenic and high titers of antibodies are generally elicited after the first injection.

p0340 The specificity of antibodies is rarely absolute, as they generally will recognize both E and 20E, as well as a series of closely related ecdysteroids. The degree of hapten binding by antibodies is primarily determined by the structure of the immunogen, especially as regards the position of the linkage between the ecdysteroid and the protein. For instance, protein esterification via the C_2 hydroxyl on the A-ring or the C_{22} hydroxyl on the side chain will generally result in antisera specificity against ecdysteroid ligands exhibiting molecular changes in either the side chain or the steroid

t0025 **Table 5** Bioassays and immunoassays

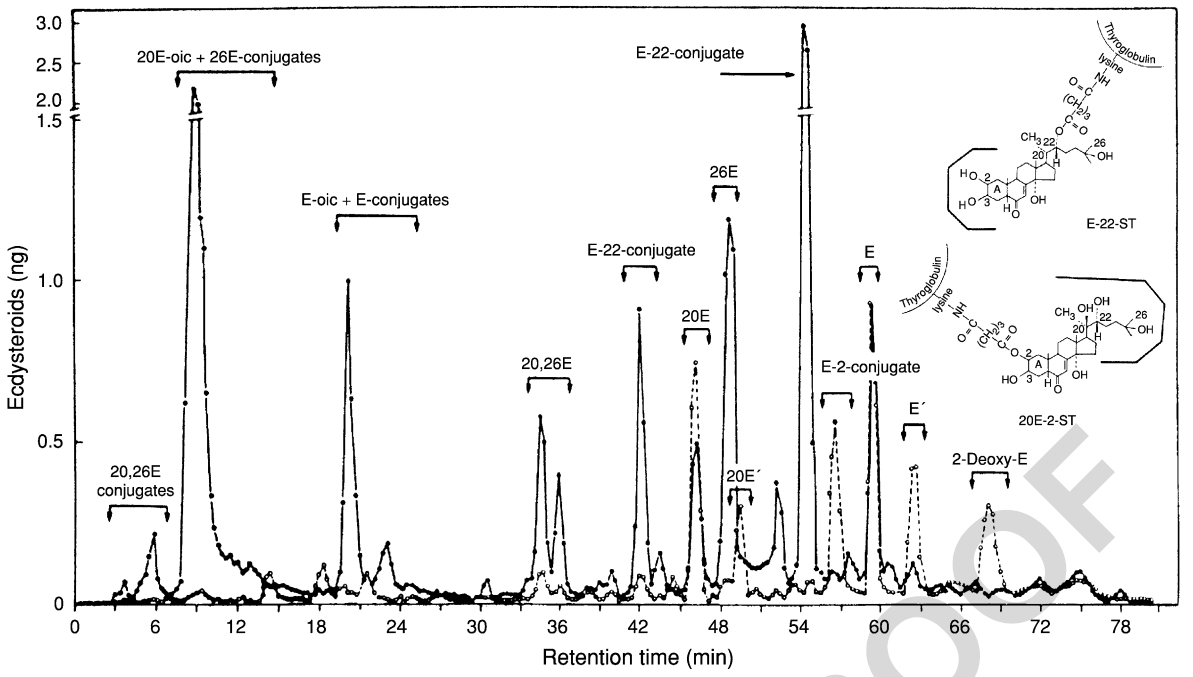
Method	*Subtypes*	*Detection modes*	*Comments*	*References*
Bioassays			Coupling with a separation method enhances the informative power	Review: Cymborowski (1989)
	In vivo	Induction of molting or development in hormone deprived systems	Fundamental role for ecdysone isolation; still used for some SAR[a] studies	*Calliphora* test: Adelung and Karlson (1969); Karlson and Shaaya (1964); Thomson *et al.* (1970); Fraenkel and Zdarek (1970) *Musca* test: Kaplanis *et al.* (1966) *Sarcophaga* test: Ohtaki *et al.* (1967) Diapausing *Samia cynthia* test: Williams (1968) *Chilo* dipping test: Sato *et al.* (1968); Koreeda *et al.* (1970) *Limulus* bioassay: Jegla and Costlow (1979) Blowfly abdominal bags: Agrawal and Scheller (1985) *Galleria* test: Sláma *et al.* (1993) *Dermestes* test: Sláma *et al.* (1993)
	In vitro	Organ development		Integument pieces: Agui (1973) Imaginal discs: Kuroda (1968); Oberlander (1974); Terentrou *et al.* (1993)
		Cell proliferation/ differentiation (s.l.)	SAR studies	Kc cells: Cherbas *et al.* (1980) Bll cells: Clément and Dinan (1991); Clément *et al.* (1993)
		Chromosome puffing	SAR studies	Richards (1978)
		Reporter gene (e.g., luciferase or GFP) activation	SAR studies	Mikitani (1995); Baker *et al.* (2000); Swevers *et al.* (2004)
		Radio receptor assay	Uses *Drosophila* Kc cells cytosol as receptor preparation	Mikitani (1996)
Immunoassays			Coupling with a separation method enhances the informative power	Reviews: Warren and Gilbert (1988); Reum and Koolman (1989)
	RIA	Radioactivity	Radioactive tracer required	Borst and O'Connor (1972)
	CLA	Chemiluminescence	Chemiluminescent tracer required	Reum *et al.* (1984)
	EIA	Colorimetry	Enzymatic tracer required	Porcheron *et al.* (1989); Royer *et al.* (1995)
	ELISA	Colorimetry	No tracer required	Kingan (1989)

[a]SAR, structure-activity relationship.

nucleus, respectively (**Figure 8**) (Warren and Gilbert, 1988).

p0345 Immunoassays need tracer molecules. In Radioimmune Assay (RIA), tracers are radiolabeled with a high specific activity. Two kinds of analogs are used: commercially available [^{3}H]-ecdysone (50–80 Ci/mmol), and laboratory-made iodinated analogs (up to 2000 Ci/mmol), which provide a higher sensitivity (Hirn and Delaage, 1980). The RIA has several drawbacks due to the utilization of a radioisotope, which requires special precautions, and for that reason other immunoassays have been developed: a chemilumino-immunoassay (CIA), which uses ecdysone-6-carboxy-methoxime aminobutylethyl-isoluminol (ABEI) to form a chemroluminescent tracer (Reum *et al.*, 1984), and enzyme immunoassays (EIA) with an ecdysone derivative coupled to an enzyme, acetylcholinesterase (Porcheron *et al.*, 1989; Royer *et al.*, 1993) or peroxidase (Delbecque *et al.*, 1995; Von Gliscynski *et al.*, 1995).

After incubation of antibodies, tracer, and p0350 biological extract (or calibration references), the separation of free and bound fractions is performed in various ways (Reum and Koolman, 1989). The lower limit of sensitivity was estimated, in the best cases, as 4.10^{-10} mol (1.6 pg) (Hirn and Delaage,



f0040 **Figure 8** RP-HPLC/RIA analysis of ecdysteroids from the gut of females during day 14 of pupal–adult development in *M. sexta*. The chromatograms show the elution from a C_{18}-silica column by a neutral buffered water/acetonitrile system of a female gut sample. Analysis is by differential RIA employing two complimentary antibodies. One antibody was elicited by an ecdysone-22-succinylthyroglobulin immunogen conjugate (E-22-ST) where the ecdysteroid hapten was conjugated via its C_{22}-hydroxyl group to the immunogenic carrier protein (solid line). The other antibody was elicited by a 20-hydroxyecdysone-2-succinylthyroglobulin immunogen conjugate (20E-2-ST) where the ecdysteroid was conjugated via the C_2 hydroxyl group (dashed line). In general, substrate discrimination by antibodies is greatest for molecules that differ in their architecture at a place far from the point of hapten attachment to the immunogenic protein carrier molecule. E′: 3α-epiE, 20E′, 3α-epi20E; 26E, 26-hydroxyE; 20,26E, 20,26-dihydroxyE; E-oic, ecdysonoic acid; 20E-oic, 20-hydroxyecdysonoic acid; E-22- (or E-2)-conjugate, E conjugated at the 22 (or 2) position. (Modified from Warren, J.T., Gilbert, L.I., **1986** Ecdysone metabolism and distribution during the pupal-adult development of *Manduca sexta*. *Insect Biochem. 16*, 65–82).

1980) but, in fact, the sensitivity currently achieved by most ecdysteroid immunoassays is in the range of 20–200 fmol (10–100 pg). An example of RIA performed after an HPLC separation is given in **Figure 8**.

s0325 **3.3.3.5. Sources of Ecdysteroids**

p0355 Only a few ecdysteroids are commercially available. All of them have been isolated from plants and they include only four insect ecdysteroids: 2d20E, E, 20E, and makisterone A. Because of its use as an anabolic substance by many bodybuilders (Lafont bib1450 and Dinan, 2003), 20E can now be purchased in large quantities at a low price. When an ecdysteroid other than the four noted above is required, there are three ways in which it can be obtained: (1) it can be requested from someone who has already isolated (or synthesized) this compound; (2) the molecule can be isolated from an adequate plant source; or (3) it can be synthesized from an available precursor. This does not always require complicated chemistry; **Table 6** lists some syntheses that are not highly complex and can be performed in the absence of an experienced chemist.

3.3.4. Ecdysteroid Biosynthesis s0330

3.3.4.1. Sterol Precursors and Dealkylation Processes s0335

p0360 Owing to the lack of a squalene synthase (and/or other enzymes), insects are unable to synthesize sterols bib0320 bib2345 *de novo* (Clayton, 1964; Svoboda and Thompson, 1985). Consequently, they rely on dietary sterols for their supply. Zoosterols are essentially cholesterol; thus, insects feeding on animal derived food get cholesterol directly. On the other hand, those feeding on plants obtain mainly phytosterols, which differ from cholesterol by the presence of various alkyl substituents (ethyl, methyl, methylene) on carbon 24. However, it should be noted that most plants also contain small but detectable amounts of cholesterol. Nevertheless, many, but not all, phytophagous insect species possess the ability to first dealkylate phytosterols to cholesterol, which is subsequently

t0030 **Table 6** Just a little bit of chemistry: total or partial synthesis (side-chain modifications) and simple modifications (e.g., oxidation at C-3, removal of -OH groups (e.g., 25-OH), conjugation to acetic acid, fatty acids, glucose, sulfate)

Starting material	*Derivative*	*References*
20E	3D20E	Koolman and Spindler (1977); Spindler *et al.* (1977); Dinan and Rees (1978); Girault *et al.* (1989)
	20E′	Dinan and Rees (1978)
	Ponasterone A	Dinan (1985)
	Inokosterone	Yingyongnarongkul and Suksamrarn (2000)
	Acetates	Horn (1971)
	Glucosides	Pis *et al.* (1994)
	Poststerone	Hikino *et al.* (1969); Petersen *et al.* (1993)
	Rubrosterone	Hikino *et al.* (1969)
	2d20E	Suksamrarn and Yingyongnarongkul (1996)
	14d20E	Harmatha *et al.* (2002)
E	3DE	Spindler *et al.* (1977); Dinan and Rees (1978); Girault *et al.* (1989)
	E′	Dinan and Rees (1978)
	25dE	Pis *et al.* (1995b)
	22-Acyl esters	Dinan (1988)
	Sulfates	Pis *et al.* (1995a)
2dE	2,25dE	Pis *et al.* (1995b)
	2,22dE	Kumpun *et al.* (in preparation)

converted in endocrine tissues to the active C_{27} molting hormone 20E. By contrast, some species, mainly Hemiptera and Hymenoptera, are unable to dealkylate phytosterols and consequently produce 24-alkylated ecdysteroids (i.e., C_{28} makisterone A and/or C_{29} makisterone C; Feldlaufer *et al.*, 1986, Feldlaufer, 1989; Mauchamp *et al.*, 1993).

p0365 In some cases, due to a combination of specific mechanisms including (1) selective concentration of the dietary cholesterol (Thompson *et al.*, 1963; Svoboda *et al.*, 1980) and/or (2) its selective delivery to steroidogenic organs, the insect can produce mainly C_{27} ecdysteroids even if cholesterol is a very minor sterol component of its diet. The ecdysteroid pool usually represents only 0.1–0.2% of total sterols, as illustrated by the low conversion rate of radioactive cholesterol when injected *in vivo* for long-term labeling studies (e.g., Beydon *et al.*, 1981, 1987).

p0370 Finally, some insects have become independent of a dietary sterol supply thanks to the production of sterols by yeast-like or fungal symbionts; this situation has been described in several insect species, particularly in aphids and beetles (Svoboda and Thompson, 1985). Yeast-like symbionts provide mainly 24-alkylated sterols (e.g., ergosta-5,7,

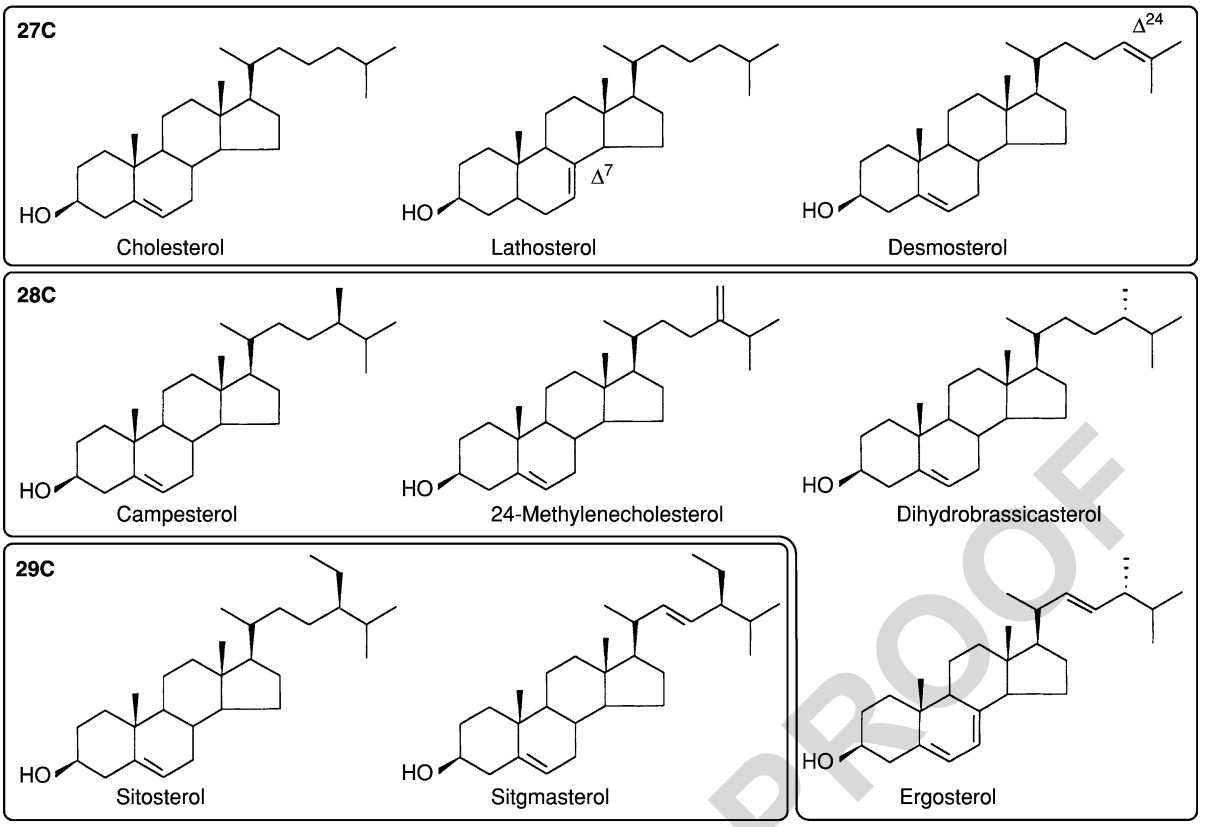
t0035 **Table 7** Relationship between dietary sterols and ecdysteroid structures

Dietary sterols	*Dealkylation*	*Major ecdysteroid*
27C	Indifferent	20-Hydroxyecdysone (or Ponasterone A)
28C/29C	+	20-Hydroxyecdysone
28C	−	Makisterone A (or 24-epi-Makisterone A)
29C	−	Makisterone C

24(28)-trienol). Thus, those insects still require a dealkylation process in order to produce cholesterol (Weltzel *et al.*, 1992; Nasir and Noda, 2003). According to the nature of their dietary sterols and their ability to dealkylate phytosterols, insects produce different ecdysteroids (**Table 7**).

s0340 p0375 **3.3.4.1.1. General scheme of cholesterol formation from phytosterols** A given species usually contains a complex sterol cocktail, with a few major compounds and many minor ones. Sterol diversity concerns (1) the number of carbon atoms (27 to 29) as discussed above and (2) the number and position of unsaturations, classically termed Δ: mainly Δ^5 and Δ^7 on the steroid nucleus, and Δ^{22} and Δ^{24} on the side-chain (**Figure 9**). In addition, sterols can be in free or conjugated form, i.e., sterol acyl esters with various fatty acids or sterol acylglucosides, where a sugar moiety is linked both to the 3β-OH of the sterol and to a fatty acid (Eichenberger, 1977; Grunwald, 1980).

p0380 Ingested sterols are subjected to the action of several types of enzymes: (1) hydrolytic enzymes (esterases or glycosidases) from the insect gut (see **Chapter 4.5**), which will release free sterols from their conjugates; (2) reductases and/or oxidases; and (3) enzymes involved in the so-called "dealkylation process." Reactions (2) and (3) have been investigated extensively from the late 1960s by a combination of several strategies: feeding experiments with diets containing one particular sterol; *in vivo* metabolic studies with [^{3}H] or [^{14}C] labeled sterols allowing the isolation of labeled intermediates; in vitro enzymatic studies using isolated tissues or cell-free preparations; and observing the effects of various pharmacological inhibitors of reactions known in mammals/humans (i.e., hypocholesterolemic agents). All the biochemical pathways have been fully elucidated, at least with regards to the metabolism of the most common Δ^5 plant sterols (reviews: Fujimoto *et al.*, 1985b; Svoboda and Thompson, 1985; Svoboda and Feldlaufer, 1991; Svoboda and Weirich, 1995; Svoboda, 1999), and are summarized in **Figure 10**.

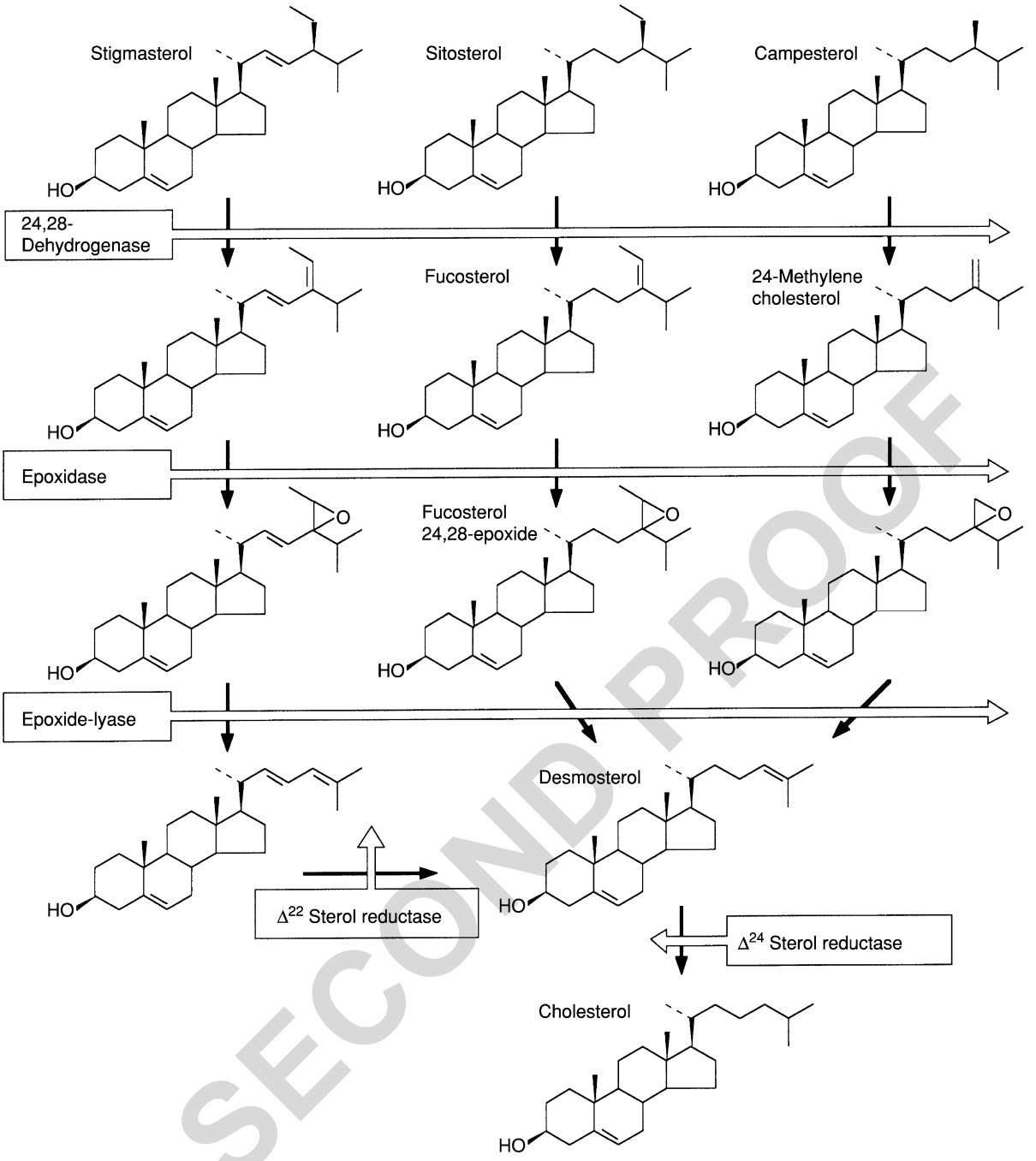


f0045 **Figure 9** Structures of cholesterol and of representative phytosterols.

s0345 p0385 **3.3.4.1.2. Dealkylating enzymes** The first evidence for dealkylation of ergosterol into Δ^{22}-cholesterol was obtained with the cockroach *Blatella germanica* (bib0305 Clark and Bloch, 1959). Further work, performed in particular with *Manduca sexta* and *Bombyx mori* larvae, allowed the identification of all intermediates. Four to five enzymes are necessary to dealkylate phytosterols. The first three reactions involve the formation of an epoxide intermediate, and the removal of the side chain is accompanied by the formation of a Δ^{24} bond, thus forming desmosterol or its $\Delta^{22,24}$ analog (**Figure 10**). The second part (one or two steps) concerns the reduction of the side chain. When starting for instance from sitosterol or campesterol, the dealkylation product (desmosterol) bears a single (Δ^{24}) double bond, and can be reduced to cholesterol by most insect species analyzed so far. In the case of insects fed on Δ^{22} sterol (e.g., stigmasterol or ergosterol), the dealkylation product bears a 22,24-diene and two reductases are required. It has been shown that the reduction at C_{22} must precede that at C_{24} and cannot be performed by all insect species (bib2345 Svoboda and Thompson, 1985).

p0390 It was expected that dealkylation takes place in the insect gut. Indeed, cell-free extracts prepared from larval silkworm guts when incubated with adequate intermediates resulted in the formation of desmosterol and cholesterol (bib0610 Fujimoto *et al*., 1985b). Midgut microsomes are the most active source of enzymes (e.g., *Spodoptera littoralis*, bib0315 Clarke *et al*., 1985), but midgut is not the only active tissue in this respect (bib0045 Awata *et al*., 1975). Unfortunately, most of these early experiments were discontinued, and as yet none of these enzymes has been fully characterized.

p0395 The desmosterol 24-reductase is present both in insects and vertebrates, catalyzing the last step in cholesterol biosynthesis. There seems to be a significant structural conservation, as the insect enzyme can be inhibited by many hypocholesterolemic agents used in humans to reduce endogenous cholesterol biosynthesis. Indeed, azasteroids and various amines are also active on the insect enzyme and will consequently inhibit insect development (bib2355 Svoboda and Weirich, 1995), but they cannot be used as insecticides due to their phytotoxicity. Moreover, there is significant sequence conservation between the human and nematode proteins and even human and plant sequences share 30% identity (bib0300 Choe *et al*., 1999; bib2605 Waterham *et al*., 2001). Unfortunately, available data on insect genomes concern



f0050 **Figure 10** From phytosterols to cholesterol: the dealkylation processes.

species that are believed to be unable to dealkylate phytosterols and thus would lack the corresponding enzyme.

s0350 p0400 **3.3.4.1.3. Variations among insect species** Not all phytophagous species that can dealkylate phytosterols use the pathway described in **Figure 10**. Thus, the Mexican bean beetle, *Epilachna varivestis*, first totally reduces sitosterol (or stigmasterol) to stigmastanol, which is then dealkylated to cholestanol and the latter is then dehydrogenated to lathosterol (Δ^7), the major sterol found in this species (Svoboda *et al.*, 1975). This example highlights the importance of unsaturations present in the steroid nucleus. In some insect species, Δ^7 or $\Delta^{5,7}$ compounds can be the major sterols (Svoboda and Lusby, 1994; Svoboda, 1999), while *Drosophila pachea* has evolved specific requirements for ingesting Δ^7

sterols in order to produce E (Heed and Kircher, 1965; Warren *et al*., 2001; see Section 3.3.4.2.1). In summary, insects' ability to modify sterol nucleus unsaturations differs greatly between species (Rees, 1985).

