

# Shade is the *Drosophila* P450 enzyme that mediates the hydroxylation of ecdysone to the steroid insect molting hormone 20-hydroxyecdysone

Anna Petryk<sup>\*†</sup>, James T. Warren<sup>\*‡</sup>, Guillermo Marqués<sup>§¶||</sup>, Michael P. Jarcho<sup>\*</sup>, Lawrence I. Gilbert<sup>‡</sup>, Jonathan Kahler<sup>‡</sup>, Jean-Philippe Parvy<sup>\*\*</sup>, Yutai Li<sup>††</sup>, Chantal Dauphin-Villemant<sup>\*\*</sup>, and Michael B. O'Connor<sup>§¶††</sup>

<sup>\*</sup>Department of Pediatrics, University of Minnesota, 516 Delaware Street SE, Minneapolis, MN 55455; <sup>‡</sup>Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280; <sup>††</sup>Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC 27599-7431; <sup>§</sup>Department of Genetics, Cell Biology, and Development and <sup>¶</sup>Howard Hughes Medical Institute, University of Minnesota, 321 Church Street SE, Minneapolis, MN 55455; and <sup>\*\*</sup>Université Pierre et Marie Curie, Laboratoire Endocrinologie Moléculaire et Évolution, 7 Quai St. Bernard, 75252 Paris Cedex 05, France

Communicated by Bruce D. Hammock, University of California, Davis, CA, September 23, 2003 (received for review March 25, 2003)

The steroid 20-hydroxyecdysone (20E) is the primary regulatory hormone that mediates developmental transitions in insects and other arthropods. 20E is produced from ecdysone (E) by the action of a P450 monooxygenase that hydroxylates E at carbon 20. The gene coding for this key enzyme of ecdysteroidogenesis has not been identified definitively in any insect. We show here that the *Drosophila* E-20-monooxygenase (E20MO) is the product of the *shade* (*shd*) locus (cytochrome p450, CYP314a1). When *shd* is transfected into *Drosophila* S2 cells, extensive conversion of E to 20E is observed, whereas in sorted homozygous *shd* embryos, no E20MO activity is apparent either *in vivo* or *in vitro*. Mutations in *shd* lead to severe disruptions in late embryonic morphogenesis and exhibit phenotypes identical to those seen in *disembodied* (*dib*) and *shadow* (*sad*) mutants, two other genes of the Halloween class that code for P450 enzymes that catalyze the final two steps in the synthesis of E from 2,22-dideoxyecdysone. Unlike *dib* and *sad*, *shd* is not expressed in the ring gland but is expressed in peripheral tissues such as the epidermis, midgut, Malpighian tubules, and fat body, i.e., tissues known to be major sites of E20MO activity in a variety of insects. However, the tissue in which *shd* is expressed does not appear to be important for developmental function because misexpression of *shd* in the embryonic mesoderm instead of the epidermis, the normal embryonic tissue in which *shd* is expressed, rescues embryonic lethality.

One principal reason for the success of insects is their rigid exoskeleton (cuticle), which in many cases protects them from desiccation and predators while also providing the substrate for the development of jointed legs and wings. Growth is ultimately restricted by the surface area of this cuticle, and the insect must synthesize a larger cuticle exterior to the old one. As this process occurs, the old cuticle is digested by specific enzymes, the products of which are recycled, and the insect finally sheds the remnants of the original cuticle (ecdysis). This molting process is therefore required for the use of this rigid exoskeleton and indirectly for the success of insects on this planet.

The trigger, or initiator of the molting process, is a polyhydroxylated steroid, 20-hydroxyecdysone (20E), the molting hormone of the vast majority of arthropods, which elicits not only molting but most of the morphogenetic processes that comprise insect growth and metamorphosis (1). That is, almost the entire insect is the target of 20E. 20E itself is the product of the hydroxylation of ecdysone (E) mediated by an E-20-monooxygenase (E20MO), i.e., ecdysone 20-hydroxylase, so E is a precursor of 20E, although it may have hormonal roles as well (2, 3). E is synthesized from cholesterol or plant sterols in the prothoracic gland cells of the ring gland (Fig. 1).

Although it has been known for several decades that the E20MO is a P450 enzyme that is localized in the endoplasmic reticulum (4) and/or mitochondria (5, 6), depending on the insect, tissue, and developmental stage (7, 8), it has not been purified to homogeneity nor has the gene coding for this enzyme been cloned. Here, we

report the cloning of *shade* (CYP314a1), a member of the Halloween gene family (9), and demonstrate that its gene product codes for the *Drosophila* E20MO.