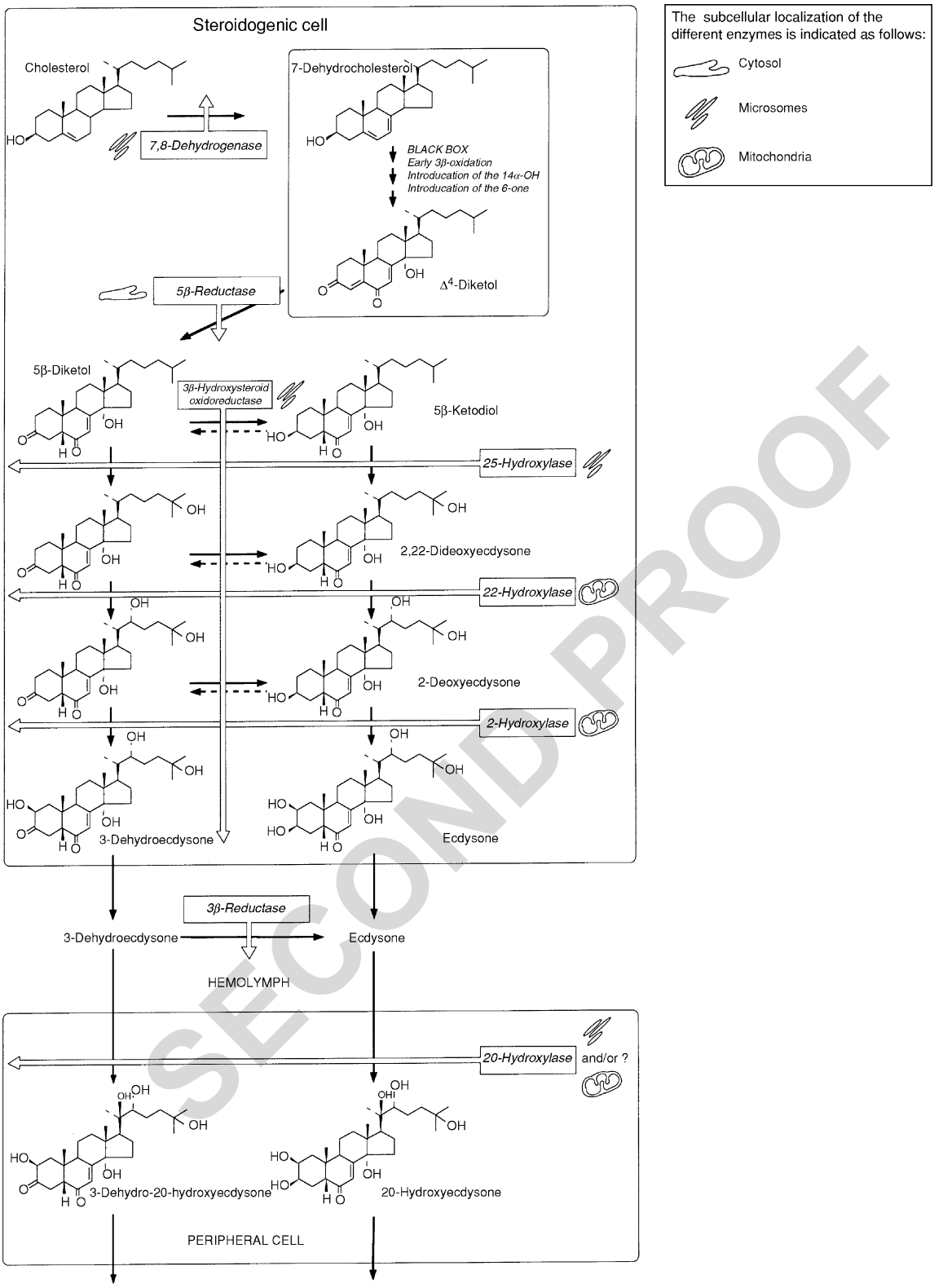
p0405 In some instances, species unable to dealkylate phytosterols may adapt their physiology to the nature of their sterol diet, e.g., *Drosophila melanogaster*. Apparently unable to dealkylate phytosterols (Svoboda *et al*., 1989), *D. melanogaster* produces a mixture of 20E and makisterone A, the proportions of which reflect the composition of its sterol diet (Redfern, 1986; Feldlaufer *et al*., 1995; Pak and Gilbert, 1987). This strategy can operate because in this species E receptors have a very similar affinity for both ecdysteroids (Dinan *et al*., 1999; Baker *et al*., 2000) (37). Bees (Hymenoptera) seem to follow a similar scheme (Svoboda *et al*., 1983; Feldlaufer *et al*., 1986). Leafcuttier ants appear to be unique in that they feed mainly on fungal sterols, which differ from plant sterols in their stereochemistry at C_{24}. As a result, they produce 24-epimakisterone A instead of makisterone A (Maurer *et al*., 1993). More generally, different enzymatic machinery and/or ecdysteroid receptor specificity among insects will result in different sterol requirements. These are revealed by feeding experiments on defined diets and so represent an aspect of the adaptation of insects to specific foods.

s0355 ### 3.3.4.2. The Biosynthetic Pathway of Ecdysteroids from Cholesterol

p0410 Although it has received considerable attention (see reviews by Rees, 1985, 1995; Warren and Hétru, 1990; Sakurai and Gilbert, 1990; Grieneisen, 1994; Dauphin-Villemant *et al*., 1998; Gilbert *et al*., 2002), the biosynthetic pathway of ecdysteroids is still not understood completely. Biochemical approaches focusing on the nature of ecdysteroid synthetic intermediates have long formed the bulk of the results. However, unlike vertebrate steroidogenesis, where all the metabolites between cholesterol and the various active steroid hormones have been isolated and identified, no intermediate between the initial dehydrogenation product of cholesterol (7-dehydrocholesterol, 7dC) and the first recognizable ecdysteroid-like product, the 5β-ketodiol (2,22, 25-trideoxyecdysone, 2,22,25dE) has ever been observed, much less identified. These are the reactions that comprise the mysterious "black box" of Dennis Horn (**Figure 11**). During invertebrate steroidogenesis, biosynthetic intermediates do not accumulate, perhaps because the proposed multistep oxidations of 7dC are either very efficient or the resulting compounds are very unstable, or both. A great step towards elucidating the synthetic mechanism in the insect was made when it was finally determined that the actual secreted product of lepidopteran prothoracic glands (Warren *et al*., 1988a,b; Sakurai *et al*., 1989; Kiriishi *et al*., 1990) and of most other insect prothoracic glands and crustacean Y-organs (Spaziani *et al*., 1989; Böcking *et al*., 1994) (see **Chapter 3.16**) was in fact not E but 3-dehydroecdysone (3DE). This finding greatly strengthened existing proposals of the early intermediacy of a 3-dehydro-Δ^4-sterol in arthropod ecdysteroidogenesis (Davies *et al*., 1981; Rees, 1985; Grieneisen *et al*., 1991), as it is a rather common moiety in mammalian steroidogenesis (Brown, 1998). However, the precise step at which the 3β-hydroxyl groups of early sterol ecdysteroid precursors become oxidized (dehydrogenated) to the ketone remains mystery. The recent molecular identification in *Drosophila* of the cytochrome P450 enzymes (CYP) catalyzing the terminal hydroxylation reactions at C_{25}, C_{22}, C_2, and C_{20} has provided new tools for studying these unresolved questions in ecdysteroid biosynthesis (see Section 3.3.4.3.1).

s0360 p0415 **3.3.4.2.1. Cholesterol 7,8-dehydrogenation** Early feeding studies showed that 7dC could support insect growth in the absence of C or phytosterols, suggesting that this compound was an early intermediate in ecdysteroid biosynthesis (Horn and Bergamasco, 1985). Subsequent kinetic studies revealed that 7dC (**Figure 11**) was the first intermediate in the formation of either E or 3DE from C, both in insects and crustaceans (Milner *et al*., 1986, Warren *et al*., 1988a; Grieneisen *et al*., 1991, 1993; Rudolph *et al*., 1992; Böcking *et al*., 1993, 1994; Warren and Gilbert, 1996). Based on labeling experiments with radioactive precursors, the mechanism of cholesterol 7,8-dehydrogenation is known to involve the stereospecific removal of the 7β- and 8β-hydrogens from the more sterically hindered "top" (β-surface) of the planar C molecule (Rees, 1985). In several arthropod species, it was shown that the reaction is basically irreversible (Grieneisen, 1994) and is catalyzed by a microsomal CYP enzyme. This assumption was based on its biochemical characteristics, such as enzyme kinetics, subcellular localization, requirement for NADPH, or inhibition by CO and fenarimol (Grieneisen *et al*., 1991, 1993; Warren and Gilbert, 1996).

p0420 Although its activity may become substrate limited, the cholesterol 7,8-dehydrogenase does not appear to be the rate-limiting enzyme in the biosynthesis of ecdysteroids. In many insect species, including *Manduca* (Warren *et al*., 1988a; Grieneisen *et al*., 1991) and *Bombyx* (Sakurai *et al*., 1986), the



f0055 **Figure 11** Putative biosynthetic pathway of ecdysteroids.

prothoracic glands always contain considerable amounts of both C and 7dC, even though both sterols undergo wild contralateral fluctuations during E biosynthesis, while in a few insect species, i.e., *Tribolium confusum, Atta cephalotes,* and *Acromyrmex octospinosus*, there is a clear preponderance of $\Delta^{5,7}$ sterols in the whole body (Grieneisen, 1994). In crabs, a ratio of 7dC:C ranging from 1:20 to 1:100 in the hemolymph was observed, while in the molting glands, the ratio was closer to 1:1 (Lachaise *et al*., 1989; Rudolph *et al*., 1992).

p0425 In the desert fly, *D. pachea*, the 7,8-dehydrogenase reaction is completely absent (Warren *et al*., 2001) and so it depends on an alternative source of 7dC. It lives on a single species of cactus that supplies it with the Δ^7-sterol lathosterol, which it alternatively dehydrogenates to 7dC (Goodnight and Kircher, 1971), i.e., similar to the synthesis of 7dC in vertebrates (Brown, 1998). Like *D. pachea*, the "low E" *Drosophila* mutant, *woc,* also shows an impairment of 7,8-dehydrogenase activity (Warren *et al*., 2001). However, in *woc*, while late third instar larval ecdysteroid production and subsequent larval–pupal metamorphosis can be rescued by the simple feeding of 7dC, continued normal development, as in *D. pachea*, is not observed in *woc* as the animals die as early pupae (see Section 3.3.5.4.3).

p0430 The 7,8-dehydrogenation of sterols appears not to have strict substrate requirements in arthropods. Cholesterol, along with the primary phytosterols campesterol, sitosterol, and stigmasterol (Sakurai *et al*., 1986), as well as the more polar synthetic substrate, 25-hydroxycholesterol (25C) (Böcking *et al*., 1994; Warren and Gilbert, 1996; Warren *et al*., 1996, 1999, 2001), are all efficiently converted into their respective 7-dehydro derivatives, both *in vivo* and *in vitro*. This is also the case for the unusual oxysterols α-5,6-epoxycholesterol (αepoC) and α-5,6-iminocholesterol, but not for their respective β-isomers (which, nevertheless, do function as competitive inhibitors of this reaction; (Warren *et al*., 1995). Only the former substrates were efficiently converted into either α-5,6-epoxy7dC, an oft-mentioned prospective "black box" intermediate (Rees, 1985) and a potentially potent alkylating agent (Nashed *et al*., 1986), or α-5,6-imino7dC, respectively. Indeed, a 46 kDa protein became increasingly radiolabeled following the incubation of ^{3}H-αepoC with *Manduca* prothoracic gland microsomes. Unfortunately, the resultant tagged product–enzyme adduct has so far proved too unstable for a classical purification and sequencing of the cytochrome. Nevertheless, the substrate specificity of the CYP enzyme for the α-isomer over the β-isomer again suggests a mechanism involving an attack by the cytochrome on the β-face of the molecule.

Even if the precise mechanism of action of the p0435
enzyme, as well as its structural characteristics, are not yet known, many studies have shown that CYPs can catalyze a great diversity of reactions, including dehydrogenation reactions. In vertebrates, for instance, dehydrogenation of the alkane CH-CH bond from testosterone is catalyzed by a CYP. The P450-dependent oxidation of this substrate not only leads to a dehydrogenated metabolite, but also to a hydroxylated metabolite, the latter not being a metabolite of the former (Mansuy, 1998). A similar mechanism might be envisioned for cholesterol 7,8-dehydrogenation, although the formation of such a hydroxylated C derivative has not been observed in insects. Nevertheless, one such possible intermediate (7β-hydroxycholesterol) did significantly inhibit the conversion of radiolabeled C to either 7dC or E (Grieneisen *et al*., 1991), but precise chemical studies are needed with a recombinant enzyme when it becomes available.

3.3.4.2.2. Early steps after the formation of 7dC: the "black box" s0365 p0440 Conversion of 7dC to the 5β-ketodiol or 5β-diketol, via the long-hypothesized 3-dehydro-Δ^4-diketol (cholesta-4,7-diene-3,6-dione-14α-ol) intermediate, constitutes the so-called "black box" (**Figure 11**). The name is most apt, as both the mechanism of this apparent multistep oxidation, as well as the subcellular location of all these reactions, is still a matter of great debate. Several modifications of 7dC appear to take place early and seemingly simultaneously, i.e., oxidation of the 3β-alcohol to the ketone (with stereospecific loss of the 3α-hydrogen), C_6 oxidation with additional loss of the 4β- and 6-hydrogens and formation of the 6-keto group, and finally 14α-hydroxylation. These observations are consistent with the intermediacy of 3-dehydro-Δ^4-diketol (Davies *et al*., 1981; Rees, 1985; Grieneisen *et al*., 1991, Dauphin-Villemant *et al*., 1998). Analogous to the rate-limiting step in mammalian steroidogenesis, it has long been thought that most or all of these reactions take place in the mitochondria and may be catalyzed by one or more P450 enzymes. That is, 7dC (or 7-dehydro25C) could not be converted into ecdysteroids by the crude microsomal fraction, only by the crude mitochondrial fraction of *Manduca* prothoracic glands. However, as no intermediates could be detected, no reaction mechanism was apparent (Grieneisen *et al*., 1993; Warren and Gilbert, 1996).

Nevertheless, some "negative" evidence has been p0445
obtained from metabolic studies concerning these

early biochemical modifications. Concerning 14α-hydroxylation, the results of incubations of *Manduca* prothoracic glands (Bollenbacher *et al*., 1977a) or *Locusta migratoria* prothoracic glands or ovaries (Haag *et al*., 1987) have suggested that this reaction must take place before, or in concert with, the introduction of the 6-keto group. That is, 3β-hydroxy-5β-cholest-7-en-6-one can be efficiently converted to 14-deoxy-ecdysone, but not to E.

p0450 Several hypotheses have also been tested concerning the introduction of the 6-keto group. Even though 6-hydroxyl-sterol derivatives have been isolated in some biological models, ecdysteroid analogs with a 6-hydroxyl group could not be oxidized to form true ecdysteroids with a 6-ketone group (Schwab and Hétru, 1991; Grieneisen, 1994; Gilbert *et al*., 2002). The possibility that either a 3-dehydro-α-5,6-epoxy-7dC molecule (Grieneisen *et al*., 1991; Warren *et al*., 1995) or a 3-dehydro-5α,8α-epidioxide (J. Warren, C. Dauphin-Villemant, and R. Lafont, unpublished data) could be converted to a 3-dehydro-Δ^4-sterol intermediate has also been investigated. However, when incubated with prothoracic glands from insects or Y-organs from crustaceans, these compounds (either nonradioactive or as radioactive tracers) were never shown to be converted into known ecdysteroids (Gilbert *et al*., 2002).

p0455 Finally, if the 3-dehydro-Δ^4-diketol (**Figure 11**) is an intermediate in ecdysteroid biosynthesis, its formation would also require an early oxidation step at C_3. However, as yet, every attempt to understand when and how this step takes place has failed. In studies with either prothoracic glands or Y-organs, the early oxidation of C (or 25C) to 3-dehydro-C (or 3-dehydro-25C) has never been directly observed. The facile conversion of these oxidized sterols, by either acid or base catalysis, to their distinctive isomerization products, 3-dehydro-Δ^4-C or 3-dehydro-Δ^4-25C, has also never been detected in vivo or in vitro (J. Warren and R. Lafont, unpublished data). Due to greater electron delocalization, 3-dehydro-7dC is very much more unstable, instantly converting into its more stable 3-dehydro-$\Delta^{4,7}$ sterol isomer when placed in an aqueous environment (Antonucci *et al*., 1951; Lakeman *et al*., 1967). However, the formation of either of these compounds (or their 25C analogues) has never been observed during ecdysteroid biosynthesis from radiolabeled precursors. Apparently, C_3 oxidation most likely occurs in concert with subsequent oxidations of these very unstable intermediates.

p0460 A hypothesis where all three modifications might possibly be performed simultaneously by a single enzyme (perhaps a CYP enzyme localized in the mitochondria) has been proposed (Gilbert *et al*., 2002). However, at present, mitochondrial CYP involvement in these hypothetical black box oxidations is not supported for *Drosophila*. All six families of putative mitochondrial CYPs have been tentatively identified on the basis of their conserved motifs associated with the specific cofactor (adrenodoxin) binding to the cytochrome (Tijet *et al*., 2001) (see **Chapter 4.1**). According to tissue expression studies in *Drosophila* (C. Dauphin-Villemant and M. O'Connor, unpublished data), no mitochondrial CYP has been localized to the ring glands, other than those already identified as catalyzing the final 2- and 22-hydroxylation steps (see Section 3.3.4.3.1). Nevertheless, even if the black box reactions do not involve mitochondrial P450 enzymes, these oxidation reactions could still occur in the mitochondria. Alternatively, it remains a possibility that these reactions are instead localized within an alternative membrane-bound subcellular organelle, that is, either another domain of the endoplasmic reticulum (ER) or an organelle known to be involved in the oxidation of numerous substrates (including some mammalian sterols), i.e., the peroxisome (see Section 3.3.6.2). In any case, regardless of the location and mechanisms of the black box reactions, they appear to be quite novel and so may be specific to many invertebrates.

s0370 p0465 **3.3.4.2.3. Reduction steps of a proposed 3-dehydro-Δ^4-ecdysteroid intermediate** Even though there is as yet no direct evidence for the intermediacy of the 3-dehydro-Δ^4-diketol in the biosynthesis of ecdysteroids, it still remains a good candidate. This is substantiated by several lines of evidence. It has been shown to be efficiently converted first to the 5β-diketol (and subsequently to 3DE and E) by a 5β-reductase present primarily in the Y-organs of crustaceans, i.e., in the crab *Carcinus maenas* (Blais *et al*., 1996) and the crayfish *Orconectes limosus* (C. Blais, C. Dauphin-Villemant, and R. Lafont, unpublished data). The reaction is quite specific for the correct oxidation state of the substrate at C_3, as no reduction of the unoxidized 3β-OH-Δ^4-sterol intermediate was observed either in crab Y-organs (Blais *et al*., 1996) or in insect prothoracic glands (Bollenbacher *et al*., 1979). However, the complete characterization of an arthropod 3-dehydro-Δ^4-steroid 5β-reductase is still lacking as all attempts to demonstrate a similar reaction in insects have so far been unsuccessful. Nevertheless, in all other vertebrates or plants investigated so far, the formation of 5β[H]-cholesterol derivatives always proceeds through such 3-dehydro-Δ^4-sterol intermediates (Brown, 1998).

p0470 In the case of species producing only 3β-hydroxylated ecdysteroids, i.e., E or 20-deoxymakisterone A or C, the postulated intermediacy of the 3-dehydro-Δ^4-diketol and diketol in the biosynthetic pathway implies the presence of a 3β-reduction step involving subterminal compounds in the molting glands (**Figure 11**). Until now, the interconversion between 3-dehydro and 3-OH compounds has essentially been documented only in the hemolymph and peripheral tissues of several arthropods (see Section 3.3.5.3.2). A cytosolic NADPH requiring enzyme has been identified. It is able to rapidly and irreversibly catalyze the conversion of 3DE into E, which, in turn, is rapidly hydroxylated to 20E. This enzyme has been cloned in the lepidopterans *Spodoptera littoralis* (Chen *et al*., 1999b) and *Trichoplusia ni* (Lundström *et al*., 2002). However, this enzyme cannot be responsible for 3β-reduction in the prothoracic glands, since it retains a rather narrow substrate specificity, is unable to reduce subterminal intermediates of ecdysteroidogenesis, and, furthermore, is not expressed in the steroidogenic tissues.

p0475 However, in the crab, *Carcinus maenas*, a microsomal enzyme similar to the 3β-hydroxysteroid dehydrogenase (3βHSD) from vertebrates has been detected specifically in the Y-organs (Dauphin-Villemant *et al*., 1997). In the presence of NADH, it can catalyze the 3β-reduction not only of the diketol, but also 3-dehydro-2,22-dideoxyecdysone and 3-dehydro-2-deoxyecdysone, but not 3DE. Interestingly, in the presence of NAD^+, the same subcellular fraction can re-oxidize 2-deoxyecdysone and 2,22-deoxyecdysone back to the original 3-dehydro substrates, suggesting a reversible reaction consistent with a true 3βHSD activity.