## Materials and Methods

***Drosophila* Strains.** *shd* mutant strains were obtained from C. Nüsslein-Volhard (Max Planck Institute, Tübingen, Germany) and cultured on standard cornmeal/yeast extract/dextrose medium. The alleles were described by Jürgens *et al.* (10). *twist*-Gal4 line 2517 and *armadillo*-Gal4 line 1560 were obtained from the Bloomington Stock Center (<http://flystocks.bio.indiana.edu>).

**Phenotypic Characterization of *shd* Embryos.** Cuticle preparations, staining for spectrin (an actin-binding cytoskeletal component) and IMP-E1 (a 20E-inducible gene) expression in embryos have been described (9).

**Gene Cloning and Identification of Mutations.** The full-length cDNA sequence was amplified from a *Drosophila* embryonic cDNA library in pNB40 (11) by PCR using the MACH amplification protocol (12). Two pairs of primers were designed on the 5' end (A + B) and 3' end (C + D): *ShdA* 5'-AGGAGCGCCCGAGGTAGAT-ATC-3'; *ShdB* 5'-GAAGAACACGCTCCTTGAGGACTTC-3'; *ShdC* 5'-GCCTGGCTCGCAGTAGTTCG-3'; and *ShdD* 5'-GGGATCCGGATACTGTTGG-3'. To identify the mutations in the *shd* mutants, genomic DNA from heterozygous animals was amplified by PCR using the primers 5'-ATAAGTGCCTCCA-AAGCGGATC-3' and 5'-AAACGCCTGAGGGTAGGCAC-3' and sequenced by using the Thermosequase cycle sequencing kit (United States Biochemical) according to the manufacturer's protocol. The mutant lesion was identified by the presence of two bases at a particular position that corresponds to either the mutant or balancer genomic sequences.

**Tissue Expression of *shd* by RT-PCR.** Total RNA was extracted from tissues of wandering third instar larvae (ring gland, brain, gut, fat body, salivary glands, and epidermis) and from adult males or females (head, gut, gonads, and carcass) by using the SV Total RNA Extraction kit (Promega). Single-stranded cDNAs for PCRs were

Abbreviations: CYP, cytochrome P450; 2dE, 2-deoxyecdysone; E, ecdysone; E20MO, E-20-monooxygenase; ESI, electrospray ionization; 20E, 20-hydroxyecdysone; HA, hemagglutinin; RP, reverse-phase; UAS, upstream activator sequence.

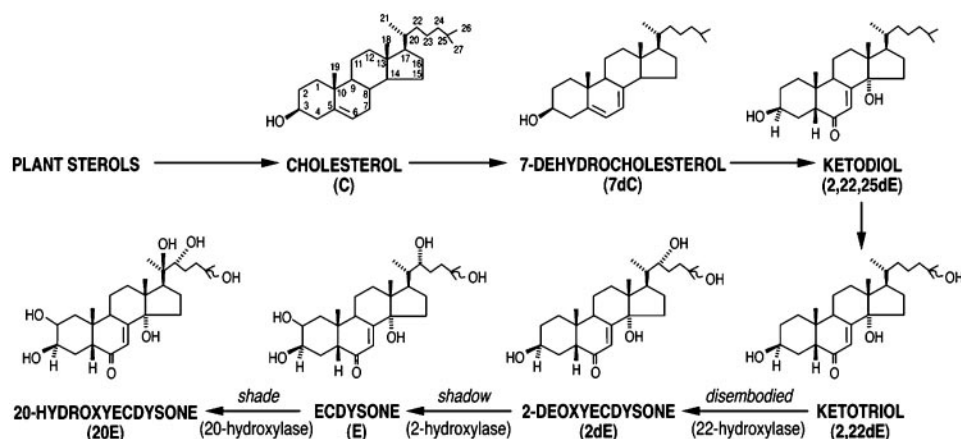
Data deposition: The sequence of *shade* cDNA has been deposited in the GenBank database (accession no. AF484414).

<sup>†</sup>A.P. and J.T.W. contributed equally to this work.

<sup>||</sup>Present address: Department of Cell Biology, University of Alabama, Birmingham, AL 35294.

<sup>††</sup>To whom correspondence should be addressed. E-mail: moconnor@mail.med.umn.edu.

© 2003 by The National Academy of Sciences of the USA



**Fig. 1.** Scheme of 20E biosynthesis. The top portion represents a theoretical pathway in which plant sterols obtained in the diet are converted to the ketodiol (23). The bottom portion of the figure shows the final three steps in the *Drosophila* pathway that involve conversion of the ketotriol intermediate to 20-hydroxyecdysone by the activity of the P450 enzymes coded for by *dib*, *sad*, and *shd* (ref. 16 and this work).

synthesized from total RNAs (100 ng for all tissues) with M-MLV reverse transcriptase (Promega). Specific primers for *shd* (*ShdUp1* 5'-CGCTCTCCATCGGCACAAAT-3' and *ShdDo1* 5'-AGCAG-CACCACCTCCATTTTC-3') and *rpL17A* (*L17Up1* 5'-GTGAT-GAACTGTGCCGACAA-3' and *L17Do1* 5'-CCTTCATT-TCGCCCTTGTTG-3') were used, giving 1.4-kb and 300-bp fragments. Thirty-five and 40 PCR cycles were carried out for *rpL17A* and *shd* amplification, respectively (94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min 30 sec), followed by a 10-min extension period at 72°C.

***shd* in Situ Hybridization.** The RNA hybridization and detection were done according to standard protocols (9). To obtain the *shd* RNA antisense probe, pNB40-*shd* was linearized with *HindIII* and transcribed with the T7 promoter. For the *shd* sense probe, pNB40-*shd* was linearized with *NotI* and transcribed with the SP6 promoter.

**Construction of Upstream Activator Sequence (UAS) Transgenes and Rescue Crosses.** The UAS-*shd* construct was generated by ligation of a *BglII/NotI* fragment, isolated from a pNB40 clone containing the full-length cDNA of *shd*, into a *BglII/NotI* site of the pUAS vector (13). Transformants were obtained by using standard protocols. Males carrying a tissue-specific Gal4 driver, either *twist* (14) or *armadillo* (15), and the mutant allele *shd*<sup>Z320</sup>/*TM6B* were crossed with virgin females carrying a UAS transgene and a different mutant allele, *shd*<sup>Z320</sup>/*TM6B*. Progeny were scored for the survival and fertility of *shd*-homozygous adults.

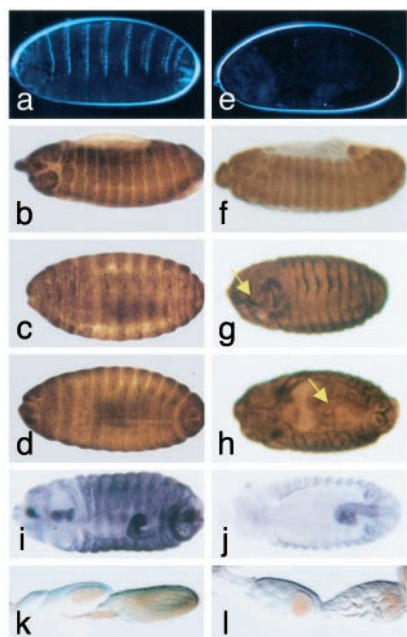
**Endogenous Embryonic Ecdysteroid Titers and Composition.** Dechorionated, wild-type embryos collected every 2 h or selected homozygous mutant *shd*<sup>Z320</sup> embryos, mechanically sorted from heterozygous mutant *shd*<sup>Z320</sup>/*TM3-armadillo*-GFP embryos (both 10–14 h old) (16) by their lack of expression of GFP fluorescence (Copas Select, Harvard Bioscience, Holliston, MA), were homogenized and extracted exhaustively with methanol. Aliquots of the pooled extracts from replicate 20-mg samples (≈2,000 embryos) were subjected to RIA with the H22 antibody (16). Results are expressed in E equivalents. The replicate extracts were then pooled, evaporated to dryness, and subjected to reverse-phase (RP)-HPLC/TLC and differential RIA analysis with both the H22 and SHO3 antibodies to determine the composition of endogenous embryonic ecdysteroids (17). High specific activity 23,24-<sup>3</sup>H]E (60 Ci/mmol; 1 Ci = 37 GBq) was purchased from NEN, E and 20E standards were purchased from Sigma-Aldrich, and 2-deoxyecdysone (2dE) was a gift from R. Lafont (Université Pierre et Marie Curie).

**Transfection of S2 Cells.** S2 cells were transfected with *shd* cDNA under the control of the actin 5C promoter or with a control construct constitutively expressing the GFP protein by using DDAB-mediated transfection (16, 18). *shd* cDNA was cloned as a *HindIII/NotI* fragment containing the full-length cDNA of *shd* into the *StuI/NotI* of the S2 expression vector pBRAcP (19). Shd, Sad, and Dib proteins were epitope-tagged at the C terminus by introducing a *NotI* site after the last amino acid. A three-copy hemagglutinin (HA) epitope was dropped into the *NotI* site and the constructs were checked by sequencing.