s0375 p0480 **3.3.4.2.4. Late hydroxylation steps** Starting from either the 5β-diketol or 5β-ketodiol (the terminal 3-dehydro or 3-OH products of the black box), conversion initially into 3DE or E and ultimately into 3D20E or 20E (**Figure 11**) is well documented. The 5β-ketodiol appears to be a true intermediate in ecdysteroid biosynthesis since at least in one insect species, *Locusta migratoria,* it has been identified as an endogenous compound of ovaries, in addition to 7dC (Hétru *et al*., 1978, 1982). It was also identified as a conversion product from [^{3}H]-cholesterol during *in vitro* incubations of ovaries (Hétru *et al*., 1982) and following [^{3}H]-cholesterol injections into nondiapause eggs of *B. mori* (Sonobe *et al*., 1999). Furthermore, it has been demonstrated in several insect and crustacean species (Grieneisen, 1994) that both prothoracic glands and Y-organs are able to convert the 5β-ketodiol very efficiently into E via sequential hydroxylations at C_{25}, C_{22}, and C_2 (**Figure 11**). Subsequent hydroxylation of circulating E at C_{20}, catalyzed by an ecdysone 20-monooxygenase present in peripheral tissues, yields the active molting hormone 20E (see Section 3.3.5.2).

p0485 It has long been accepted that the enzymes catalyzing these reactions are four different monooxygenases belonging to the CYP superfamily (see **Chapter 4.1**), based on their biochemical characteristics, i.e., membrane subcellular localization, NADPH cofactor, and O_2 requirement and inhibition of the reaction by CO or by classical CYP inhibitors like fenarimol, metyrapone, or piperonyl butoxide observed in various arthropods (Grieneisen, 1994). In this respect, the 2-hydroxylase presents some peculiar features, since it is insensitive to CO and apparently can function with some Krebs cycle intermediates (succinate or isocitrate) in addition to NADPH (Kappler *et al*., 1986, 1988).

p0490 The subcellular distribution of these terminal hydroxylases has been studied in several arthropod models. While the tissue locations of the enzymatic activities of the first three enzymes in insects (Kappler *et al*., 1988; Grieneisen *et al*., 1993) and crustaceans (Blais *et al*., 1996; Dauphin-Villemant *et al*., 1997) are not clear-cut, it is nevertheless widely accepted that the 25-hydroxylase is a microsomal enzyme whereas the 2- and 22-hydroxylases are strictly mitochondrial. In contrast, the 20-hydroxylase has been shown to be microsomal, mitochondrial, or, in some instances, both (Bollenbacher *et al*., 1977b; Feyereisen and Durst, 1978; Smith *et al*., 1979; Smith, 1985; Mitchell and Smith, 1986; Smith and Mitchell, 1986; see Section 3.3.5.2). The subcellular localization of all these CYP enzymes has now been directly demonstrated in *Drosophila* S2 cells (see Section 3.3.4.3.1).

p0495 By analogy with steroid hormone biosynthesis in vertebrates, the identified hydroxylases might be expected to be expressed specifically in the endocrine tissues (or peripheral tissues for the 20-monooxygenase) and perhaps act through a preferential sequence of hydroxylations. Yet, early metabolic studies (Rees, 1985) have reported 2-, 22-, and 25-hydroxylase activities in a number of tissues, not only larval prothoracic glands and adult ovaries, but also in fat body, Malpighian tubules, gut, and epidermis. However, the specific activity of the observed enzymatic activities was seldom mentioned and a closer examination always revealed a substrate conversion that was much more efficient in true steroidogenic organs (prothoracic glands, Y-organs, ovaries) than in other tissues, at least in terms of the C_{22}- and C_{25}-hydroxylations (Meister *et al*., 1985; Rees, 1985, 1995; Grieneisen, 1994). While the

evidence for C_2-hydroxylation activity outside the ring gland remains strong, the C_{25} and C_{22} activities may have been due to the use of crude preparations with the possibility that other CYPs where present in the subcellular fractions. Cytochromes with broader substrate specificities (detoxification enzymes, for instance) may, to some extent, have been able to convert intermediates into ecdysteroids.

p0500 Another characteristic of the enzymes involved in vertebrate steroidogenesis is that the reactions generally occur in a preferred sequence, both due to their precise substrate specificities and their subcellular compartmentalization (Brown, 1998). This appears to be at least partly true in the case of ecdysteroid biosynthesis, even though additional enzymological studies are necessary. In fact, accumulation of many intermediates has been repeatedly reported in insect and crustacean systems when using labeled 5β-ketodiol as an exogenous precursor (Rees, 1985; Grieneisen, 1994). However, these compounds have not been identified as endogenous intermediates and might therefore be artifacts. More precisely, if hydroxylation at C_2 occurs first in either the 5β-ketodiol (2,22,25dE) or ketotriol (2,22dE), it leads to a dead end. This has been shown both in insects (Dollé *et al*., 1991; Rees, 1995) and crustaceans (Lachaise *et al*., 1989; Pís *et al*., 1995b) since both 22,25-dideoxyecdysone and 22-deoxyecdysone (Warren *et al*., 2002) are only poorly converted into E via hydroxylation at C_{22}. On the other hand, C_{25}-hydroxylation does not appear to be the last biosynthetic step, since 25-deoxyecdysone is a major secretory product in several crab species (Lachaise *et al*., 1989; Rudolph and Spaziani, 1992), but has not been found in insects. Taken together, these results suggest that the last steps in E biosynthesis within the endocrine gland also follow a preferential sequence of hydroxylations.

p0505 The present hypothesis suggests that the first terminal oxidation reaction of the newly reduced black box product (i.e., the 3-dehydro-Δ^4-diketol), whether localized in the mitochondria, ER, the peroxisome, or in the cytosol around these organelles, nevertheless occurs at C_{25} within the ER. Then, after translocation of the resulting 5β-ketotriol product to the mitochondria, sequential hydroxylations take place, the first necessarily at C_{22} and the last at C_2, followed by the secretion of the product from the cell as either 3DE and/or E. Circulating E is then converted to 20E in the peripheral tissues. Thus, in arthropods, the apparent preferential sequence of biosynthetic steps may be due more to their particular subcellular compartmentalization than to any strict substrate specificity. That is, the more convenient access of this cytosolic substrate to the 25-hydroxylase in the ubiquitous ER may determine the first reaction. Curiously, of the last two mitochondrial enzymes, the 22-hydroxylase apparently must function first, as the formation of the alternative 2-hydroxylated product (22dE), while not prohibited by any strict substrate specificity, nevertheless is not normally observed *in vivo*. Specific compartmentalization of these two enzymes within the mitochondria may somehow restrict initial substrate (2,22dE) access to the 2-hydroxylase.

3.3.4.2.5. Various secretion products: single or multiple metabolic pathways? s0380 p0510 Ecdysteroid biosynthesis has been studied in various model systems, i.e., steroidogenic tissues originating from animals producing different and sometimes exclusive ecdysteroids. There is a general implicit postulate that production of the same ecdysteroid proceeds through a common pathway in all investigated arthropods. This means that orthologous enzymes are expected to be identified in the various species. Until now, however, comparative structural data has been lacking. On the other hand, the occurrence of different secretory products in different species raises the question of whether they are the result of profound changes in ecdysteroid biosynthesis or just the effect of minor differences in pathways. The reason for the different oxidation state at C_3 in the two major ecdysteroid products identified in many arthropod species, i.e., E or 3DE, might involve just variability in the activity or expression of a terminal reductase in or near the steroidogenic tissue. Such a hypothesis is substantiated by the fact that, generally, in 3DE-producing species, the conversion of the 5β-diketol leads preferentially to 3DE, while the conversion of the 5β-ketodiol is also efficient but leads to E (Böcking *et al*., 1993; Grieneisen, 1994). Similarly, the preferential production of 25-deoxy ecdysteroids by crab Y-organs may be due simply to the absence or the very low expression of the 25-hydroxylase in crustaceans. However, the mechanisms for these differences may be more complex and could involve different substrate specificities of the corresponding enzymes.

3.3.4.3. Molecular Analysis of Ecdysteroid Biosynthesis s0385

p0515 Different approaches have been engaged in recent years in order to gain structural information about the genes and proteins involved in ecdysteroid biosynthesis. With regard to the biosynthetic enzymes, a first approach has been to take advantage of expected sequence homologies and look for CYP enzymes expressed specifically in prothoracic glands (Snyder *et al*., 1996). Using this strategy, CYP

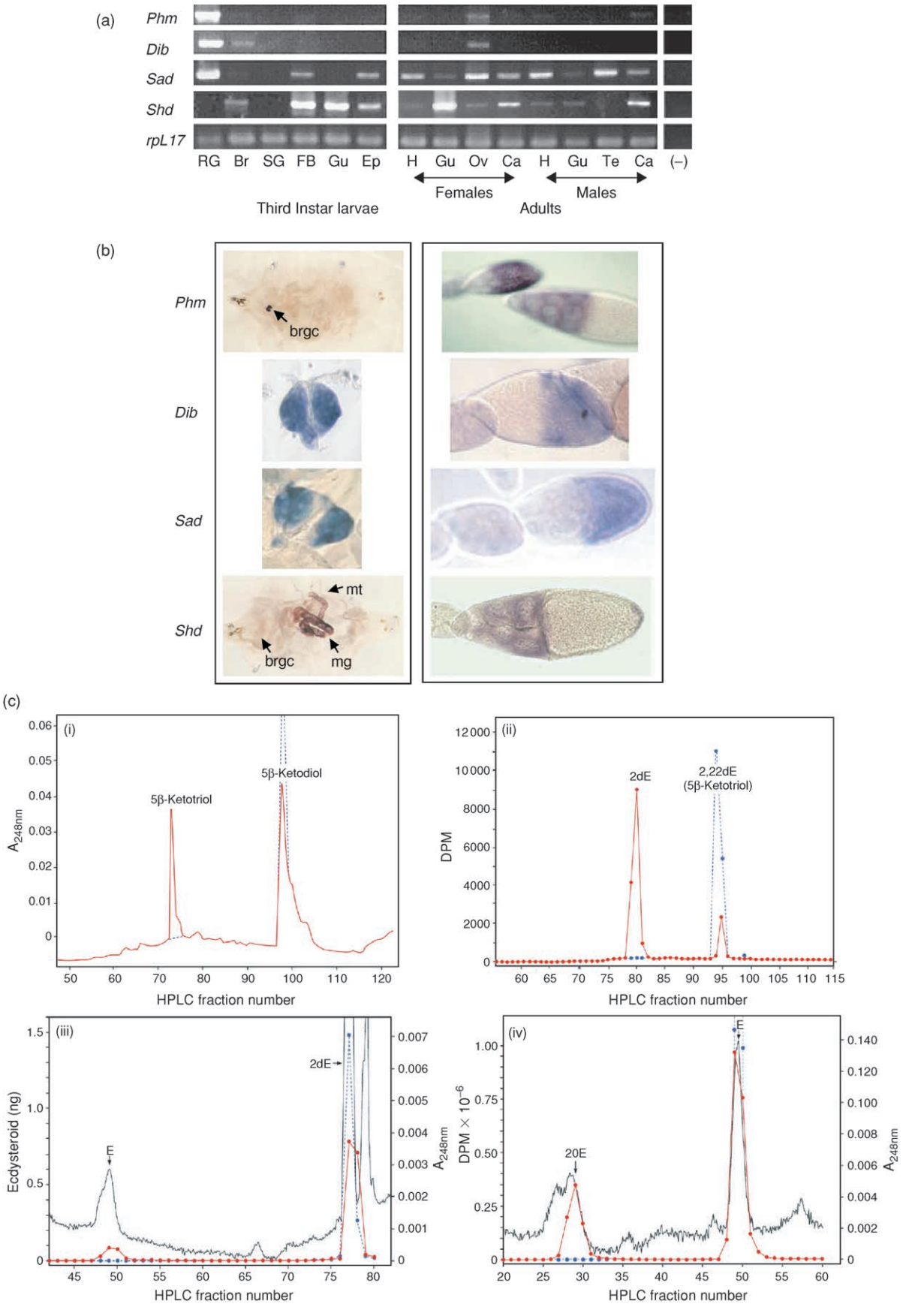
enzymes specific to *Manduca* prothoracic glands (see **Chapter 4.1**) and crayfish (*Orconectes limosus*) Y-organs (Dauphin-Villemant *et al*., 1997, 1999) were identified. Curiously, both were of the CYP4 family, but their precise function has not yet been elucidated.

p0520 In *Drosophila*, genetic approaches as well as the recent sequencing of the genome represent powerful tools to identify genes involved in ecdysteroid biosynthesis. Several mutations, including *ecdysoneless* (*ecd*^{1}), *DTS3, dre4, giant*, and *woc*, have been shown to affect E titers (Garen *et al*., 1977; Walker *et al*., 1987; Schwartz *et al*., 1984; Sliter and Gilbert, 1992; Warren *et al*., 1996; Wismar *et al*., 2000) and consequently development and metamorphosis. However, the corresponding genes appear to be more linked either to the transcriptional regulation of ecdysteroid production or to the efficient delivery of ecdysteroid intermediates to the enzymatic machinery rather than to the biosynthetic enzymes themselves. For instance, *giant* encodes a bZIP (basic-leucine zipper) transcription factor (Capovilla *et al*., 1992), *ecd*^{1} may impair sterol availability (Warren *et al*., 1996) while *woc* encodes a transcription factor with eight zinc fingers (Wismar *et al*., 2000) that appears (in the least) to regulate expression of the cholesterol 7,8-dehydrogenase (Warren *et al*., 2001). An enhancer-trap approach has also been used in recent years (Harvie *et al*., 1998) in order to begin characterizing the function of *Drosophila* ring glands during larval development. Several genes have been identified as specifically expressed in the lateral cells of the ring gland (i.e., the prothoracic gland cells), but there is presently no evidence that they are involved in ecdysteroid biosynthesis

s0390 p0525 **3.3.4.3.1. Identification of steroidogenic cytochrome P450 enzymes** While many of the early oxidation steps in ecdysteroid biosynthesis are still incompletely understood, it nevertheless appears to involve a cascade of sequential hydroxylations catalyzed by several cytochrome P450 enzymes. A combination of molecular genetics and biochemistry was used recently to further elucidate these reactions in the biosynthetic pathway in *Drosophila*. One phenotype predicted for mutations that disrupt the production of ecdysteroids would be the inability to produce a cuticle at any stage, the earliest being the synthesis of embryonic cuticle during the second half of embryogenesis. Among the mutants screened by Nüsslein-Volhard and colleagues in their landmark characterization of embryonic patterning (Jürgens *et al*., 1984; Nüsslein-Volhard *et al*., 1984; Wieschaus *et al*., 1984), several were later grouped under the name of the *Halloween* mutants (Chavez *et al*., 2000). They were selected for their similar abnormal cuticular patterning and low ecdysteroid titers, suggestive of a defect in the ecdysteroid pathway. In all of them, several developmental and physiological defects (i.e., absence of head involution, dorsal closure, and gut development) were associated with a very poor differentiation of embryonic cuticle and which led to lethality before the end of embryonic development.

p0530 While the first Halloween gene, *disembodied* (*dib*) (CYP302A1) was located by classical molecular techniques (Chavez *et al*., 2000), in order to select the remaining genes, the *Drosophila* genome was searched for the presence of cytochrome P450 enzyme-type sequences near the known map location of these genes. At least four other genes, *phantom (phm), spook (spo), shadow (sad)*, and *shade (shd)* were shown to also code for CYP enzymes, CYP 306A1, CYP307A1, CYP315A1, and CYP314A1, respectively (Warren *et al*., 2002; Petryk *et al*., 2003; Warren *et al*., 2004), as they retained the conserved domains characteristic of this superfamily (particularly the high conservation of the heme-binding domain at the C-terminal end). Owing to a high divergence in their overall sequence as compared to other already known sequences, these enzymes are members of a new CYP family (see drnelson for nomenclature) (see **Chapter 4.1**). Four of them, *phm*, *sad*, *dib*, and *shd*, have now been fully characterized, as their corresponding enzymes, when expressed in *Drosophila* S2 cells, have been shown to catalyze the last four reactions in ecdysteroid biosynthesis, i.e., the terminal 25-, 22-, 2-, and 20-hydroxylations (see below).

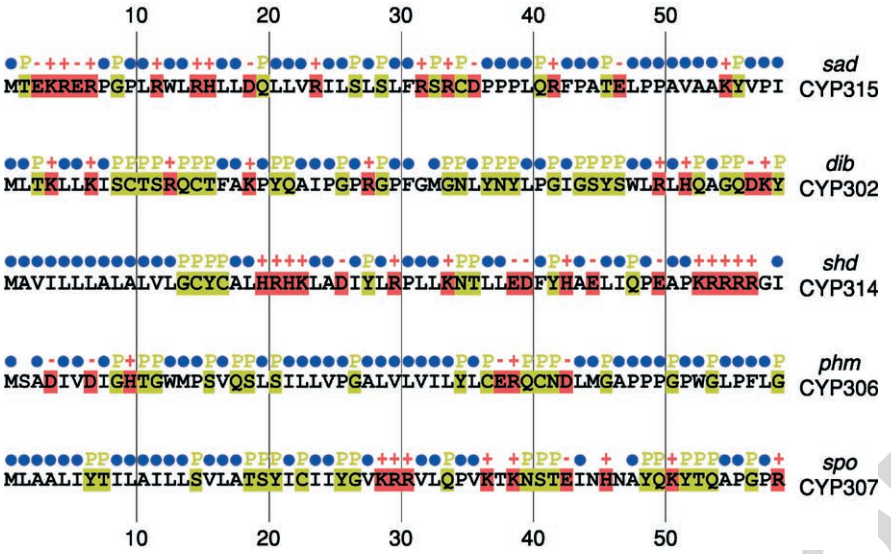
p0535 *Dib* and *sad* mRNAs are initially expressed in the epidermis during early embryogenesis and then later in classical steroidogenic tissues, i.e., in ring glands from late embryogenesis to metamorphosis and in the nurse and/or follicle cells of adult female ovaries. Their expression patterns were studied using *in situ* hybridization on different tissues in addition to RT-PCR analysis (**Figure 12 a** and **b**). More recently, it was shown that *phm* retains an almost identical expression pattern to *dib* (Warren *et al*., 2004), while *sad* actually exhibits a much broader pattern, consistent with prior tissue activity studies, including not only expression in the steroidogenic tissues (Warren *et al*., 2002), but also significant expression in the gut and epidermis in larvae and adults (**Figure 12 a** and **b**). In contrast, while *shd* is also expressed in the early embryonic epidermis, it is not expressed in late embryonic or larval ring glands, but instead is prominent in peripheral larval tissues and adult ovaries (Petryk *et al*., 2003).


(a)
Phm
Dib
Sad
Shd
rpL17
RG Br SG FB Gu Ep H Gu Ov Ca H Gu Te Ca (–)
Females
Males
Third Instar larvae
Adults
(b)
Phm
Dib
Sad
Shd
brgc
mt
brgc
mg
(c)
(i)
5β-Ketodiol
5β-Ketotriol
A248nm
HPLC fraction number
(ii)
2dE
2,22dE
(5β-Ketotriol)
DPM
HPLC fraction number
(iii)
E
2dE
Ecdysteroid (ng)
A248nm
HPLC fraction number
(iv)
E
20E
DPM × 10−6
A248nm
HPLC fraction number

p0540 The initial hypothesis that *dib* and the other Halloween gene CYPs might catalyze steps in the ecdysteroid biosynthetic pathway has now been confirmed by the biochemical characterization of the Phm, Dib, Sad, and Shd CYP enzymes. The cDNA coding sequences of these genes have been transiently transfected into *Drosophila* S2 cells as expression plasmids under the control of the actin 5C promoter. When the transfected cells are incubated with specific ecdysteroid precursor substrates, they efficiently and selectively convert them to product (**Figure 12c**), indicating the 25-, 22-, 2-, and 20-hydroxylase functions for Phm, Dib, Sad, and Shd respectively. Additional information on the substrate specificities of these enzymes was obtained in a similar manner. For instance, *dib*-transfected cells converted 2,22-dideoxyecdysone (the 5β-ketotriol) to 2-deoxyecdysone (**Figure 12c**), but could not convert 22-deoxyecdysone to E, confirming the strict substrate requirement (i.e., the absence of a 2-hydroxy function) for the 22-hydroxylase enzyme, at least in this expression system (Warren[bib2970] *et al*., 2002). On the other hand, cells transfected with *sad* hydroxylated not only 2-deoxyecdysone and 2,22-dideoxyecdysone at C_2 (Warren[bib2570] *et al*., 2002), but also 2,22-dideoxy-20-hydroxyecdysone (C. Blais, C. Dauphin-Villemant, and R. Lafont, unpublished data), suggesting that modifications on the side chain do not necessarily interfere with the active site of this enzyme. Similarly, cells transfected with *phm* converted not only 2,22,25-trideoxyecdysone (the 3β,5β-ketodiol) to 2,22-dideoxyecdysone, but also 25-hydroxylated both the 3β,5α-ketodiol and the 3α,5α-ketodiol to their respective isomeric ketotriol products (Warren[bib9165] *et al*., 2004)). Apparently, in this case, A/B-ring and C_3 stereochemistry are relatively unimportant determinants for Phm activity. However, Phm could not metabolize 25dE to E or ponasterone A to 20E, indicating an inability (as was observed with Dib) to recognize substrates prematurely hydroxylated at C_2. Detailed enzymological and mechanistic studies are now possible following the stable, heterologous expression in nonsteroidogenic cell lines of both single and multiple (i.e., linked) steroidogenic P450 enzymes targeted to either the mitochondria or the endoplasmic reticulum, or both.

p0545 Cytochrome P450 enzymes, as membrane-bound enzymes, have so far been found to be either localized in the endoplasmic reticulum or in the mitochondria of eukaryotes (see **Chapter 4.1**). Their amino acid sequences at the N-terminus present conserved structural features that are hypothesized to direct their subcellular localization (Omura[bib1830] and Ito, 1991; Von Wachenfeldt[bib2515] and Johnson, 1995; Van den[bib2500] Broek *et al*., 1996) (see **Chapter 4.1**). The N-terminal sequences deduced from *dib* and *sad* cDNAs (**Figure 13**) are consistent with their mitochondrial localization previously determined for the C_2- and C_{22}-hydroxylases from subcellular fractionation studies in *Locusta*[bib1105] (Kappler[bib1110] *et al*., 1986; 1988) and *Manduca*[bib0745] (Grieneisen *et al*., 1993). Both contain numerous positively charged residues, in addition to many polar groups within the first 20–30 amino acids. In contrast, the N-terminus of Shd (the 20-monooxygenase) and Spo (which remains unidentified) consists of a string of about 20 or more hydrophobic amino acids devoid of charged

f0060 **Figure 12** Identification of *Halloween* genes of *Drosophila*. Abbreviations: *phantom, phm; disembodied, dib; shadow, sad; and shade, shd*. (a) RT-PCR analysis showing larval and adult tissue expression of *phm*, *dib*, *sad*, and *shd*. RpL17 expression was used as a control, since this gene is expressed similarly in all tissues. RG, ring gland; Br, brain; SG, salivary gland; FB, fat body; Gu, gut; Ep, epidermis; H, head; Ov, ovary; Te, testis, Ca, carcass; (−) negative control. (b) *In situ* hybridization antisense digoxygenin-labeled RNA probes were used. Left part: total larvae or ring gland labeling of late L3 larvae; brcg, brain ring gland complex; mg, midgut; mt, Malphighian tubules. Right part: staining of adult egg chambers (stages 8–10). (c) CYP Enzyme characterization: Halloween genes or GFP were transfected into *Drosophila* S2 cells using DDAB. Three days later, the cells were incubated with either radiolabeled or nonradiolabeled substrates for 4–6 h prior to analysis by RP-HPLC (C_{18}-silica based column, 30–100% methanol gradient over 2 h, 1 ml min^{-1}, 1 ml/HPLC fraction). Specific ecdysteroid detection by UV absorbance (A_{248nm}) and/or radioimmunoassay (RIA, SHO3 antibodies, pg ecdysteroids) for non-labeled substrates and DPM for radiolabeled substrates. (i) S2 cells + *Phm* (red line – A_{248nm}). Substrate: non-radiolabeled 5β-ketodiol (2,22,25-trideoxyecdysone = 2,22,25dE). Control: S2 cells + GFP (blue line – A_{248nm}). Product: 5β-ketotriol (2, 22-dideoxyecdysone = 2,22dE). (ii) S2 cells + *Dib* (red line – DPM). Substrate: [^{3}H]-5β-ketotriol (2,22dE). Control: S2 cells + GFP (blue line – DPM). Product: [^{3}H]-2-deoxyecdysone (2dE). (iii) S2 cells + *Sad* (red line – pg ecdysteroids RIA; black line – A_{248nm}). Substrate: nonradiolabeled 2-deoxyecdysone (2dE). Control: S2 cells + GFP (blue line – pg ecdysteroid RIA). Product: ecdysone (E). (iv) S2 cells + *Shd* (red line – DPM; black line – A_{248nm}). Substrate: [^{3}H]-ecdysone (E). Control: S2 cells + GFP (blue line – A_{248nm}). Product: [^{3}H]-20-hydroxyecdysone (20E). (Modified from Chávez, V.M., Marqués, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., *et al.*, **2000**. The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126; Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., *et al.*, **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 99*, 11043–11048; Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., *et al.*, **2004**. Shade: the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778).



f0065 **Figure 13** Predicted N-terminal amino acid sequence of the Halloween P450 genes. Red represents charged (+/−) residues, yellow shows polar (P) residues, and blue circles represent nonpolar amino acids. Prominent charged residues in the N-terminal 20–30 amino acids of P450 enzymes are thought to be involved in the targeting of the protein to the inner mitochondrial matrix. Alternatively, predominantly nonpolar N-terminal residues are thought to help anchor the protein in the membrane of the endoplasmic reticulum. (Modified from Chávez, V.M., Marqués, G., Delbecque, J.P., Kobayashi, K., Hollingsworth, M., Burr, J., Nätzle, J.E., O'Connor, M.B. **2000**. The *Drosophila disembodied* gene controls late embryonic morphogenesis and codes for a cytochrome P450 enzyme that regulates embryonic ecdysone levels. *Development 127*, 4115–4126; Warren, J.T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., Dauphin-Villemant, C., O'Connor, M.B., Gilbert, L.I. **2002**. Molecular and biochemical characterization of two P450 enzymes in the ecdysteroidogenic pathway of *Drosophila melanogaster*. *Proc. Natl Acad. Sci. USA 99*, 11043–11048; Petryk, A., Warren, J.T., Marqués, G., Jarcho, M.P., Gilbert, L.I., Parvy, J.-P., Dauphin-Villemant, C., O'Connor, M.B. **2003**. Shade: the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone. *Proc. Natl Acad. Sci. USA 100*, 13773–13778).

residues, suggesting that both these CYP enzymes are targeted to the endoplasmic reticulum.

p0550 The mitochondrial localization of the Dib and Sad cytochromes has been confirmed by *in situ* localization of the corresponding C-terminal, epitope-tagged (His or HA) proteins expressed in S2 cells (Warren *et al*., 2002; Petryk *et al*., 2003). However, in apparent contradiction of the above considerations, when a similarly tagged *shd* construct was transfected into S2 cells, it was determined that it, like *dib* and *sad*, also localized in the mitochondria and not in the ER (Petryk *et al*., 2003). When phylogenetic trees with various CYPs are constructed, *shd* always clusters (along with *dib* and *sad*) with the mitochondrial CYPs, primarily because of shared sequences associated with binding to its redox partner adrenodoxin (Tijet *et al*., 2001) (see **Chapter 4.1**). However, upon closer examination of the Shd N-terminus (**Figure 13**), a possible mitochondrial import sequence containing numerous charged residues is present immediately downstream from the apparent microsomal targeting sequence. Protease-mediated cleavage of the N-terminus of Shd, thereby eliminating the initial string of hydrophobic residues, could result in the retargeting of this enzyme to the mitochondria. Such differential posttranslational modification of Shd could explain the observed distribution of ecdysone 20-monoxygenase activity into either the mitochondria or ER or both, depending on the insect, tissue, or stage (see Section 3.3.5.2). A similar situation has been reported for mammalian CYP1A1, which has a chimeric N-terminal signal that facilitates the targeting of the protein to the ER and, following protease action, to the mitochondria (Addya *et al*., 1997). Not clear, however, is how the P450 enzyme manages to interact with apparently separate reductase cofactors when it is present in these two different membrane environments (see Section 3.3.4.3.2).

p0555 The predicted N-terminal sequence of Phm (**Figure 13**) could present a similar anomaly, as there is no typical hydrophobic stretch in the first 20 amino acids. However, if one considers the second methionine at position 14 as being the real start of the protein, then the following 20 amino acids are mainly hydrophobic and so would suggest an ER import sequence. Indeed, a similarly tagged Phm enzyme was found to localize in the ER of transfected S2 cells (Warren *et al*., 2004), consistent with

prior biochemical characterizations of the 25-hydroxylase in arthropods (Kappler[bib1110] *et al*., 1988).

p0560 Several points should be noted regarding the molecular evolution of CYP genes and their relation to steroidogenesis. It is postulated that all cytochrome P450 enzymes are derived from a few ancient CYPs (Nelson,[bib1780] 1998) (see **Chapter 4.1**). As additional genomes are deciphered, genes putatively orthologous to *phm*, *dib*, *sad*, and *shd* will soon be identified in *Anopheles gambiae, Drosophila pseudoobscura*, and *Bombyx*. Comparative studies and rapid progress in insect genomics will allow confirmation that these orthologous genes actually catalyze the same reactions in mosquitoes and other insects as they do in *Drosophila*. Indeed, following similar heterologous expression in *Drosophila* S2 cells, it was recently determined that a *Bombyx* gene analogous to *phm* also codes for a P450 enzyme that catalyzes specific ecdysteroid 25-hydroxylation (Waren[bib9165] *et al*., 2004). Furthermore, as was shown for the *Drosophila* ring gland-specific genes *dib*, *sad* and *phm* (Warren[bib2270] *et al*., 2002; Petryk[bib1890] *et al*., 2003; Warren[bib9165] *et al*., 2004), *Bombyx phm* expression in the prothoracic gland cells during the last larral-larval molt undergoes a dramatic down-regulation by the beginning of the last larval stadium (Warren[bib9165] *et al*., 2004), perhaps the result of feedback inhibition by the product, 20E, operating via its nuclear receptor (see **Chapter 3.8**).

p0565 Finally, recent advances in the knowledge of insect genomes will likely enable the identification of all the P450 enzymes involved in ecdysteroid biosynthesis (including the cholesterol 7,8-dehydrogenase) in the near future. For instance, all putative CYP genes have been identified in *Drosophila* (Tijet[bib2465] *et al*., 2001) (see **Chapter 4.1**). There is no doubt that a systematic analysis of the tissue expression of all CYP genes will enable us to determine the best candidates coding for the remaining enzymes involved in ecdysteroid biosynthesis. More integrated approaches using microarray technology approaches are presently available and should constitute new tools to identify the large set of genes specifically expressed in steroidogenic cells and so potentially linked to ecdysteroidogenesis.

s0395 p0570 **3.3.4.3.2. Electron transport involved in cytochrome P450 reactions (see Chapter 4.1)** Monooxygenations catalyzed by cytochrome P450 enzymes require molecular oxygen and two electrons during their catalytic cycle. In vertebrate steroidogenesis, the electron transfer systems associated with the mitochondrial and microsomal CYP enzymes are different. In the mitochondrial system, reduction occurs with FAD as the flavoprotein (adrenodoxin reductase), which transfers electrons to an iron-sulfur protein (adrenodoxin) and finally to the CYP. In contrast, in the microsomal system, cytochrome P450 reductase, a flavoprotein containing both FMN and FAD, passes electrons directly from NADPH to the CYP. The second electron may alternatively arise from NADPH via cytochrome b_5, and a number of forms of cytochrome P450 show an obligatory requirement for cytochrome *b5* (Miller,[bib1690] 1988; Schenkman and Jansson,[bib2120] 2003)

p0575 There is some evidence in arthropods that ecdysteroid hydroxylations catalyzed by CYPs involve electron transfer systems analogous to those used in vertebrates (see **Chapter 4.1**). The obligatory function of cytochrome P450 reductase and the adrenodoxin/adrenodoxin reductase system in the function of microsomal and mitochondrial CYPs, respectively, has been indicated by immunoinhibition studies. Anti-adrenodoxin and anti-adrenodoxin reductase antibodies effectively inhibited ecdysone 20-monooxygenase activity in *Spodoptera* fat body mitochondria (Chen[bib0265] *et al*., 1994a). Similarly, antibodies raised against cytochrome P450 reductase inhibited microsomal E 20-monooxygenase activity in *B. mori* embryos (Horike[bib0885] and Sonobe, 1999). EPR spectroscopic studies have corroborated the presence of ferredoxin (adrenodoxin) and cytochrome P450 enzyme in the foregoing mitochondria (Shergil *et al*.,[bib2200] 1995) and in *Manduca* fat body mitochondria (Smith *et al*., 1980).

p0580 The electron transfer systems appear to be structurally and functionally conserved. The cytochrome P450 reductase has been cloned in several insect species (Koener[bib1240] *et al*., 1993; Hovemann[bib0925] *et al*., 1997; Horike[bib0890] *et al*., 2000). Similarly, the adrenodoxin reductase gene (*dare*) has been identified in *Drosophila*[bib0590] (Freeman *et al*., 1999). Taken together, these data underline the similarity of cytochrome P450 enzyme function in both vertebrates and invertebrates. As there are numerous cytochrome P450 enzymes involved in various metabolic processes, the expression patterns of the electron transfer proteins are not specific to steroidogenic tissues. However, specific transcriptional or posttranscriptional regulation may occur in steroidogenic tissues, as has been shown in vertebrates. Molecular studies are needed to substantiate such hypotheses.

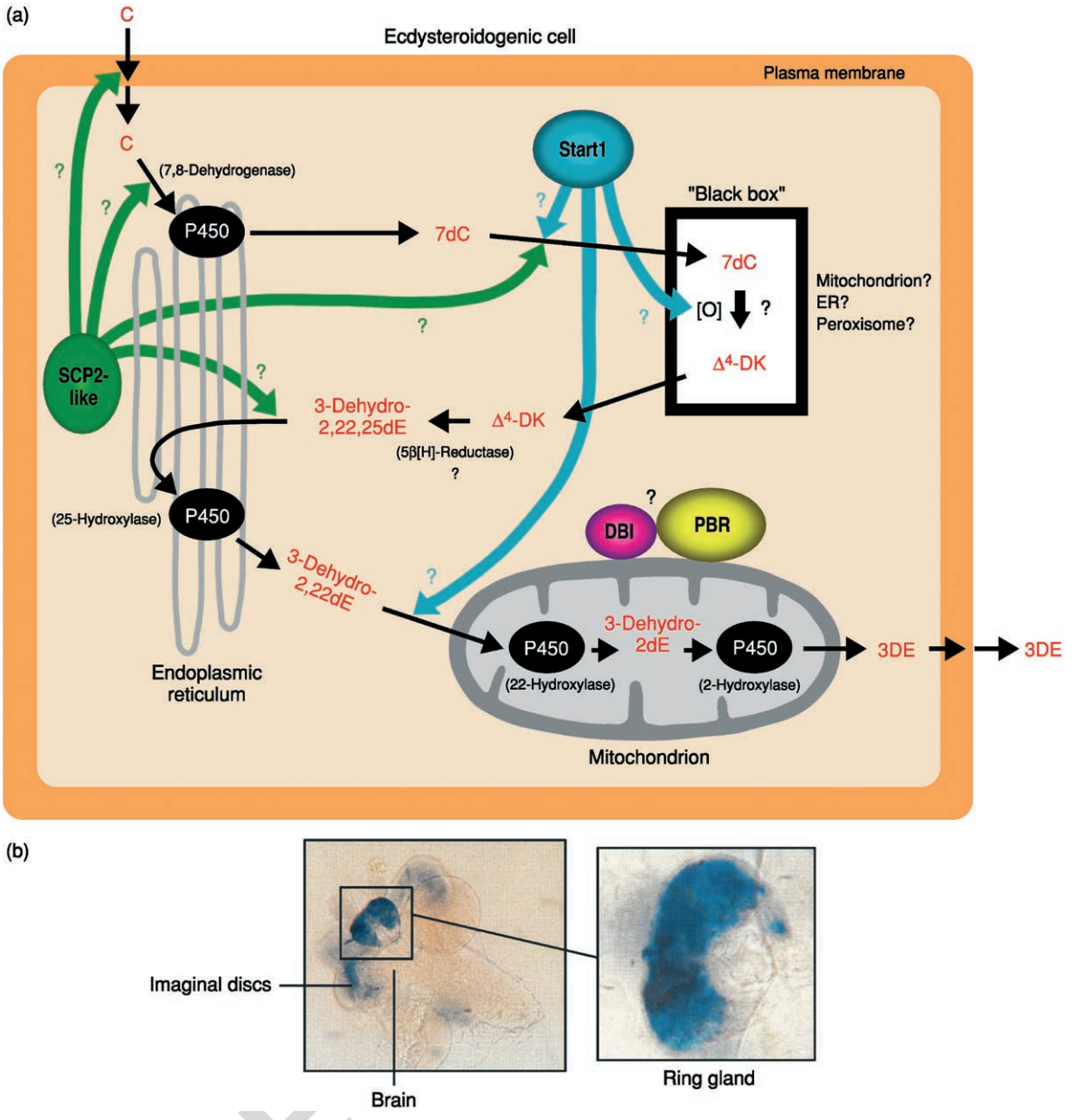
s0400 p0585 **3.3.4.3.3. Intracellular steroid movements** During ecdysteroid biosynthesis, intracellular movements of all sterol intermediates (and not just C) must necessarily occur, as the various enzymes involved in their interconversion have been localized to different subcellular compartments. In vertebrates, several proteins (in addition to the enzymatic

machinery) are expressed more or less specifically in the steroidogenic tissues, where they appear to act to promote cholesterol movements inside the cells. These proteins play complementary roles during the acute regulation of steroidogenesis. Three major steps are thought to be involved, although the mechanisms are still not fully understood. First, a sterol carrier protein (SCP-2) mediates the transfer of C, whether dissolved in the cell membrane, the ER, or within intracellular lipid droplets, to the outer mitochondrial membrane (Gallegos *et al*., 2001). Then the binding of a ligand, diazepam binding inhibitor (DBI), to a peripheral benzodiazepine receptor (PBR) is involved in the formation of a pore complex, thereby creating contact sites between the outer and inner membranes of the mitochondria (Papadopoulos, 1993). Finally (at least in the adrenal) the steroidogenic acute regulatory protein (StAR), by means of its StAR-related lipid transfer domain (START), enables a rapid increase in the transport of cholesterol from the outer to the inner mitochondrial membrane (Stocco, 2001) where the first enzyme of steroidogenesis, the cholesterol side chain cleavage cytochrome P450 (CYP11A), resides (Miller, 1988; Stocco and Clark, 1996; Black *et al*., 1994). A similar function has been attributed to the vertebrate cholesterol transporter MLN64 (metastatic lymph node 64) within the placenta (Moog-Lutz *et al*., 1997). However, while it shares the START domain with the StAR protein, MLN64 also has an additional transmembrane region.

In arthropods, the situation is somewhat different since the first step of ecdysteroid biosynthesis is microsomal instead of mitochondrial, but intracellular sterol (and ecdysterol) trafficking may nevertheless require facilitation by mechanisms similar to those observed in vertebrates (**Figure 14a**). It would first seem necessary to increase the access of C, whether localized in the cell membrane or present in cytosolic vesicles, to the 7,8-dehydrogenase in the ER. Thus, an SCP2-like protein could be involved in these sterol movements into and maybe out of the ER. Subsequently, the newly synthesized and very nonpolar 7dC must then translocate from the ER through the cytosol to the unknown organelle where it undergoes the black box oxidations, i.e., to the mitochondria, to another domain of the ER, to the peroxisome, or to some other place. If there is mitochondrial involvement at this stage of the synthesis (or later), then a StAR-like protein, i.e., Start1 (see below), may well be involved, perhaps along with DBI/PBR. Alternatively, if peroxisomal, then an SCP2-like carrier might be indicated. This movement of 7dC out of the ER and into the location of its subsequent oxidation, in analogy with the cholesterol side chain cleavage reaction of mammalian steroidogenesis, has long been hypothesized to be the rate-limiting step of ecdysteroid biosynthesis (Rees, 1985, 1995; Warren and Hétru, 1990; Warren and Gilbert, 1996) and ultimately under the control of the brain molting factor prothoracicotropic hormone (PTTH) (see **Chapter 3.2**). The final envisioned Δ^4-diketol product could then undergo hypothesized reduction by a cytosolic or membrane-bound 5β-reductase. In any case, the resulting ecdysteroid-like product, 3-dehydro-2,22,25dE (or its 3β-OH reduction product, see Section 3.3.4.2.3), must necessarily travel back to the ER to undergo 25-hydroxylation, prior to its final sequential 22- and 2-hydroxylations within the mitochondria. The now very polar, almost sugar-like steroid product 3DE (or E) is then somehow finally secreted through the cell membrane and into the hemolymph for subsequent 3β-OH reduction (if needed, see Section 3.3.5.3.2) and/or conversion to 20E in the peripheral tissues.

Homologs of the above-mentioned proteins are found across the animal kingdom, including insects. Recent studies dealing with their role in insects are consistent with their putative implication in the regulation of ecdysteroid biosynthesis (**Figure 14a**). A SCP-2-like protein has recently been identified in *Aedes aegypti* (Krebs and Lan, 2003). It has a sequence (about 40% amino acid identity with mammalian sequences) and three-dimensional structure similar to the mammalian SCP-2/SCP-X proteins (Stolowich *et al*., 2002; Dyer *et al*., 2003), suggesting a conserved role as a cholesterol transporter. In *Drosophila*, a SCP-X gene has been reported (Kitamura *et al*., 1996), but only a 1.6 kb transcript was mentioned with high expression levels in the midgut of embryos. On the other hand, an independent gene (AeSCP-2) has been identified in *Aedes* and is similar to the vertebrate SCP-2 transcript. The *Aedes* SCP-2-like protein also has a high affinity towards cholesterol, suggesting its role as a cholesterol transporter (Krebs and Lan, 2003). It presents a broad tissue distribution, but further detailed studies are needed of the expression of a SCP-2-like protein in arthropod steroidogenic tissues.

In various vertebrate steroidogenic cell models, the activation of the mitochondrial 18 kDa peripheral-type benzodiazepine receptor (PBR) by its ligand diazepam binding inhibitor (DBI) or endozepine, confers the ability to take up and release cholesterol (Papadopoulos *et al*., 1998). The diazepam-binding inhibitor (DBI) is a 10-kDa protein, which has been identified in *M. sexta* (Snyder and Feyereisen, 1993) and *D. melanogaster* (Kolmer *et al*., 1993, 1994). Homologous sequences are also



f0070 **Figure 14** Proposed involvement of the hypothetical sterol carrier proteins SCP2, DBI, and StAR in insect ecdysteroidogenesis. (a) Ecdysone biosynthesis: localizations of subcellular enzymes and hypothetical intracellular movements of intermediates within the ecdysteroidogenic cell. C, cholesterol; 7dC, 7-dehydrocholesterol; D4-DK, D4-diketol; 3-dehydro-2,22,25dE, 3-dehydro-2,22,25-trideoxyecdysone (diketol); 2,22dE, 2,22-dideoxyecdysone (ketotriol); 3-dehydro-2dE, 3-dehydro-2-deoxyecdysone; 3DE, 3-dehydroecdysone; SCP2-like, sterol carrier protein 2-like; Start1, steroidogenic acute regulatory protein related lipid transfer domain protein 1; DBI, diazepam binding inhibitor; PBR, peripheral benzodiazepine receptor. (b) *In situ* hybridization of *Drosophila* L3 larvae brain ring gland complex employing an antisense *Drosophila* StAR (Start1) digoxigenin-labeled RNA probe (C. Dauphin-Villemant, A. Mesneau, and J.-P. Parvy, unpublished data).

found in other dipteran and lepidopteran species. In *M. sexta* prothoracic glands, DBI expression varies concomitantly with ecdysteroid production, and *in vitro* ecdysteroid production is stimulated by a diazepam analog (bib9135Snyder and Antwerpen, 1998). Changes in prothoracic glands of both mRNA and DBI protein levels suggest a tissue-specific regulation of this gene, consistent with a role in the regulation of ecdysteroid biosynthesis.

The capacity of steroidogenic cells during development is highly variable in arthropods, as in vertebrates, and peptide hormones induce very rapid increases in steroid production (within minutes) (see **Chapter 3.2**). The acute regulation of vertebrate p0605

steroidogenesis seems to be the predominant role of the labile proteins StAR and its close analog MLN64 (Stocco, 2000, 2001). Similarly, stimulation of insect steroidogenesis is rapidly inhibited by cycloheximide, an inhibitor of protein synthesis, indicating the requirement of a labile protein (Keightley *et al*., 1990; Dauphin-Villemant *et al*., 1995) (see **Chapter 3.2**). Analysis of genome databases revealed related sequences in invertebrates, including *C. elegans*, *D. melanogaster*, and *A. gambiae*. One, *Start1*, is homologous not with *StAR*, but rather with *MLN64*. However, in addition to the four putative transmembrane domains (helices) of MLN64, it also contains a 122 amino acid insert of unknown function. The spatial and temporal pattern of both *Start1* message and Start1 protein expression in *Drosophila* is consistent with it being the rate-limiting factor in ecdysteroidogenesis (Roth *et al*., 2004). For instance, *in situ* hybridization using an antisense Digoxygenin labeled probe corresponding to the sequence for *Start1* isolated from third instar total RNAs shows a strong labeling of ring glands (**Figure 14b**; C. Dauphin-Villemant, unpublished data). In a similar manner, a carotenoid binding protein (CBP) presenting similarities with the StAR family was recently identified in the *Bombyx mori* silk gland (Tabunoki *et al*., 2002). However, as it binds carotenoids rather than cholesterol, it is possible that several StAR related proteins may exist in arthropods. Clearly, further studies are needed to demonstrate the involvement of the *Drosophila* Start1 protein or any of these other proteins, in the regulation of steroidogenesis.

s0405 **3.3.4.4. The Ecdysteroidogenic Tissues**

s0410 p0610 **3.3.4.4.1. Prothoracic glands and gonads** Classically, prothoracic glands are considered as the source of ecdysteroids during postembryonic development, but they usually degenerate prior to the early adult stage. The development of new analytical techniques such as radioimmunoassay (Borst and O'Connor, 1972) allowed direct evidence to be obtained for ecdysone secretion by prothoracic glands. Using *in vitro* incubations of these organs, several authors could demonstrate that they actually secrete measurable amounts of ecdysone, and that the variations of this secretory activity are consistent with the fluctuations of hemolymph ecdysteroid levels (Chino *et al*., 1974; King *et al*., 1974; Borst and Engelmann, 1974). However, this was not a full demonstration for *de novo* synthesis. Only the *in vitro* conversion of radiolabeled cholesterol or 25-hydroxycholesterol by prothoracic glands and Y-organs provided the final argument for the *de novo* synthesis of ecdysteroids by these endocrine organs (Hoffmann *et al*., 1977; Warren *et al*., 1988a,b; Böcking *et al*., 1994).