**In Vitro E20MO Activity.** Conversion of [<sup>3</sup>H]E to [<sup>3</sup>H]20E was measured 3 days after the transfection of S2 cells with *shd* or GFP (control) expression constructs. The cells (8 × 10<sup>6</sup>) were collected by centrifugation (1,200 × *g* for 5 min) and homogenized into medium (1 ml) containing [<sup>3</sup>H]E (1.0 μCi), nonradiolabeled E (1 μg, 2 μM), and NADPH (0.5 mM), and incubated at 25°C for 8 h. Alternatively, the cells were transferred to fresh medium (11 ml) containing [<sup>3</sup>H]E (1.0 μCi) and E (0–2 μM), but without NADPH, and incubated as before. Methanol extracts were analyzed by RP-HPLC, TLC, and electrospray ionization (ESI)-MS (16).

In a similar manner, to assay E20MO activity during *Drosophila* development, 20 mg of dechorionated wild-type embryos, 2,000 machine-sorted homozygous or heterozygous mutant *shd*<sup>Z320</sup> embryos (10–14 h old), or whole bodies or selected tissues from *yw*, heterozygous, or homozygous (rescued) UAS-*shd*/*twist*Gal4 wandering late third instar mutant larvae or 3-day-old adults were homogenized in Grace's medium (1 ml, pH 7.0) containing [<sup>3</sup>H]E (0.5 μCi) and NADPH (0.5 mM) and incubated at 25°C for 6 h. Samples were extracted repeatedly with methanol, the pooled solvents were evaporated, and the residues were subjected to RP-HPLC and TLC analysis along with added E and 20E standards (1 μg).

**Subcellular Localization of Halloween Gene Products.** S2 cells were cotransfected with HA-tagged versions of the protein of interest and an endoplasmic reticulum (mSpitz-GFP) marker (20). Three days after transfection the cells were plated on Con A-coated Lab-Tek II slides (21). After 2 h, the cells were washed, treated with 250 nM MitoTracker Red (Molecular Probes), fixed with 3.7% paraformaldehyde, and permeabilized with methanol. 12CA5 anti-HA (Roche) and goat anti-mouse Alexa 488 or 633 (Molecular Probes) were used to detect the HA-tagged proteins. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI), mounted in PermaFluor (Thermo Shandon, Pittsburgh), and ana-



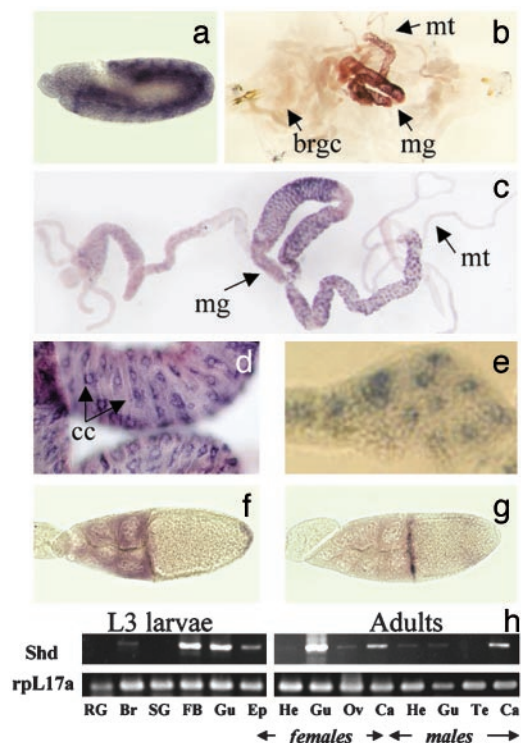
**Fig. 2.** Phenotype of homozygous *shd* embryos and egg chambers. (a) Normal cuticle development in wild-type embryos. (b–d) Normal embryonic development of wild-type embryos. (e–h) Homozygous *shd* embryos. (e) Lack of cuticle differentiation in *shd* mutant embryos. (f) Normal embryonic development at stage 14. (g) Failure of head involution at embryonic stages 15–16 (see arrow). (h) Defect in dorsal closure and aberrant gut looping at embryonic stages 15–16 (see arrow). Embryos in b–d and f–h were stained with spectrin antibody. (i) Stage-15 wild-type embryo showing normal *IMP-E1* expression. (j) Reduced *IMP-E1* epidermal expression in stage 13 *shd* mutant embryos. The remaining gut staining is presumably under the influence of a nonecdysone response enhancer element (9, 16). (k) Wild-type egg chambers. (l) Egg chambers showing arrest and degeneration at stages 8–9 of oogenesis in rescued females of the genotype *twist>gal4, shd<sup>Z329</sup>/UAS-shd, shd<sup>Z320</sup>*.

lyzed by confocal microscopy (Axioplan 2, Zeiss). Individual optical sections are shown.