Adult insects also contain ecdysteroids, as was found in *Bombyx* (Karlson and Stamm-Menendez, 1956). The same methods used for prothoracic glands established that ovaries are a temporary ecdysteroid source, usually in adults and/or pharate adults, depending on the stage when the oocytes develop (Hagedorn *et al*., 1975; Hagedorn, 1985). Careful dissections allowed demonstration that the follicle cells surrounding terminal oocytes are able to produce these compounds *de novo* from cholesterol (Goltzené *et al*., 1978). Labeling was only observed in vitellogenic follicles (stage 10), suggesting local effects of ecdysteroids (paracrine), as expected from the inhibitory effect of ecdysteroids on selective stages of oocyte maturation (Soller *et al*., 1999). These maternal ecdysteroids can also be stored as conjugates by developing oocytes and/or are released into the hemolymph for distribution within the adult. There, they may retain specific endocrine functions such as the control of vitellogenesis, of sexual pheromone biosynthesis, or of ovarian cyclic activity. p0615

Insect testes also contain ecdysteroids and it has been proposed that the testis interstitial tissue plays a role similar to the Leydig cells of vertebrates. When cultured *in vitro*, testes indeed release small amounts of a complex mixture of immunoreactive compounds (Loeb *et al*., 1982), and this release can be stimulated by an ecdysiotropic peptide originating in the brain (Loeb *et al*., 1988). They efficiently convert 2,22,25-trideoxyecdysone into E and 20E (Jarvis *et al*., 1994b). However, the direct evidence for a complete *de novo* biosynthetic pathway in testes is still lacking, and thus a partial synthesis starting from some downstream biosynthetic intermediate or ecdysteroid conjugate cannot be excluded at present. The amounts of ecdysteroids involved are always low as compared to those produced by ovaries, and the absence of a prominent component within the ecdysteroids released *in vitro* does not strongly argue for an endocrine function. Consistent with this hypothesis is the lack of *dib* and *phm* expression in testes, as noticed in the RT-PCR study of various *Drosophila* tissues (**Figure 12a**), although much further study is needed to affirm this result. p0620

s0415 p0625 **3.3.4.4.2. Other tissues?** The idea of alternative sites of ecdysteroid production has arisen from much experimental data that do not fit with the classical scheme (Redfern, 1989; Delbecque *et al*., 1990). Prothoracic glands of *Tenebrio molitor* (Coleoptera) degenerate during the pharate pupal stage, so the pharate pupal and pupal ecdysteroid

peaks must arise from another source (Glitho *et al*., 1979). *In vitro* cultivated epidermal fragments of *Tenebrio* pupae release significant amounts of ecdysteroids, and they convert 2,22,25-trideoxyecdysone (but not C) into E. This release is connected with the epidermis cell cycle, as it can be abolished by treatment with mitomycin (Delbecque *et al*., 1990 and references therein). The prothoracic glands of larvae and pupae of the mosquito, *Ae. aegypti*, do not secrete any ecdysteroid when cultured *in vitro*, whereas fragments of thorax and abdomen do secrete ecdysteroids (Jenkins *et al*., 1992). In the same way, epidermal fragments from locust last instar nymphs secrete ecdysteroids *in vitro* (Cassier *et al*., 1980), and cell lines derived from imaginal discs display a rhythmic ecdysteroid production (Mesnier *et al*., 2000). It should be emphasized that prothoracic glands have an ectodermal origin. Therefore, the general epidermis is expected to be the source of molting hormones (see, e.g., Bückmann, 1984; Lachaise *et al*., 1993). Interestingly, it was recently demonstrated that skin is also a primary steroidogenic tissue in humans (Thiboutot *et al*., 2003).

p0630 Other approaches have raised similar questions. Insects deprived of molting glands can still molt (e.g., *Periplaneta americana*, Gersch, 1979; *M. sexta*, Sakurai *et al*., 1991); ovariectomized adult insects still contain/produce ecdysteroids (Delbecque *et al*., 1990). Thus, there are data that cannot be explained, unless an alternative ecdysteroid source is postulated. At this level, we should clearly distinguish between primary and secondary sources (Delbecque *et al*., 1990). Primary sources can be defined as tissues that are capable of the *de novo* synthesis of ecdysteroids starting from C or related phytosterols. Secondary sources release ecdysteroids following the hydroxylation of late intermediates or the hydrolysis of conjugates, and therefore do not contain the whole set of enzymes required for de novo synthesis. It is expected, with the complete characterization of all the various *Drosophila* steroidogenic enzymes, that these molecular tools will become available for other insect species as well (e.g., *Aedes*, *Tenebrio*) and they will allow the identification of the tissues that express steroidogenic enzymes in these species.

p0635 Finally, the importance and mechanism of ecdysteroid biosynthesis during early embryonic development is still not well understood. Early metabolic studies performed in *Locusta* (Koolman 1989) suggested that ecdysteroids were provided by maternal stores at this stage. However, the very existence of the lethal embryonic Halloween mutations and the results of *in situ* hybridizations using *dib*, *sad*, and *phm* probes argue for *de novo* ecdysteroid biosynthesis during early embryogenesis in *Drosophila*, as has now been described in *Bombyx* embryos (Sonobe *et al*., 1999), and not simply from the hydrolysis of maternally derived ecdysteroid stores within the developing embryo (Bownes *et al*., 1988; Grau and Gutzeit, 1990; Kozlova and Thummel, 2003).

3.3.5. Ecdysteroid Metabolism

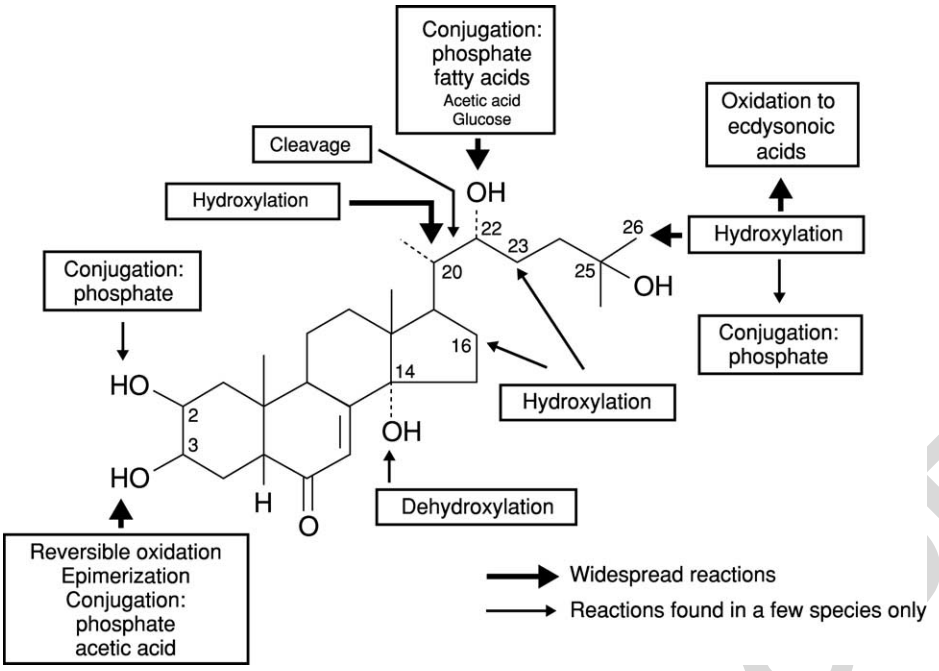
3.3.5.1. Introduction

Early reviews are available on ecdysteroid metabolism (Rees and Isaac, 1984, 1985; Lagueux *et al*., 1984; Lafont and Koolman, 1984), including two on synthetic (Smith, 1985) and degradative (Koolman and Karlson, 1985) aspects of regulation of ecdysteroid titer in the first edition of this series, together with several articles in the book edited by Koolman (1989) and a more recent one (Rees, 1995).

In view of the diverse nature of the insect class, there is always a danger of attempting to unify their metabolic processes into common schemes. Certainly, there is considerable species variation in ecdysteroid metabolic pathways. The pathways have been primarily deduced from examination of the fate of [^{3}H]-ecdysteroids in various species of whole insects at various stages of development, or in various tissues incubated *in vitro*. Although in many cases, the metabolic products have been incompletely characterized, such studies have been complemented by the isolation and physical characterization of metabolites.

Defined changes in the ecdysteroid titer are mandatory for correct functioning of the hormone system during development. Regulation of the ecdysteroid titer involves the rates of ecdysone biosynthesis, conversion into the generally more active 20E, inactivation, and excretion. The presence of ecdysteroid carrier proteins, which may protect the hormone to a certain extent from inactivating enzymes, may influence the fate of circulating ecdysteroids. From the limited information available, there is an apparent species-dependent heterogeneity in ecdysteroid transport, with some species lacking binding proteins, whilst others differ in relation to the specificity of protein binding (Karlson, 1983; Whitehead, 1989; Koolman, 1990).

One difficulty is ensuring that some ecdysteroids that have been generally regarded as hormonally inactive metabolites do not possess biological activity in particular systems. For example, although 20E is regarded as the principal active ecdysteroid in most species, there is evidence that E, 3-dehydro-, and 26-hydroxy-ecdysteroids may have hormonal



f0075 **Figure 15** Major reactions of ecdysone metabolism. (Modified from Lafont, R., Connat, J.-L. **1989**. Pathways of ecdysone metabolism. In: Koolman, J. (Ed.), Ecdysone, From Chemistry to Mode of Action. Georg Thieme Verlag, Stuttgart, p. 167.

activity in certain systems (see Lafont and Connat, 1989; Dinan, 1989). The tissues that are most active in metabolizing ecdysteroids are the fat body, Malpighian tubules and the midgut. In larvae, ecdysteroids are excreted via the gut or Malpighian tubules and occur in feces as unchanged hormones and various metabolites including conjugates, ecdysteroid acids, and 3-epiecdysteroids (Lafont and Koolman, 1984; Koolman and Karlson, 1985).

p0660 Over the years, there have been a plethora of studies on the metabolism of E in various species and the major modifications that have been detected are summarized in **Figure 15**. This concentrates on insects and does not include some more specialized transformations such as found in vertebrates. Since most of the metabolic studies have utilized [^{3}H]-ecdysone labeled in the side chain, the importance of side chain cleavage may well have been underestimated, since the labeled side chain fragment may not have been detected under the conditions used. This section will concentrate on work published since 1985. Studies *in vitro* on E metabolism in different tissues of various species (Koolman and Karlson, 1985), together with the enzymes catalyzing the reactions (Weirich, 1989), have been tabulated and reviewed.

s0430 **3.3.5.2. Ecdysone 20-Monooxygenase**

p0665 E, either produced directly by the prothoracic glands or in many Lepidoptera via reduction of 3dE by hemolymph 3-dehydroecdysone 3β-reductase, undergoes ecdysone 20-monooxygenase-catalyzed hydroxylation to yield 20E (**Figure 11**). The 20-hydroxylation reaction is important in the production of 20E, which is generally far more active than E. Earlier work on ecdysone 20-monooxygenase (ecdysone 20-hydroxylase; EC 1.14.99.22) is covered in several reviews (Weirich *et al.*, 1984; Lafont and Koolman, 1984; Smith, 1985; Weirich, 1989, Grieneisen, 1994; Rees, 1995).

3.3.5.2.1. Properties s0435

3.3.5.2.1.1. Species and tissues Early studies on ecdysone 20-monooxygenase in certain tissues of various insect species, together with the biochemical properties of some of these enzymes have been reviewed (Weirich *et al.*, 1984; Smith, 1985). Recently, the gene coding for ecdysone 20-monooxygenase activity in *Drosophila* (*shd*, CYP314A1) has been identified and characterized (Petryk *et al.*, 2003; see Section 3.3.4.3.1). The enzyme is expressed in several peripheral tissues, primarily in the fat body, Malpighian tubules, and midgut. Although generally lower activity may occur in certain other tissues including the ovaries and integument, it is absent from prothoracic glands. s0440 p0670

3.3.5.2.1.2. Subcellular distribution Depending on species and tissue, ecdysone 20-monooxygenase may be either mitochondrial or microsomal or occur s0445 p0675

in both subcellular fractions. For example, a thorough study has established the occurrence of both high mitochondrial and microsomal activities in midgut from *M. sexta* (Weirich *et al*., 1985). Although the mitochondrial enzyme has the higher V_{max}, since it also has the higher apparent K_M (E) (1.63×10^{-5} M versus 3.67×10^{-7} M for the microsomal one), at physiological E concentrations (10^{-7}–10^{-8} M), it is only one-eighth to one-tenth as active as the microsomal enzyme. Thus, the microsomal ecdysone 20-monooxygenase is the primary enzyme activity in *Manduca* midgut (Weirich *et al*., 1996). Similarly, in larvae of *Drosophila* and fed adult females of *Aedes aegypti*, dual localization of enzymatic activity has been shown (Smith and Mitchell, 1986; Mitchell and Smith, 1986; see Section 3.3.4.3.1). Furthermore, in larvae of the housefly, *Musca domestica*, although appreciable ecdysone 20-monooxygenase activity occurs in both mitochondrial and microsomal fractions, pretreatment of larvae with E results in an increase in V_{max} and a decrease in K_M values in mitochondria, but not in microsomes. This suggested that the mitochondrial E 20-monooxygenase is under regulatory control by E in the larval stage and that only such activity has a physiological role during development in *M. domestica* (Agosin *et al*., 1988). Since one of the mitochondrial cytochrome P450 species fractionated from E-induced mitochondria catalyzed the reaction at significantly higher rates than the other five species in a reconstituted system, it was postulated that the 20-monooxygenase is a mitochondrial process that requires the induction of a low K_M P450 species by ecdysone (Agosin and Srivatsan, 1991). In adults of both sexes of the cricket, *Gryllus bimaculatus*, E 20-monooxygenase activity is located primarily in the microsomal fraction of the midgut, with peak activity at day 4 following ecdysis; no detectable activity was observed in fat body, ovaries, Malpighian tubules, and carcass tissues (Liebrich *et al*., 1991; Liebrich and Hoffmann, 1991).

s0450 p0680 *3.3.5.2.1.3. Cofactor requirements and cytochrome P450 properties* Ecdysone 20-monooxygenase requires NADPH as a source of reducing equivalents. As in the case of vertebrates, the mitochondrial system can use other secondary sources of reducing equivalents such as NADH or Krebs cycle intermediates, presumably by intramitochondrial generation of NADPH via transhydrogenation of $NADP^+$ (Greenwood and Rees, 1984; Smith, 1985). Similarly, a requirement of the 20-monooxygenase for oxygen has been firmly established. Evidence for involvement of cytochrome P450 has been obtained by inhibition of activity with carbon monoxide, its maximal reversal with light at 450 nm as revealed by a photochemical action spectrum, use of a range of specific inhibitors of cytochrome P-450, together with demonstration of the presence of the protein by CO difference spectroscopy (see Smith, 1985).

s0455 p0685 *3.3.5.2.1.4. Substrate specificity* Although reconstituted pure vertebrate steroid hydroxylating cytochrome P450s exhibit substrate specificity, it is not absolute and may hydroxylate more than one position on the steroid substrate albeit at different rates (Sato *et al*., 1978). Since pure cytochrome $P450_{ecdysone}$ has not been available for such reconstitution experiments (see Section 3.3.4.3.1), only preliminary data are available using mitochondrial or microsomal preparations where multiple P450s may occur. *Locusta migratoria* microsomes do not react with 2β-acetoxy- or 3-dehydroecdysone, nor with the 5α-epimer of E; neither will they hydroxylate 20E nor 3d20E at any other position (Feyereisen and Durst, 1978). In the case of *Manduca* fat body mitochondria, the results of a competition assay, which is presumed to reflect enzyme–substrate binding, surprisingly showed that the most effective competitor was not E, but 2,25-dideoxyecdysone, followed in order of decreasing competition by E, 22-deoxyecdysone, 2-deoxyecdysone, 22,25-dideoxyecdysone, and 2,22,25-trideoxy-ecdysone (Smith *et al*., 1980). Interestingly, 20-hydroxylation of 26-hydroxyecdysone, ecdysonoic acid, and 3-epiecdysone has been observed in *P. brassicae* (Lafont *et al*., 1980).

s0460 p0690 *3.3.5.2.1.5. Kinetics and product inhibition* The K_M and V_{max} values determined for the various mitochondrial and microsomal E 20-monooxygenase systems show substantial differences (Weirich *et al*., 1984; Smith, 1985). However, the extent to which these differences are real or reflect different detailed methodologies is uncertain (see Smith, 1985). However, several studies have demonstrated competitive inhibition of the enzyme system by its product, 20E.

s0465 p0695 **3.3.5.2.2. Control during development** Early studies on regulation of E 20-monooxygenase activity have been comprehensively reviewed (Smith, 1985).

s0470 p0700 *3.3.5.2.2.1. Developmental changes in ecdysone 20-monooxygenase activity* Although many early studies on changes in the metabolism of E *in vivo* suggested that E 20-monooxygenase activities vary during development, definitive demonstration of this required *in vitro* studies, since several factors

could conceivably complicate the former (see Smith, 1985).

p0705 In the migratory locust, *Locusta migratoria*, peaks in E 20-monooxygenase activity, microsomal cytochrome P450 levels, and NADPH cytochrome *c* reductase activity in both fat body and Malpighian tubules coincided with the peak of 20E in the hemolymph (Feyereisen and Durst, 1980). This suggested that the increases in E 20-monooxygenase activities were due to increased amounts of the monooxygenase components. However, in several species, the peak of E 20-monooxygenase activity preceded that of the hormone titer during the last larval instar (*Schistocerca gregaria* Malpighian tubules: Johnson and Rees, 1977; Gande *et al*., 1979; *Calliphora vicina*: Young, 1976). Similarly, in the fifth larval instar of *Manduca*, fat body and midgut monooxygenase activities exhibited 10-fold and 60-fold fluctuations, respectively, that were not temporally coincident with one another, or with the major hemolymph ecdysteroid titer peak (Smith *et al*., 1983). These peak activities for fat body and midgut monooxygenases were temporally coincident and succedent, respectively, with the small hemolymph peak of ecdysteroid titer on day 4 that is responsible for the change in commitment during larval–pupal reprogramming; both activities were at basal levels during the major hemolymph ecdysteroid titer peak on day 7–8.

p0710 The exact significance of these E 20-monooxygenase developmental profiles remains obscure. Since the apparent K_M values for the fat body and midgut systems were fairly constant during the instar, whereas the apparent V_{max} values in each tissue fluctuated in a manner that was temporally and quantitatively coincident with the fluctuations in monooxygenase activity, it suggested that the changes in these activities were due to changes in amounts of the enzymes. More recently, the peak in *Manduca* midgut E 20-monooxygenase activity at the onset of wandering on day 5 has been further examined (Weirich, 1997). During the day preceding the peak, the microsomal activity increased 60-fold (total activity) or 115-fold (specific activity) and decreased gradually within 2 days after the peak. In contrast, the mitochondrial activity increased only 1.3- to 2.4-fold (total and specific activities, respectively) before the peak, but declined more rapidly than the microsomal activity after the peak. This indicates that mitochondrial and microsomal E 20-monooxygenase activities are controlled independently and that changes in the physiological rate of E 20-hydroxylation in the midgut are affected primarily by changes in the microsomal E 20-monooxygenase activities. Further studies on *M. sexta* showed that basal levels of E 20-monooxygenase activity occurred during the later stages of embryogenesis and that a peak of monooxygenase activity occurred late in the fourth instar in both fat body and midgut at the time of spiracle apolysis (Mitchell *et al*., 1999). Both the fat body and midgut hydroxylase activities were basal during the pupal stage and only rose late in pharate–adult development, just prior to adult eclosion.

p0715 In another lepidopteran, the gypsy moth *Lymantria dispar*, preliminary studies with homogenates of various tissues taken at different times in the fifth instar surprisingly showed that E 20-monooxygenase activity in fat body, midgut, and Malpighian tubules exhibited peak activity coinciding with the peak in the hemolymph ecdysteroid titer on the penultimate day of the instar (Weirich and Bell, 1997). In *P. brassicae*, E 20-hydroxylase activity occurs in microsomes of pupal wing discs, with an apparent K_M of 58 nM for E. The activity varied during pupal–adult development with a maximum on day 4, a time when ecdysteroid titers are high (Blais and Lafont, 1986). Although the activity is low, the peak activity is sufficient to hydroxylate 25% of endogenous E in pupae. In eggs of the silkworm, *B. mori*, during embryonic development, E 20-monooxygenase activity was primarily microsomal and remained low in diapause eggs, whereas that in nondiapause eggs increased from the gastrula stage (Horike and Sonobe, 1999). This increase in 20-monooxygenase activity was prevented by actinomycin D and α-amanitin, suggesting the requirement for gene transcription. In the last larval stage of *Diploptera punctata*, maximal 20-monooxygenase activity of midgut homogenates, which was primarily microsomal, occurred on days 5–9, whereas the hemolymph ecdysteroid titer exhibited a small peak on day 10 with a major one on day 17 (Halliday *et al*., 1986).

p0720 In *Drosophila*, E 20-monooxygenase activity has been assayed in homogenates throughout the life cycle (Mitchell and Smith, 1988; Petryk *et al*., 2003; see Section 3.3.4.3.1). There was a small peak of enzymatic activity in the egg that was temporally coincident with the major peak in the egg ecdysteroid titer. This was followed by several larger fluctuations in activity during the first, second, and early third larval stadia, with a large peak during the wandering stage that remained high during early pupariation, before dropping to basal levels by pupation and remaining at these low levels through to adults. The major peak in E 20-monooxygenase activity during the wandering stage and early pupariation is temporally coincident with the major ecdysteroid titer peak that occurs at puparium

formation. Similarly, in the Diptera *Neobellieria bullata* and *Parasarcophaga argyrostoma* fat body microsomal E 20-monooxygenase activity exhibits a peak at the beginning of wandering behavior in the third instar (Darvas *et al*., 1993). Clearly, the exact significance of the developmental profiles in E 20-monooxygenase activities during development and, particularly, in relation to the ecdysteroid titer in various species is far from clear.

3.3.5.2.2.2. Factors affecting ecdysone 20-monooxygenase activity Not only is little known about the physiological significance of the developmental fluctuations in ecdysone 20-monooxygenase during development, but the factors and mechanisms responsible for such changes are ill understood. As alluded to earlier, the product of the reaction, 20E, is a competitive inhibitor, although on the basis of K_i values and hormone titers, there is doubt as to whether 20E could affect the monooxygenase activity under physiological conditions in all species examined (see Smith, 1985).

In certain species, there is evidence that E or 20E may induce E 20-monooxygenase systems. For example, in last instar larvae of *L. migratoria*, where the peak in ecdysteroid titer and E 20-monooxygenase activity are coincident, injection of E or 20E at an earlier time led to selective induction of cytochrome P450 systems, including E 20-monooxygenase, lauric acid ω-hydroxylase, NADPH-cytochrome *c* reductase, and cytochrome P450 (Feyereisen and Durst, 1980). This induction is apparently a specific effect of active molting hormones since 22-isoecdysone and 22,25-dideoxyecdysone were inactive. Such induction apparently requires protein synthesis, since it is abolished after simultaneous injection of E and actinomycin D, puromycin, and cycloheximide. Furthermore, involvement of the brain in this induction by E is ruled out, since it was observed in isolated abdomens.

Studies on housefly larvae corroborate regulation of E 20-monooxygenase activity by E (Srivatsan *et al*., 1987). Interestingly, although both mitochondria and microsomes catalyze the reaction, only the former E 20-monooxygenase appears to be regulated by E.

In the midgut of final larval instar tobacco hornworm, *Manduca*, the 50-fold increase in E 20-monooxygenase activity at the onset of the wandering stage, is prevented by actinomycin D and cycloheximide, indicating a requirement for transcription and protein synthesis (Keogh *et al*., 1989). This increase in 20-monooxygenase activity could also be elicited in head (but not thoracic) ligated animals by a brain-retrocerebral complex factor(s) released at the time of prothoracicotropic hormone (PTTH) release (see **Chapter 3.2**). It was reported that E or 20E could elicit the increase in monooxygenase activity in both head and thoracic ligated animals, suggesting the operation of a neuroendocrine–endocrine axis (PTTH → prothoracic glands → E → midgut) in the induction. Furthermore, it was reported that the ecdysteroid receptor agonist, RH 5849, could also elicit the increase in midgut E 20-monooxygenase activity in head or thoracic ligated larvae (Keogh and Smith, 1991). However, the finding that neither 20E nor RH5849 induced E 20-monooxygenase activity, but rather ecdysteroid 26-hydroxylase activity, in the cotton leafworm, *Spodoptera littoralis* (Chen *et al*., 1994b; see Section 3.3.5.3.2) prompted the re-investigation of the situation in *Manduca* midgut. In this study, it was shown that although 20E and RH5849 could induce E 20-monooxygenase in subcellular fractions of midgut of *Manduca*, there was a much stronger induction of ecdysteroid 26-hydroxylase activity (Williams *et al*., 1997). In the original studies (Keogh *et al*., 1989; Keogh and Smith, 1991), 26-hydroxylation was not detected, presumably because the thin-layer chromatography system used for the assay may not have resolved 20E and 26-hydroxyecdysone.

In fat body of sixth larval instar *S. littoralis*, E 20-monooxygenase activity is predominantly mitochondrial, with much less activity in the microsomes (Hoggard and Rees, 1988). The former activity exhibits a peak of activity during the instar at 72 h, when the larvae stop feeding (Chen *et al*., 1994a). To ask the question as to what is the molecular mechanism underlying this increased activity, various antibodies raised against components of vertebrate mitochondrial steroidogenic enzyme systems have been used as potential probes for the corresponding insect proteins. Since correlation was observed between developmental changes in mitochondrial E 20-monooxygenase activity and the abundance of polypeptides recognized by cytochrome $P450_{11\beta}$ antibody and a polypeptide recognized by the adrenodoxin reductase antibody, it suggests that developmental changes in the abundance of components of the monooxygenase system may be important in developmental regulation of the enzyme expression (Chen *et al*., 1994a).

There is evidence that E 20-monooxygenase activity may also be influenced by dietary factors, including plant flavonoids and other allelochemicals. For example, assay of activities *in vitro* of E 20-monooxygenase from certain species has demonstrated that a range of plant flavonoids and other allelochemicals, including azadirachtin, significantly inhibit the activity in a dose-dependent manner,

whereas certain flavonols and other allelochemicals stimulate activity (Smith and Mitchell, 1988; Mitchell *et al*., 1993). However, none of the compounds tested elicited effects at very low concentrations. Furthermore, studies on the influence of dietary allelochemicals on midgut microsomal E 20-monooxygenase activity in the fall armyworm, *Spodoptera frugiperda*, have shown that various indoles, flavonoids, monoterpenes, sesquiterpenes, coumarins, methylenedioxyphenyl compounds, and ketohydrocarbons all caused significant stimulation of activity (Yu, 1995, 2000). Significantly, enzyme inducibility was different between E 20-monooxygenase and xenobiotic-metabolizing cytochrome P450 monooxygenases.

p0755 In addition, there have been numerous studies on the effects of a plethora of synthetic inhibitors on E 20-monooxygenase activity in various species (e.g., Kulcsar *et al*., 1991; Darvas *et al*., 1992; Jarvis *et al*., 1994a).

s0480 p0760 **3.3.5.2.3. Potential modulation by covalent modification** The initial indication that hormonal factors might regulate ecdysteroid metabolism was furnished by the demonstration that a forskolin-induced increase in intracellular cyclic AMP in *Calliphora* fat body *in vitro* led to decreased rates of conversion of E into 20E and of the latter to other metabolites (Lehmann and Koolman, 1986, 1989). Thus, it was suggested that since forskolin mimics the action of peptide hormones by activating the adenylate cyclase system (see **Chapter 3.2**), a peptide hormone, possibly a neuropeptide, might be involved in the regulation of E metabolism. Such activation of adenylate cyclase leads to an increase in intracellular cAMP, which enhances protein (enzyme) phosphorylation via cAMP-dependent protein kinases (see **Chapter 3.2**). Such a process as part of a reversible phosphorylation-dephosphorylation of enzymes provides a mechanism for rapid modulation of enzymatic activities. In the case of vertebrate mitochondria, such a control mechanism has been reported only for a few enzyme complexes. In such systems, relevant endogenous mitochondrial protein kinase and phosphoprotein phosphatase activities have been demonstrated (see Hoggard and Rees, 1988). In fact, experimental support for such modulation of E 20-monooxygenase activity by phosphorylation-dephosphorylation has been obtained. In the case of both the microsomal and broken mitochondrial fractions from fat body of *S. littoralis*, experiments involving treatments with phosphatase inhibitors, exogenous phosphatase, protein kinase, or the adenylate cyclase activator, forskolin, provide indirect evidence that the enzyme system may exist in an active phosphorylated state and inactive dephosphorylated state (Hoggard and Rees, 1988; Hoggard *et al*., 1989). However, confirmation of this notion requires more definitive direct evidence, including direct demonstration of the incorporation of labeled phosphate into components of the E 20-monooxygenase, together with demonstration of the occurrence of an appropriate mitochondrial protein kinase and protein phosphatase.

p0765 There are many reports of the phosphorylation of cytochrome P450s (Koch and Waxman, 1991), including steroidogenic $P450_{11\beta}$ from bovine adrenal mitochondria (Defaye *et al*., 1982), with limited evidence for the physiological significance of the phenomenon. The activity of the microsomal cytochrome P450-dependent cholesterol 7α-hydroxylase may also be modulated by phosphorylation-dephosphorylation (Goodwin *et al*., 1982).

p0770 Since the pharmacologically inactive forskolin derivative, 1,9-dideoxy-forskolin, as well as forskolin, induced a dose-dependent inhibition of E 20-monooxygenase, the effect must be direct rather than via activation of adenylate cyclase (Keogh *et al*., 1992). Thus, care must be exercised in the design of such experiments to avoid possible misinterpretation of data.

s0485 3.3.5.3. Ecdysteroid Inactivation and Storage

p0775 Insect development is absolutely dependent on defined changes in ecdysteroid titer. We have already considered the major hormone activation step, the E 20-monooxygenase-catalyzed reaction, and will now consider the major pathways contributing to hormone inactivation. As alluded to in Section 3.3.1, there are many reviews covering ecdysteroid metabolism and inactivation. It is difficult to establish unequivocally that a particular compound is an inactivation product, since an ecdysteroid that is essentially hormonally inactive in one assay may have activity in other systems (Dinan, 1989; Lafont and Connat, 1989).

p0780 Early studies on [^{3}H]-ecdysteroid metabolism in insects and other arthropods frequently reported the formation of polar products that were hydrolyzable with various hydrolytic enzymes, especially the so-called "*Helix pomatia* sulfatase," releasing free ecdysteroids. However, unless the enzyme is absolutely pure, no conclusion can be drawn regarding the nature of the conjugate.

p0785 It is convenient to consider metabolism and inactivation of ecdysteroids in relation to (1) potential re-utilization of the products from adult females during embryogenesis, or (2) removal of hormonal activity and excretion.

s0490 **3.3.5.3.1. Ecdysteroid storage, utilization, and inactivation**

s0495 p0790 *3.3.5.3.1.1. Phosphate conjugates* Ecdysteroid "storage" has been reviewed (Isaac and Slinger, 1989). Initially, a relatively high concentration of polar conjugated ecdysteroids was reported in *Bombyx* eggs (Mizuno and Ohnishi, 1975). However, the demonstration that ecdysteroids in locust ovaries/eggs occurred almost exclusively as polar conjugates (Dinan and Rees, 1981a) and their identification as the 22-phosphate derivatives in *S. gregaria* (Rees and Isaac, 1984) and *L. migratoria* (Lagueux *et al*., 1984) opened the way for more definitive investigations in various species. Significantly, these maternal conjugates are phosphorylated at C_{22} and are presumably inactive, since a free 22*R* hydroxyl group is important for high hormonal activity. The ovarian ecdysiosynthetic tissue has been shown to be the follicle cells (see Lagueux *et al*., 1984) and the cytosolic phosphotransferase from *S. gregaria* follicle cells has been characterized (Kabbouh and Rees, 1991a). The enzyme is dependent on ATP/Mg^{2+} for high activity, and activity varies during ovarian development, reaching a peak at the end of oogenesis in agreement with the titer of ecdysteroid 22-phosphates in the oocytes. Furthermore, enzymatic activity can be induced in terminal oocytes by E or 20E treatment, which suggests a physiological mechanism of increasing conjugation as biosynthesis of ecdysteroids in follicle cells proceeds (Kabbouh and Rees, unpublished data).

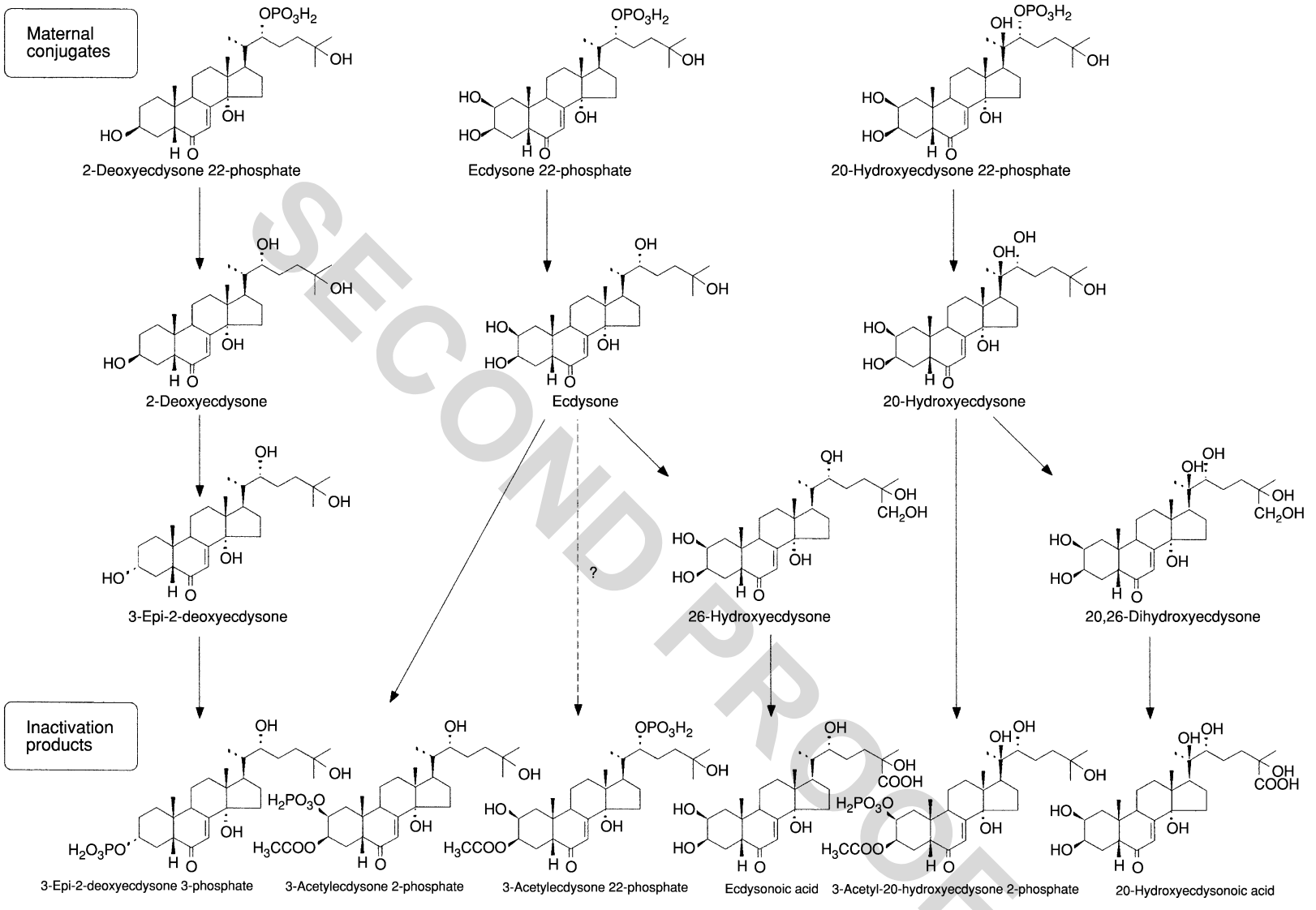
p0795 The majority of the ovarian ecdysteroid conjugates, together with some free hormone, was passed into the oocytes (Dinan and Rees, 1981a; see Rees and Isaac, 1984; Hoffmann and Lagueux, 1985). During embryogenesis, locusts undergo several molts and cycles of cuticulogenesis coincident with peak titers of free ecdysteroids (Sall *et al*., 1983). Support for the hypothesis that the ecdysteroid 22-phosphates, following enzymatic hydrolysis, could at least provide one source of hormone in embryogenesis, particularly before differentiation of the prothoracic glands, was provided by demonstration of enzymatic hydrolysis of conjugates in a cell-free system from embryos (Isaac *et al*., 1983). Significantly, activity of the enzyme increases around the time of greater utilization of the conjugates, which also corresponds to an increase in free ecdysteroid titer (Scalia *et al*., 1987). Moreover, an ecdysteroid-phosphate phosphatase was recently purified and cloned from *Bombyx* embryos and shown to be active only in nondiapausing eggs (Yamada and Sonobe, 2003). In several species, there is no doubt that embryonic synthesis of ecdysteroids occurs in the later stages. Reviews on the role of ecdysteroids in reproduction and embryonic development are available (Hagedorn, 1985; Hoffmann and Lagueux, 1985).

p0800 The egg is a closed system and, therefore, decreases in the embryonic hormone titer occur largely by metabolic inactivation, with accumulation of products prior to emergence (Dinan and Rees, 1981b). This accumulation facilitated the identification of a number of new ecdysteroid derivatives, presumed to be inactivation products, including ecdysteroid 2-phosphate 3-acetate derivatives, 3-epi-2-deoxyecdysone 3-phosphate, and ecdysteroid 26-oic acids (see Rees and Isaac, 1984; Hoffmann and Lagueux, 1985; Rees and Isaac, 1985). **Figure 16** shows probable metabolic pathways of the major ecdysteroid 22-phosphates in developing eggs of *S. gregaria* (Rees and Isaac, 1984).

p0805 In *S. gregaria*, at the beginning of embryonic development, the ecdysteroids (conjugates and free) occur only in the yolk, whereas after blastokinesis, they occur in the embryo (Scalia *et al*., 1987). *Locusta migratoria* produce diapause eggs under short-day (SD) conditions and nondiapause eggs under long-day (LD) conditions. The demonstration that the total detected ecdysteroids (primarily phosphate conjugates) in newly laid eggs was more than three times higher in nondiapause eggs than in diapause eggs suggests that ecdysteroids may be involved in the control of embryonic diapause in this species (Tawfik *et al*., 2002).

p0810 Surprisingly, in *Manduca*, the major conjugate in newly laid eggs is 26-hydroxyecdysone 26-phosphate (Thompson *et al*., 1985b). This compound undergoes hydrolysis during embryogenesis, with 20-hydroxylation of the released hormone occurring, yielding 20,26-dihydroxyecdysone, followed by reconjugation of 26-hydroxyecdysone to produce the 22-glucoside derivative; formation of 3α-epimers and 26-oic acids also occurs during embryogenesis (Warren *et al*., 1986; Thompson *et al*., 1987b, 1988). The proposed pathways of metabolism of 26-hydroxyecdysone 26-phosphate in the developing embryos of *Manduca* are given in **Figure 17**.

p0815 Early in embryogenesis, much of the 26-hydroxyecdysone 26-phosphate is hydrolysed, resulting in a peak of 26-hydroxyecdysone just prior to the appearance of the first serosal cuticle (Dorn *et al*., 1987). The 20,26-dihydroxyecdysone titer reaches a peak at about the time of deposition of the first larval cuticle late in embryogenesis, raising the possibility that this ecdysteroid may have a hormonal role at this stage of development (Warren *et al*., 1986). Significantly, the concentrations of E and 20E are minimal throughout embryogenesis. That the foregoing metabolic changes are related to



f0080 **Figure 16** Probable metabolic pathways of the major ecdysteroid 22-phosphates in developing eggs of *Schistocerca gregaria*. (Modified from Rees, H.H., Isaac, R.E. **1984**. Biosynthesis of ovarian ecdysteroid phosphates and their metabolic fate during embryogenesis in *Schistocerca gregaria*. In: Hoffmann, J.A., Porchet, M. (Eds.), Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones. Springer-Verlag, Berlin, pp. 181–195.)

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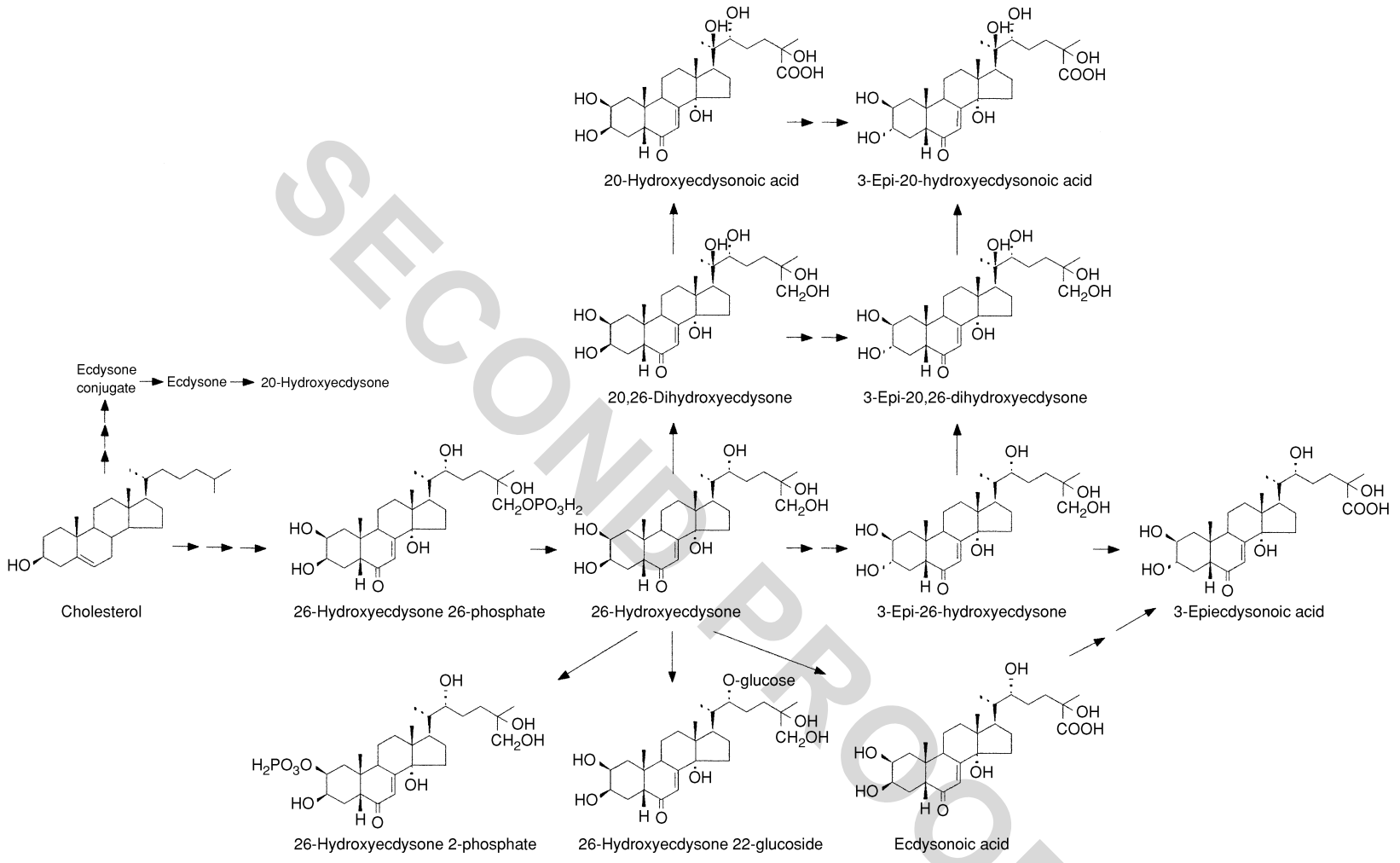


f0085 **Figure 17** Proposed pathways of metabolism of 26-hydroxyecdysone 26-phosphate during embryogenesis of *M. sexta* (Thompson, M.J., Svoboda, J.A., Lozano, R., Wilzer, K.R., **1988**. Profile of free and conjugated ecdysteroids and ecdysteroid acids during embryonic development of *Monduca sexta* (L.) following maternal incorporation of [^{14}C] cholesterol. *Arch. Insect Biochem. Physiol. 7*, 157–172. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

embryogenesis is indicated by their virtual absence in incubated unfertilized eggs of *Manduca* (Feldlaufer *et al*., 1988). A plethora of free ecdysteroids and phosphate conjugates have been isolated from ovaries of the silkworm, *B. mori*, and include the following: E, 20E, 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, 2,22-dideoxy-20-hydroxyecdysone and their 22-phosphates, bombycosterol and bombycosterol 3-phosphate (Ohnishi, 1986; Ohnishi *et al*., 1989), 22-deoxy-20-hydroxyecdysone and its 3-phosphate (Kamba *et al*., 1994), 3-epi-22-deoxy-20-hydroxyecdysone and its 2-phosphate (Mamiya *et al*., 1995), 3-epi-22-deoxy-20,26-dihydroxyecdysone, plus 3-epi-22-deoxy-16β, 20-dihydroxyecdysone and their 2-phosphates (Kamba *et al*., 2000b), as well as 2,22-dideoxy-23-hydroxyecdysone and its 3-phosphate (Kamba *et al*., 2000a). Although the metabolic relationships of many of the foregoing ecdysteroids can be surmised, they await experimental verification. The occurrence of 23- and 16β-hydroxy ecdysteroids is unusual. An ATP: ecdysteroid phosphotransferase that is active on a number of ecdysteroid substrates has been demonstrated in *B. mori* ovaries (Takahashi *et al*., 1992).

p0820 Evidence has been furnished suggesting that 20E, acting via the receptor (see **Chapter 3.5**), is responsible for the developmental difference between diapause and nondiapause in *B. mori* embryos and that a continuous supply of 20E may be required to induce embryonic development (Makka *et al*., 2002). In agreement with this is evidence suggesting that 20-hydroxylation of E may be a rate-limiting step in diapause silkworm eggs (Sonobe *et al*., 1999). Furthermore, dephosphorylation of ecdysone 22-phosphate and its subsequent hydroxylation at C_{20} and C_{26} are prominent in nondiapause eggs, whereas phosphorylation of E is a major process in diapause eggs (Makka and Sonobe, 2000). Surprisingly, in *S. littoralis*, the newly laid eggs contain primarily 2-deoxyecdysone 22-phosphate (Rees, 1995).

s0500 p0825 *3.3.5.3.1.2. Fatty acyl conjugates* Quite different types of conjugates, viz. apolar ecdysteroid 22-fatty acyl esters, originally found in tick ovaries and eggs (Wigglesworth *et al*., 1985; Crosby *et al*., 1986a) (see **Chapter 3.16**), also occur in such tissues in certain Orthoptera. In the tick, *Boophilus microplus*, such conjugates appear to function as inactive, storage forms of maternal hormone, being utilized in early embryogenesis and undergoing subsequent inactivation by 20E-oic acid formation in conjunction with re-conjugation (Crosby *et al*., 1986b). However, the situation is complicated, since eggs of all tick species do not contain significant ecdysteroid 22-fatty acyl esters, some containing appreciable free hormones.

p0830 Analogous apolar ecdysone 22-fatty acyl esters, produced by the ovaries, have been identified in newly laid eggs of the house cricket, *Acheta domesticus* (Whiting and Dinan, 1989). The rate of fatty acylation by ovaries is developmentally regulated, increasing as the ovaries increase in size (Whiting *et al*., 1997). Evidence suggests that the ecdysone 22-fatty acyl esters undergo hydrolysis during embryogenesis, with the resulting E being converted to 20E, which is then inactivated by double conjugation, first with an uncharacterized polar moiety, followed by 22-fatty acylation (Whiting *et al*., 1993; Dinan, 1997).

p0835 The apolar esters in eggs of another orthopteran, the cockroach, *Periplaneta americana*, differ slightly in chromatographic properties from ecdysteroid 22-fatty acyl esters, although free ecdysteroids are released by hydrolysis with *Helix pomatia* enzymes or esterases (Slinger *et al*., 1986; Slinger and Isaac, 1988a). A storage role is suggested for these apolar conjugates, since they undergo hydrolysis during the first third of embryogenesis when there is also an increase in free and polar conjugated ecdysteroids (Slinger and Isaac, 1988b). Synthesis of such apolar esters by an ovary microsomal fraction from *P. americana* requires fatty acyl-CoA as cosubstrate, indicating that the enzyme is an acyl-CoA: ecdysone acyltransferase and not a hydrolase (Slinger and Isaac, 1988c).

p0840 Interestingly, significant levels of apolar or polar ecdysteroid conjugates do not occur in newly laid eggs of another cricket species, *Gryllus bimaculatus* (Espig *et al*., 1989). Similarly, it is noteworthy that significant amounts of detectable maternal ecdysteroid conjugates have not been reported in newlylaid eggs of many insect species (see e.g., Hoffmann and Lagueux 1985; Isaac and Slinger, 1989).

p0845 Comparison of the sequence similarity of *Drosophila* vitellins and part of the porcine triacylglycerol lipase, led to the intriguing hypothesis that ecdysteroid fatty acyl ester may bind to vitellin in the eggs as storage forms, the conjugates being made available for enzymic hydrolysis as the vitellin is degraded during embryogenesis (Bownes *et al*., 1988; Bownes, 1992). Such candidate ecdysteroid 22-fatty acyl esters are produced in adult female *Drosophila*, together with ecdysone 22-phosphate (Grau and Lafont, 1994b; Grau *et al*., 1995). However, the significance of binding of ecdysone 22-phosphate to an approx. 50 kDa protein in D*rosophila* ovaries is unclear at present (Grau *et al*., 1995).

s0505 p0850 **3.3.5.3.2. Inactivation and excretion in immature stages** Ecdysteroids such as E and 20E may be excreted *per se* or as metabolites via the gut or Malpighian tubules. Ecdysteroid metabolizing enzymes, particularly in the fat body, midgut, and Malpighian tubules, may facilitate excretion, in addition to inactivating the hormone. In fact, variation in the rate of excretion during development appears to be an important factor in determining the hormone titer. Furthermore, hormone inactivation by the gut may be important in protection of the insect from any ingested phytoecdysteroids (see below). In closed stages of insects, where excretion is impossible (egg and pupae), irreversible inactivation of ecdysteroids is important, with metabolites accumulating in the gut (bib1295 Koolman and Karlson, 1985; bib0995 Isaac and Slinger, 1989; bib1440 Lafont and Connat, 1989).

p0855 A comparative study of the metabolism of E and 20E in representatives of arthropods, mollusks, annelids, and mammals has led to the conclusion that the metabolic pathways differ strongly between species and that each class has evolved specific reactions (bib1415 Lafont *et al*., 1986).

s0510 p0860 *3.3.5.3.2.1. Conjugates and overall metabolism* Aspects of conjugate formation will be considered initially, since such compounds feature prominently in the metabolism and inactivation of ecdysteroids in immature stages of most insect species. Early studies on enzymatic aspects of ecdysteroid conjugation/hydrolysis have been reviewed (bib2640 Weirich, 1989). Originally, the identification of various conjugated ecdysteroid inactivation products (e.g., 3-acetylecdysone 2-phosphate), together with the ecdysteroid 26-oic acids from developed eggs of locusts before hatching facilitated elucidation of inactivation pathways in other systems (bib1495 Lagueux *et al*., 1984; bib2000 Rees and Isaac, 1984). In fact, in larval stages of locusts, the inactivation pathways (bib0655 Gibson *et al*., 1984; bib1735 Modde *et al*., 1984) proved to be very similar to the embryonic routes (see **Figure 16**), with pathways in other species having much in common (bib1440 Lafont and Connat, 1989). Some features of ecdysteroid metabolic pathways in various stages of a few species that have been reported since the first edition in 1985 will be considered initially. In *Manduca*, when labeled C was injected into 5th instar larvae and the metabolites isolated from day 8 pupae, the predominant ecdysteroid metabolites were 20E, 20,26-dihydroxyecdysone, 20-hydroxyecdysonoic acid, and 3-epi-20-hydroxyecdysonoic acid. Smaller amounts occurred as conjugates (phosphates) of 26-hydroxyecdysone, 3-epi-20-hydroxyecdysone, 20, 26-dihydroxyecdysone, and its 3α-epimer (bib1570 Lozano *et al*., 1988a,b). At various stages throughout pupal–adult development, the predominant metabolites were 3-epi-20-hydroxyecdysonoic acid and the phosphate conjugates of 3-epi-20-dihydroxyecdysone (bib1580 Lozano *et al*., 1989).

p0865 In feeding last larval stage of another lepidopteran, *Pieris brassicae*, injected 20E was converted primarily into 20-hydroxyecdysonoic acid, 3-dehydro-20-hydroxyecdysone, and 3-epi-20-hydroxyecdysone 3-phosphate. Use of E as substrate gave the same metabolites, together with ones lacking the 20-hydroxyl group (bib0100 Beydon *et al*., 1987). Similarly, in *P. brassicae* eggs, [^{3}H]-ecdysone metabolism is similar to that in larvae and pupae viz. hydroxylation at C_{20} and C_{26}, with further oxidation to ecdysteroid 26-oic acids. Injection of [^{3}H]-cholesterol into female pharate adults indicated that the egg ecdysteroids/metabolites consisted of 20-hydroxyecdysonoic acid, ecdysonoic acid, 20E, and E (bib0110 Beydon *et al*., 1989). In another lepidopteran, the tobacco budworm, *Heliothis virescens*, incubation of testes with labeled E yielded a range of products including 20, 26-dihydroxyecdysone, 3-epi-20-hydroxyecdysone, 20E, and highly polar metabolites (bib1555 Loeb and Woods, 1989).

p0870 In female adults of the cricket, *Gryllus bimaculatus*, injected [^{3}H]-ecdysone was converted into at least 24 metabolites, the highest number being in the gut and feces, and included 26-hydroxyecdysone and 14-deoxyecdysone amongst the free ecdysteroids. In addition, polar (phosphate) and apolar (fatty acyl) conjugates were also observed. Following feeding of [^{3}H] ecdysone, most of the label remained within the gut and was excreted within 48 h, with 14-deoxyecdysone being a major metabolite (bib2395 Thiry and Hoffmann, 1992). Both apolar and polar ecdysteroid conjugates are widely distributed in tissues of males of this species (bib0865 Hoffmann and Wagemann, 1994). In vitellogenic females of *Labidura riparia* (Dermaptera), [^{3}H] 20-E was metabolized primarily through conjugation, in particular acetylation at C_3 and phosphate ester formation at C_2 (bib2110 Sayah and Blais, 1995).

p0875 Incubation of midgut cytosol of *Manduca*, where prominent 3-epimerization of ecdysteroid occurs, with [^{3}H]-ecdysone and Mg^{2+}/ATP, results in detection of four E phosphoconjugates and two 3-epiecdysone phosphoconjugates (bib2675 Weirich *et al*., 1986). ATP- and Mg^{2+}-dependent ecdysteroid phosphotransferase activities have been demonstrated in cytosolic extracts of ovaries and fat body from mature female pupae of *B. mori* (bib2365 Takahashi *et al*., 1992). The midgut cytosol of the cotton leafworm, *S. littoralis*, contains Mg^{2+}/ATP-dependent ecdysteroid 2- and 22-phosphotransferase activities, with a predominance of the former (bib2620 Webb *et al*., 1995). The

phosphotransferase activities were high early in the final larval instar and then declined. The relatively high K_M value (21 μM) of the E 2-phosphotransferase (Webb *et al*., 1996), together with its expression during the feeding period when the hemolymph ecdysteroid titer is low, may suggest that these phosphotransferase enzymes are involved in inactivation of any dietary ecdysteroids.

p0880 The enzymes catalyzing formation of E 3-acetate and its phosphorylation in production of 3-acetylecdysone 2-phosphate have been characterized in larval tissues of *Schistocerca*. 3-Acetylation of E and 20E was characterized in gastric caeca from final larval instar *S. gregaria*, since high activity occurred in that tissue. The microsomal enzyme had a somewhat stronger affinity for 20E (K_M 53.5 μM) than E (71 μM) and utilized acetyl coenzyme A, but not acetic acid, as cosubstrate, indicating that the enzyme is an acetyl coenzyme A (CoA): ecdysteroid acetyltransferase and not a hydrolase. Furthermore, esterification of E was not observed when long-chain fatty acylCoA derivatives were substituted as cosubstrates (Kabbouh and Rees, 1991b).

p0885 In final instar *S. gregaria* larvae, ATP: E 3-acetate 2-phosphotransferase activity was low in gastric caeca and gut, but was somewhat higher in fat body than in Malpighian tubules. Thus, the soluble fat body enzyme (*ca.* 45 kDa) was characterized. The Mg^{2+}/ATP dependent phosphotransferase utilized E 3-acetate as the preferred substrate (K_M 10.5 μM), with no significant phosphorylation of E and E 2-acetate. The physiological significance of the increase in phosphotransferase activity during the second half of the instar, well before the rise in ecdysteroid titer, is unclear (Kabbouh and Rees, 1993).

p0890 In some lepidopteran species, e.g., *Heliothis armigera* (cotton bollworm, Robinson *et al*., 1987) and *Heliothis virescens* (cotton budworm, Kubo *et al*., 1987), a major fate of ingested ecdysteroids is formation of 22-fatty acyl esters in the gut for fecal excretion. Characterization of the ecdysteroid-22-*O*-acyltransferase activity in crude homogenates of midgut from *H. virescens* has shown that the enzyme can use fatty acyl CoA as cosubstrate, but not phosphatidylcholine or free fatty acid, indicating that the enzyme differs appreciably from the cholesterol acyltransferase, where all such cosubstrates can be utilized (Zhang and Kubo, *et al*., 1992a). Furthermore, unlike the situation for the latter enzyme, divalent cations inhibit the ecdysteroid acyltransferase. Subsequently, it has been demonstrated that the ecdysteroid 22-*O*-acyltransferase is located in the plasma membrane of the gut epithelial cells (gut brush border membrane; Kubo *et al*., 1994). The acyltransferase was active only during feeding stages, its activity decreased as the larvae became committed to pupation and was not enhanced by feeding ecdysteroids to the fifth instar larvae (Zhang and Kubo *et al*., 1992b). It has been suggested that dietary phytoecdysteroid detoxification is the major function of the acyltransferase (Zhang and Kubo, 1992b, 1994). In both *H. virescens* and *B. mori*, injected E was converted into 20E, 26-hydroxyecdysone, and 20, 26-dihydroxyecdysone, which was oxidized to 20E 26-oic-acid for excretion. These transformations also occurred in the case of ingested E in these species, but as already seen in *H. virescens*, an additional prominent route involved E–22-O-acyl ester formation, which was largely excreted. In contrast, in *B. mori*, the latter pathway was replaced by formation of 3-epi-E and a polar conjugate tentatively identified as the sulfate, which was excreted, in the case of ingested E (Zhang and Kubo, 1993). In the tomato moth, *Lacanobia overacea*, and the cotton leafworm, *Spodoptera littoralis*, the situation is similar to that in *H. virescens*, where injected/endogenous ecdysteroids undergo conversion into 20-hydroxyecdysonoic acid, with ingested ecdysteroids undergoing detoxification yielding apolar 22-fatty acyl esters (Blackford and Dinan, 1997a; Blackford *et al*., 1997). Surprisingly, the Death's head hawkmoth, *Aclerontia atropos*, is unaffected by dietary 20E, which it excretes in an unmetabolized form (Blackford and Dinan, 1997b). Interestingly, in four other Lepidoptera (*Aglais urticae, Inachis io*, *Cynthia cardini*, and *Tyria jacobaeae*) ingested and injected 20E followed the same fate, with polar compounds being prominent and no detectable formation of 22-fatty acyl esters (Blackford and Dinan, 1997c).

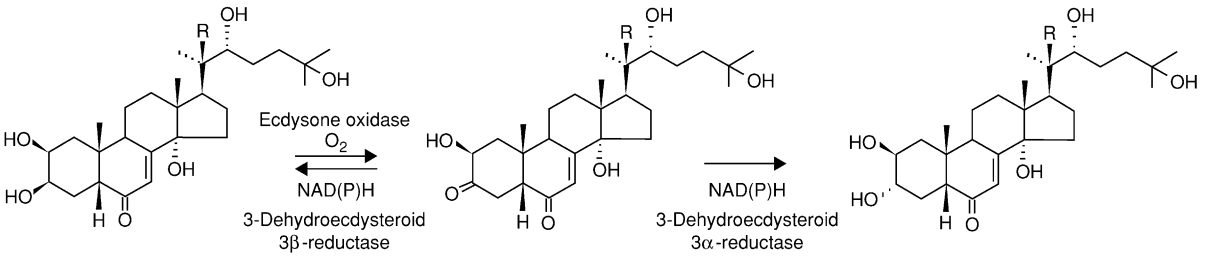
p0895 In adult *Drosophila melanogaster*, following injection of [^{3}H]-E, E-22-fatty acyl esters were the major metabolites, followed by 3dE, 26-hydroxyecdysone, ecdysonoic acid, 20-hydroxyecdysone, and E 22-phosphate (Grau and Lafont, 1994a; Grau *et al*., 1995). 20E was metabolized in a similar manner (Grau and Lafont, 1994a). The E-22-phosphate was produced in the ovaries, whereas E was efficiently converted into 3DE and ecdysone 22-fatty acyl esters by gut/Malpighian tubule complexes (Grau and Lafont, 1994b). In third instar *D. melanogaster* larvae, although E metabolism proceeds through different pathways including C_{20} hydroxylation, C_{26} hydroxylation, and oxidation to 26-oic acids, C_3 oxidation and epimerization followed by conjugation, 3-dehydroecdysteroids are the major metabolites, with C_3 oxidation occurring in various tissues (Sommé-Martin *et al*., 1988).

s0515 p0900 *3.3.5.3.2.2. 26-Hydroxylation and ecdysonoic acid formation* Ecdysonoic acid and 20-hydroxyecdysonoic acid were originally identified from developing eggs of *Schistocerca*, pupae of *Spodoptera* (Isaac *et al*., 1983), and *Pieris*, where their formation has been shown to occur in several tissues (Lafont *et al*., 1983). Since that time, it has become apparent that 26-oic acid formation is a widespread and prominent pathway of ecdysteroid inactivation, as alluded to in several studies considered above.

p0905 It has been shown that administration of E, 20E, or an ecdysteroid agonist, RH-5849, to last-instar larvae of the cotton leafworm, *S. littoralis*, leads to induction of ecdysteroid 26-hydroxylase activity in fat body mitochondria (Chen *et al*., 1994b). This induction occurred in both early last instar larvae and in older larvae that had been head-ligated to prevent the normal developmental increase in E 20-monooxygenase activity. The induction of hydroxylase activity requires both RNA and protein synthesis. E-26-oic acid and a compound tentatively identified as the 26-aldehyde derivative of E were also formed from E in the RH 5949 induced systems. Direct demonstration in a cell-free system of the conversion of 26-hydroxyecdysone into the aldehyde and the corresponding 26-oic acid (ecdysonoic acid), established the following inactivation pathway: ecdysteroid → 26-hydroxyecdysteroid → ecdysteroid 26-aldehyde → ecdysteroid 26-oic acid. Whether these three reactions are catalyzed by a single enzyme (as is the case of rabbit liver cytochrome P450 CYP27, which catalyzes the complete conversion of 3α,7α,12α-trihydroxy-5β-cholestane into 3α,7α,12α-trihydroxy-5β-cholestanoic acid (see Betsholtz and Wikvall, 1995) remains an open question.

p0910 Induction of the 26-hydroxylase activity might be expected to result from enhanced cytochrome P450 gene transcription and protein synthesis (Chen *et al*., 1994b). Similarly, both 20E and RH-5849 caused RNA- and protein-synthesis-dependent induction of ecdysteroid 26-hydroxylase activity in midgut mitochondria and microsomes in *Manduca* (Williams *et al*., 1997). This induction of an ecdysteroid inactivation pathway (and others, see following section) by 20E and RH-5849 is reminiscent of the regulation of vitamin D inactivation in vertebrates where 1α,25-dihydroxyvitamin D_3 stimulates its own degradation by induction of a vitamin D response element to initiate degradation to excretory metabolites (see Williams *et al*., 1997 for references). However, the possibility cannot be discounted that at least some of the observed induction events may be indirect. Evidence has been furnished that the 26-hydroxylase activity is a typical NADPH-requiring cytochrome P450-dependent monooxygenase that is sensitive to inhibition by CO and imidazole/triazole-based fungicides (Kayser *et al*., 1997; Williams *et al*., 2000). Furthermore, indirect evidence suggested that the *Manduca* mitochondrial and microsomal 26-hydroxylases could exist in a less active dephosphorylated state or more active phosphorylated state (Williams *et al*., 2000). Interestingly, long-term culture of a *Chironomus tentans* (Diptera) cell line in the presence of 20E resulted in selection of subclones that are resistant to the steroid, but respond normally to the agonist, tebufenozide (RH-5992) (see **Chapter 3.4**). Evidence was obtained that ecdysteroid resistance of the cell clones is due to high metabolic inactivation of the hormone by 26-hydroxylation (Kayser *et al*., 1997). In this cell system, 20,26-dihydroxyecdysone is further metabolized to two compounds that are slowly interconvertible geometrical isomers, which presumably arise from hemiacetal formation between the 26-aldehyde and the 22*R*-hydroxyl groups to yield a tetrahydropyrane ring in the side chain (Kayser *et al*., 2002). Induction of ecdysteroid 26-hydroxylase by the agonists RH-5849, RH-5992, and RH-0345 appears universal in lepidopteran species that show susceptibility to the agonists (Williams *et al*., 2002). It appears that the more potent ecdysteroid agonists in Lepidoptera, RH-5992, and RH-0345 show in general a greater induction of 26-hydroxylase than does RH-5849. Feeding RH-5849 to the dipteran *Musca domestica* results in induction of an ecdysteroid phosphotransferase. The low toxicity of the ecdysteroid agonists in orthopteran and coleopteran orders also correlates with a lack of induction of ecdysteroid 26-hydroxylase activity. In fact, it has been proposed that in species where ecdysteroid agonists are effective in stimulating an untimely premature molt, a response to a state of apparent hyperecdysonism elicited by the agonists is induction of enzymes of ecdysteroid inactivation (Williams *et al*., 2002) (see **Chapter 3.4**).

s0520 p0915 *3.3.5.3.2.3. 3-Epimerization* An oxygen and NAD(P)H-dependent "E epimerase" activity was originally reported in *Manduca* midgut cytosol which irreversibly converted E into its 3α-isomer (3-epiecdysone; Nigg *et al*., 1974; Mayer *et al*., 1979). Subsequently, this transformation has been shown to occur via the oxygen-dependent E oxidase catalyzed formation of 3DE, which is then irreversibly reduced to 3-epiecdysone, catalyzed by NAD(P)H-dependent 3-dehydroecdysteroid 3α-reductase (**Figure 18**) (*Pieris*, Blais and Lafont, 1984; *Spodoptera*, Milner and Rees, 1985; *Manduca*, Weirich, 1989).



f0090 **Figure 18** Enzymatic interconversion of 3β-ecdysteroids, 3-dehydroecdysteroids, and 3α-ecdysteroids. R = H: Ecdysone (metabolites); R = OH: 20-hydroxyecdysone (metabolites). (Modified from Rees, H.H. **1995**. Ecdysteroid biosynthesis and inactivation in relation to function. *Eur. J. Entomol. 92*, 9–39.)

p0920 The 3-epiecdysteroid product may also undergo phosphoconjugation. However, many tissues also contain an NAD(P)H-dependent 3-dehydroecdysteroid 3β-reductase, which reduces the 3DE back to E (see bib1460 Lafont and Koolman, 1984); the exact significance of this apparent futile cycle is unclear. The foregoing pathways occur with E and 20E and have been reviewed (bib1460 Lafont and Koolman, 1984; bib1295 Koolman and Karlson, 1985; bib2450 Thompson *et al*., 1985a; bib2640 Weirich, 1989; bib1995 Rees, 1995). It is apparent from the foregoing sections of this chapter that 3-epiecdysteroid formation is a widespread ecdysteroid metabolic pathway amongst species, with the 3-dehydroecdysteroid 3α-reductase being apparently restricted to gut tissues. 3-Epiecdysteroid formation is certainly emphasized in Lepidoptera.

p0925 The kinetic properties and developmental expression of E oxidase, 3-dehydroecdysteroid 3α-reductase and 3β-reductase enzymes from midgut cytosol of *Manduca* (bib2680 Weirich *et al*., 1989, bib2685 1991, bib2655 1993) and *Spodoptera* have been investigated (bib2620 Webb *et al*., bib2625 1995, 1996). In the former species, the larval midgut was the only organ exhibiting substantial specific activities of E oxidase and 3α-reductase, with both activities increasing up to the seventh day after ecdysis (bib2680 Weirich *et al*., 1989). Hemolymph and fat body had only moderate to high 3β-reductase and low E oxidase activities, whereas the epidermis did not contain significant activities of any of the enzymes. Thus, apparently, only the larval midgut has a role in the inactivation of ecdysteroids by 3-epimerization in *Manduca*.

p0930 It has been shown that administration of the hormone agonist, RH5849, but not of 20E, to *Manduca* results in RNA- and protein-dependent induction of the midgut cytosol E oxidase and 3-epiecdysone phosphotransferase activities (bib2740 Williams *et al*., 1997). Clearly, elucidation of the significance of these results requires further work.

p0935 Ecdysone oxidase from *Calliphora* was amongst the first enzymes catalyzing ecdysteroid transformations to be investigated extensively (bib1265 Koolman, 1978; bib1290 Koolman and Karlson, 1978; bib1275 Koolman, 1985) and has been purified considerably (bib1290 Koolman and Karlson, 1978). In *Calliphora vicina* also, injected 3DE is rapidly reduced *in vivo*, but relative formation of E and 3-epiecdysone cannot be ascertained since the TLC fractionation system employed lacks resolution (bib1130 Karlson and Koolman, 1973). The reaction catalyzed by E oxidase (EC 1.1.3.16) may be written as:

$$E + O_2 = 3\text{-dehydroecdysone} + H_2O_2$$

p0940 The enzyme would be expected to possess a prosthetic group, but none has been identified so far. E oxidase efficiently oxidizes a number of ecdysteroids, but not 22,25-dideoxyecdysone nor cholesterol (bib1290 Koolman and Karlson, 1978; bib1275 Koolman, 1985). E oxidase represents the only oxidase in eukaryotic animals known to catalyze oxygen-dependent oxidation of steroids; in contrast, oxidation of steroids in vertebrates occurs via NAD(P) $^{+}$-linked dehydrogenases.

p0945 The native enzyme from *Calliphora* has an apparent molecular mass of approximately 240 kDa (bib1275 Koolman, 1985). However, native E oxidase purified from midgut cytosol of *Spodoptera* might be a trimer (approximately 190 kDa), since the apparent Mr of the oxidase subunit was 64 kDa by SDS-PAGE (bib0275 Chen *et al*., 1999a). The latter was corroborated by cloning the cDNA (2.8 kb) encoding a 65 kDa protein (bib2375 Takeuchi *et al*., 2001). Northern blotting demonstrated that the mRNA transcript was expressed in midgut during the prepupal stage of the last larval instar at a time corresponding to an ecdysteroid titer peak. Conceptual translation of the cDNA followed by data base searching indicated that the enzyme is an FAD flavoprotein that belongs to the glucose-methanol-choline oxidoreductase superfamily. Injection of the agonist, RH-5992, induced the transcription of E oxidase, suggesting that it is an ecdysteroid responsive gene. The gene encoding the enzyme consists of five exons and

sequences similar to the binding motifs for Broad-Complex and FTZ-F1 occur in the 5′ flanking region, consistent with ecdysteroid regulation. Southern blotting indicated that E oxidase is encoded by a single-copy gene.

p0950 Interestingly, the entomogenous fungus, *Nomuraea rileyi*, inhibits molting of its host, *B. mori*, by production of an enzyme that oxidizes the C_{22} hydroxyl group of hemolymph ecdysteroids (Kiuchi *et al*., 2003). Whether this enzyme is an oxidase or an $NAD(P)^+$-dependent dehydrogenase has not been established.

p0955 One major 3-dehydroecdysteroid 3α-reductase (active with both NADPH and NADH) together with three major 3-dehydroecdysteroid 3β-reductases (active only with NADPH as cosubstrate) have been fractionated from *Manduca* midgut cytosol (Weirich and Svoboda, 1992). In the case of *Spodoptera*, two forms of 3-dehydroecdysone 3α-reductase have been observed during purification, a 26 kDa form that may be a trimer with an apparent Mr of approximately 76 kDa and a second 51 kDa form that appears to be a monomer by gel filtration (Chen *et al*., 1999a). The cDNA (1.2 kb) encoding the 26 kDa protein has been cloned and Northern blotting showed that the mRNA transcript was expressed in Malpighian tubules during the early stage of the last larval instar (Takeuchi *et al*., 2000). Conceptual translation of the cDNA followed by database searching revealed that the enzyme belongs to the Short-chain-Dehydrogenases/Reductases (SDR) superfamily. Furthermore, the enzyme is a novel eukaryotic 3-dehydrosteroid 3α-reductase member of that family, whereas vertebrate 3-dehydrosteroid 3α-reductases belong to the Aldo-keto Reductase (AKR) superfamily. Surprisingly, no similarity was observed between the 3-dehydroecdysone 3α-reductase and a reported 3-dehydroecdysone 3β-reductase from the same species (Chen *et al*., 1999b), which acts on the same substrate and belongs to the AKR family.

p0960 As alluded to earlier, 3-dehydroecdysteroid 3β-reductase activity has been demonstrated in the cytosol of various tissues of many species. In addition, hemolymph 3-dehydroecdysteroid 3β-reductase is involved in reduction of the 3DE produced by prothoracic glands of many lepidopterans (see Section 3.3.4.2.; Warren *et al*., 1988b; Sakurai *et al*., 1989; Kiriishi *et al*., 1990).

p0965 Hemolymph NAD(P)H-dependent 3-dehydroecdysone 3β-reductase activity has been shown to undergo developmental fluctuation in several species, exhibiting high activity at the time of the peak in ecdysteroid titer near the end of the last larval instar (*Manduca*, Sakurai *et al*., 1989; *Spodoptera* Chen *et al*., 1996; *Periplenta americana*, Richter *et al*., 1999). In *P. americana*, some enzyme is located on the outer surface of the prothoracic gland cells as well. The exact source of the hemolymph 3-dehydroecdysone 3β-reductase and the mechanism regulating its expression during development have not been elucidated. The 3β-reductase has a wide tissue expression (*Pieris*, Blais and Lafont, 1984; European corn borer, *Ostrinia nubilalis*, Gelman *et al*., 1991; *Bombyx*, Nomura *et al*., 1996), including embryonated eggs of the gypsy moth, *Lymantria* (Kelly *et al*., 1990). Of significance to ecdysteroid biosynthesis is the demonstration that various 3-dehydroecdysteroid precursors may be reduced to the corresponding 3β-hydroxy compounds in prothoracic glands and follicle cells of *Locusta* (Dollé *et al*., 1991; Rees, 1995).

The 3-dehydroecdysteroid 3β-reductases have p0970 been purified from hemolymph of *Bombyx* (Nomura *et al*., 1996) and *Spodoptera* (Chen *et al*., 1996), demonstrating that the native enzyme is a monomer of Mr of approximately 42 kDa and 36 kDa, respectively. In the case of both enzymes, a lower K_m was observed with NADPH than with NADH as cosubstrate (*B. mori*, 0.90 μM versus 5.4 μM; *S. littoralis*, 0.94 μM versus 22.8 μM). Kinetic analysis of the *S. littoralis* hemolymph 3β-reductase, using either NADPH or NADH-as cofactor, revealed that the enzyme exhibits maximal activity at low 3DE substrate concentrations, with a drastic inhibition of activity at higher concentrations (>5 μM). Similar substrate inhibition was observed for the cytosolic NADPH-dependent 3-dehydroecdysone 3α-reductase of *Manduca* midgut which showed apparent 3DE substrate inhibition above 10 μM (Weirich *et al*., 1989). Although the K_M for the *S. littoralis* hemolymph 3-dehydroecdysone 3β-reductase has not been determined due to the substrate inhibition, it is conceivable that the K_M value could be less than 5 μM. The results suggest that the 3β-reductase has a high-affinity (low K_M) binding site for the 3-dehydroecdysteroid substrate, together with a lower affinity inhibition site (Chen *et al*., 1996). Cloning of the full-length 3-dehydroecdysone 3β-reductase cDNA from *S. littoralis* and conceptual translation yield a protein with a predicted Mr of 39 591 of which the first 17 amino acids appear to constitute a signal peptide to yield a predicted Mr for the mature protein of 37 689 (Chen *et al*., 1999b), which agrees with the approx. Mr (36 kDa) of the purified protein on SDS-PAGE. Similarly, database searching suggested that the enzyme is a new member of the third (AKR) superfamily of oxidoreductases.

Northern blot analysis revealed that highest ex- p0975 pression is detected in Malpighian tubules, followed

by midgut and fat body, with low-level expression in hemocytes and none detectable in central nervous system. Surprisingly, preliminary results of Western blotting experiments revealed 3β-reductase detection in hemolymph and hemocytes, with little in midgut and none in Malpighian tubules, in agreement with the results for *B. mori* (Nomura *et al.*, 1996). This suggests that selective translation of mRNA may contribute to regulation of expression of 3-dehydroecdysteroid 3β-reductase (Chen *et al.*, 1999b). The developmental profile of the mRNA revealed that the gene is only transcribed in the second half of the sixth instar essentially at the time of increasing enzymatic activity and ecdysteroid titer. Thus, it appears that the change in 3β-reductase activity is, at least in part, regulated by gene expression at the transcriptional level, although posttranscriptional regulation may also be important in certain tissues. Southern analysis indicates that the 3-dehydroecdysone 3β-reductase is encoded by a single gene, which probably contains at least one intron (Chen *et al.*, 1999b). Recently, a gene encoding a protein that shows 42.5% identity to *S. littoralis* 3-dehydroecdysteroid 3β-reductase has been cloned from the cabbage loofer, *Trichoplusia ni* and the protein has been shown to possess low 3β-reductase activity (Lundström *et al.*, 2002). Northern blotting has indicated that highest expression of the gene is detected in fat body, the expression being highly induced after bacterial challenge. However, by immunohistochemistry, the protein was localized exclusively in the epidermis and the cuticle, but its significance is unclear.

s0525 p0980 *3.3.5.3.2.4. 22-Glycosylation* The formation of 26-hydroxyecdysone 22-glucoside in late embryos of *Manduca* has already been alluded to (Thompson *et al.*, 1987a).

p0985 In the baculovirus, *Autographa californica* nuclear polyhedrosis virus, expression of the *egt* gene produces UDP-glycosyl transferase, which apparently inactivates the host ecdysteroids by catalyzing production of ecdysteroid 22-glycoside. Thus, *egt* gene expression allows the virus to interfere with insect development by blocking molting in infected larvae of the fall armyworm, *Spodoptera frugiperda* (O'Reilly and Miller, 1989; O'Reilly *et al.*, 1991). Although kinetic analysis indicates that the ecdysteroid UDP-glucosyltransferase (EGT) has broadly similar specificities for UDP-galactose and UDP-glucose (Evans and O'Reilly, 1998), there is evidence that *in vivo*, ecdysteroids are conjugated with galactose (O'Reilly *et al.*, 1992; review: O'Reilly, 1995). EGT appears to be an oligomer of 3–5 subunits (Mr approx 56 kDa) and, surprisingly, E seems to be the optimal substrate, whereas 3DE is seven times less favorable, with 20E being conjugated very poorly, more than 34 times less readily than 3DE (Evans and O'Reilly, 1998). The physiological significance of this finding is uncertain. Analogous EGT enzymes and mechanisms have been established for the gypsy moth *Lymantria* nuclear polyhedrosis virus (Park *et al.*, 1993; Burand *et al.*, 1996), *Epiphyas postvittana* nucleopolyhedrovirus (Caradoc-Davies *et al.*, 2001), and *Mamestra brassicae* multinucleocapsid nucleopolyhedrovirus (Clarke *et al.*, 1996).

s0530 p0990 *3.3.5.3.2.5. Neglected pathways?* Given the huge diversity of insects, there is no reason to consider that metabolic pathways are restricted to those described above. A few examples will illustrate that the story is not finished:

- Side chain cleavage between C_{20} and C_{22} resulting in the formation of poststerone was described long ago in *Bombyx* by Hikino *et al.* (1975); moreover, other experiments have demonstrated the ability of certain insects to produce C_{21} steroids from cholesterol in exocrine glands of aquatic beetles (Schildknecht, 1970) and also ovaries of *Manduca* (Thompson *et al.*, 1985c), providing the evidence for a desmolase activity in some insect species at least. The fact that available [^{3}H]-ecdysteroids are labeled on the side chain has precluded further analysis of this problem, as the resulting poststerone would not be labeled and the radioactivity would become "volatile". This problem awaits reexamination when nuclear labeled ecdysteroids are available.
- Minor ecdysteroids have been isolated from *Bombyx* that bear an –OH group in positions 16β (Kamba *et al.*, 2000b) or 23 (Kamba *et al.*, 2000a), which indicates that during some developmental stages at least (here in adults) additional CYPs must be active.
- 14-Deoxy metabolites of E and 20E have been identified in *Gryllus bimaculatus* feces, and their formation is probably due to bacterial metabolism in the insect gut (Hoffmann *et al.*, 1990).
- Labeling studies with *Drosophila* adults have shown very complex patterns that include some as yet unidentified metabolites (Grau and Lafont, 1994a,b). Their chromatographic behavior is consistent with the presence of one additional –OH group in position 11β (?), but positions 16β and 23 as observed in *Bombyx* (see above) are also plausible.
- Finally, it should be noted that sulfate conjugates, although never isolated as endogenous

metabolites, can be formed *in vitro* by insect/tissue homogenates incubated with ecdysteroids and a sulfate donor (e.g., *Prodenia eridania* gut: Yang and Wilkinson, 1972; *Aedes togoi* whole larvae/pupae/adult: Shampengton and Wong, 1989; *Sarcophaga peregrina* fat body: Matsumoto *et al*., 2003). In *Sarcophaga*, a 43-kDa protein responsible of this reaction was purified by affinity chromatography on a 3′-phosphoadenosine 5′-phosphate (PAP)-agarose column (Matsumoto *et al*., 2003). However, the physiological significance of such activities *in vivo* in ecdysteroid metabolism remains to be firmly established, since it is conceivable that the activity observed might be ascribed to another primary activity, with low specificity for ecdysteroids.

3.3.6. Some Prospects for the Future

3.3.6.1. Evolution and Appearance of Ecdysteroids

Zooecdysteroids are not restricted to arthropods. Trace amounts of E, 20E, and other ecdysteroids have been detected by HPLC-immunoassays or GC-MS in annelids, nematodes, helminths, and mollusks (Spindler, 1988; Franke and Käuser, 1989; Barker *et al*., 1990). More recently, ecdysteroids were also found in nemerteans (Okazaki *et al*., 1997). It must be emphasized that at the moment there is no evidence that these animals are able to produce ecdysteroids themselves, as they seem to lack some key biosynthetic enzymes, a conclusion based on metabolic studies performed with biosynthetic intermediates efficiently converted in arthropods (**Table 8**, Lafont *et al*., 1995; Lafont, 1997). An evolutionary scenario (see **Chapter 3.6**) has been proposed for the progressive appearance of enzymes, which resulted in an increasing number of hydroxyl groups on the sterol molecule (the last ones being the three mitochondrial enzymes, i.e., the 22-, 2-, and the 20-hydroxylases). A similar phylogenetic emergence seems to have operated in the case of vertebrate-type steroid hormones (Bolander, 1994).

Several findings, however, do not fit with this scheme. In particular, large amounts of ecdysteroids have been isolated from Zoanthids (e.g., Sturaro *et al*., 1982; Suksamrarn *et al*., 2002) and even from various sponges (Diop *et al*., 1996; Cafieri *et al*., 1998; Costantino *et al*., 2000). The large amounts seem to preclude a hormonal role and rather fit with an allelochemical (protective) role, perhaps against predation, as in the case of pycnogonids (Tomaschko, 1995). In the case of Zoanthids, it is conceivable that ecdysteroids arise from their food (e.g., copepods), but this does not seem so evident in the case of sponges, which are expected to feed on bacteria, protozoa, or dissolved matter, none of which are known to contain ecdysteroids. This awaits additional experiments to establish whether or not sponges are able to produce ecdysteroids themselves. It should be noted that sponges also contain an incredible number of steroids (Aiello *et al*., 1999), including steroidal $\Delta^{4,7}$-3,6-diketones (Malorni *et al*., 1978) or 4,6,8-triene-3-ones (Kobayashi *et al*., 1992), which resemble putative ecdysteroid precursors (Grieneisen *et al*., 1991; Grieneisen, 1994; Gilbert *et al*., 2002). We expect that molecular approaches will allow a search for homologs of insect biosynthetic enzymes that will provide an adequate answer to this fundamental problem in comparative endocrinology.

Table 8 Presence/absence of some key hydroxylases involved in ecdysteroid biosynthesis and/or metabolism in invertebrates (After Lafont *et al*., 1995)

	Hydroxylations at positions				
Animals	*C2*	*C20*	*C22*	*C25*	*Other*
Arthropods					
Insects	+	+	+	+	16β, 23, 26
Crustaceans	+	+	+	+	26
Ticks	+	+	+	+	26
Myriapods	+	+	+	+	?
Nonarthropods					
Mollusks (Gastropods)	−	+	−	+	16β
Annelids					
Achaetes	−	+	−	+	16β
Polychaetes	−	+	−	+	26
Oligochaetes	−	−	−	+	18
Nematodes	−	?	?	?	?

?, does not mean that the reaction is absent, but only that there is at present no direct demonstration for it.

3.3.6.2. Biosynthetic Pathway(s)

The last 2 years have resulted in a spectacular improvement of our knowledge of ecdysteroidogenic enzymes, thanks to the *Drosophila* Halloween mutants. Up to now, however, this has only led to the characterization of enzymes involved in already identified reactions (i.e., hydroxylations at positions 25, 2, 22, and 20), due to the expression of recombinant enzymes and the determination of their catalytic activity. Whether this approach will suffice in the identification of the remaining unknown reactions (the so-called "black box") is debatable. Subsequent to the conversion of C to 7dC by the 7,8-dehydrogenase present in the ER, oxidized early intermediates do not accumulate under normal conditions. In theory we may expect to increase their

concentrations by using one (or a combination) of the following strategies: (1) the use of mutants defective in one or several terminal enzymes; (2) the use of specific inhibitors of these enzymes (Burger *et al*., 1988, 1989; Mauvais *et al*., 1994); or (3) the use of subcellular fractions instead of whole cells/tissues in order to have only a few biosynthetic enzymes present in the preparation. An additional technical problem to overcome will be the instability of such intermediates. This problem has already been encountered when using a tritiated $\Delta^{4,7}$-3,6-diketone (Blais *et al*., 1996) or α-5,6-epoxy7dC (Warren *et al*., 1995). Furthermore, recent experiments (see Section 3.3.4.2.2) suggest that the long-hypothesized, rate-limiting, mitochondrial P_{450}-mediated "black box" oxidations of 7dC, leading to the Δ^4-diketol, cannot be catalyzed by a mitochondrial P450 enzyme and may not even occur in the mitochondria. Studies implicating the mitochondria in these novel transformations employed crude membrane fractions from *Manduca* prothoracic glands (Warren and Gilbert, 1996) and there was clear evidence for the co-sedimentation of a large proportion of the microsomes with the crude mitochondrial pellet, as has been observed with other insect prothoracic gland tissues (Kappler *et al*., 1988). However, insect peroxisomes also normally sediment with the mitochondrial pellet (Kurisu *et al*., 2003) and mammalian peroxisomes are known to participate in the oxidation of both steroids and fatty acids (Breitling *et al*., 2001). Thus, it is possible that the oxidation of 7dC (or 3-oxo7dC) may instead occur in this membrane-bound subcellular organelle. In addition, the peroxisome has been shown to be a target of sterol carrier protein 2 (Schroeder *et al*., 2000). Also, at least in *Spodoptera frugiperda* cells, the organelle has been shown to be devoid of catalase activity, a normal marker enzyme in mammalian peroxisomes that acts to destroy hydrogen peroxide (Kurisu *et al*., 2003). If a normal synthesis of hydrogen peroxide is also observed in insect peroxisomes, then the result could be an ideal source of oxygen for the hypothesized black box oxidations (Gilbert *et al*., 2002).

p1010 Up to now, the black box has been investigated through trial-and-error experiments analyzing the metabolic fate of radiolabeled putative intermediates, but this has mainly led to the elimination of several hypotheses (Grieneisen, 1994). In an alternative strategy, the so-called "isotope trap," steroidogenic organs would be incubated with a labeled early precursor in the presence of an excess of an unlabeled putative downstream intermediate. If the latter is indeed an intermediate (and is stable enough to survive the incubation), it is expected to trap radioactivity due to isotopic dilution and saturation of the subsequent steps. It could also be possible to use systems capable of producing larger amounts of ecdysteroids (other than what can be achieved through the tedious dissection of thousands of prothoracic glands or ring glands), e.g., perhaps embryos or suitable plant cell or organ cell cultures, which would allow the use of precursors labeled with stable isotopes (^{2}H, ^{13}C) and subsequent NMR analysis of terminal ecdysteroid products (Fujimoto *et al*., 1997, 2000).

Thus, the problem of the early steps remains a very open question. In addition, it may well be that different biosynthetic pathways have been developed between insects and plants (Fujimoto *et al*., 1989, 1997; Reixach *et al*., 1999), and perhaps also within the diverse array of insects and other arthropods. Nevertheless, the black box reactions appear to be quite specific to arthropods (and maybe a few plants). As such, they make a very appealing target for the development of novel biochemical strategies for the control of insect pests. p1015

3.3.6.3. Additional Functions for Ecdysteroids? s0550

p1020 We have discussed that tissues other than prothoracic glands and gonads may produce ecdysteroids, possibly as autocrine factors (see Section 3.3.4.4.2). Whatever the biosynthetic site, this corresponds to an involvement of ecdysteroids in the control of development and reproduction processes. The recent discovery of large ecdysteroid concentrations in the defensive secretions of chrysomelid beetles (Laurent *et al*., in press) suggests a possible allelochemical role of ecdysteroids in certain cases. The situation described in chrysomelid beetles is similar to that of the pycnogonids, where ecdysteroids have been demonstrated conclusively to represent an efficient protection against predation by crabs (Tomaschko, 1995). Another example is the phytoecdysteroid accumulation by many plant species, seen as a protection against phytophagous insects (Dinan, 2001). However, it should be emphasized that previous analyses of defensive secretions of Chrysomelid beetles allowed the isolation of 2,14,22-trideoxyecdysone 3-sophorose and other ecdysteroid related compounds lacking the Δ^7 bond (Pasteels *et al*., 1994). Thus, the story of ecdysteroid biosynthesis could turn out to be considerably more complex than it already appears to be.

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http://drnelson.utmem.edu – A free database for cytochrome P450 homepage by Dr. David Nelson, Assistant professor at the Department of Biochemistry, from the University of Tennessee.

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