## Results

**Confirmation of *shade* as CYP314a1.** The Halloween genes *dib* and *sad* code for the ecdysteroid C<sub>22</sub>-monooxygenase (CYP302a1) and C<sub>2</sub>-monooxygenase (CYP315a1), respectively (Fig. 1 and ref. 16). Because both *dib* and *sad* proved to code for ecdysone biosynthetic pathway components, we analyzed other Halloween mutants characterized by similar abnormal cuticular patterning (9, 10). We determined that *shade* (*shd*), which mapped to 70D2-E8 (10), was in the vicinity of CYP314a, a P450 located at 70E4 (<http://flybase.bio.indiana.edu>; ref. 22). To determine whether *shd* corresponds to this P450, CYP314a genomic DNA was amplified by PCR from the appropriate heterozygous mutant stock and sequenced. We found that *Shd<sup>Z320</sup>* has a stop codon at position 136 (a change in the first base from C to T), *shd<sup>Z383</sup>* has a point mutation resulting in an amino acid change (glutamic acid to lysine) at position 225 (a change in the first base from G to A), and *shd<sup>Z329</sup>* has a mutation in the acceptor site of intron 1 confirming that CYP314a is the product of the *shd* gene.

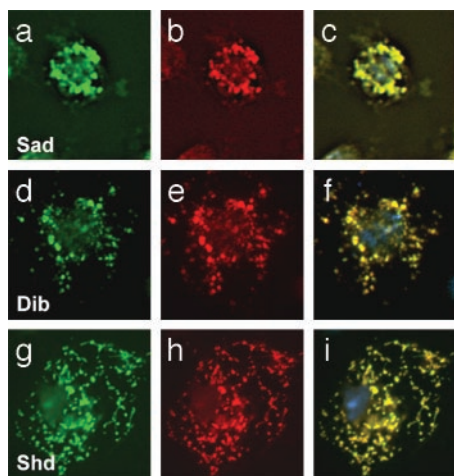
**Phenotype of *shd* Mutants.** Like other mutants of the Halloween class, *shd* mutants do not produce differentiated cuticle (Fig. 2 a vs. e). Spectrin staining (to highlight the general morphology of embryos) reveals that early embryonic development of *shd* mutants appears normal until approximately stage 14 (10.3–11.3 h) (Fig. 2 b vs. f). At stages 15–16 (11.3–16.0 h) (Fig. 2 c and d vs. g and h), abnormal morphogenetic movements become apparent that in-



**Fig. 3.** *In situ* expression pattern of *shd*. (a) *shd* expression in the epidermis at the stage of germband extension (stage 10, 4:20–5:20 h). (b) *shd* expression in midgut (mg) and Malpighian tubules (mt) in third instar larval whole body but not in the brain-ring gland complex (brgc). (c) *shd* expression in third instar larval mg and mt. (d) Copper cells (cc) of the third instar larval midgut. (e) Third instar larval fat body. (f) Nurse cells of the adult ovary. (g) Centripetally migrating follicle cells. (h) RT-PCR study of *shd* tissue expression (Upper) and a control gene, *rpl17a* (Lower), in third instar larvae (Left) and adults (Right). RG, ring gland; Br, brain; SG, salivary glands; FB, fat body; Gu, gut; Ep, epidermis; He, head; Ov, ovary; Te, testis; Ca, carcass.

volve failure of head involution (indicated by an arrow in Fig. 2g), defects in dorsal closure, and aberrant gut looping (indicated by an arrow in Fig. 2h), a phenotype very similar to that of *dib* and *sad* mutants (9, 10). All *shd* alleles exhibit phenotypes equivalent to *shd<sup>Z320</sup>*, which we assume is a null allele because the stop codon eliminates the critical catalytic heme-binding domain. As with *dib* and *sad* mutants (9, 16), *shd* mutants lack epidermal expression of the ecdysone responsive gene *IMP-E1* in stage 15 (11.3–13.0 h) embryos relative to wild type (Fig. 2 i and j), consistent with our hypothesis that this gene is also involved in ecdysteroid synthesis.

**Expression of *shd*.** To determine whether the expression pattern of *shd* is consistent with a role for *shd* in ecdysteroidogenesis, *in situ* hybridization of embryos, third instar larvae, and adult ovaries was performed. Unlike *dib* and *sad*, *shd* is not expressed early in embryogenesis (data not shown), but by the time of maximum germband extension (Fig. 3a, stage 10, 4:20–5:20 h), strong *shd* expression is observed in the epidermal cells. Expression of *shd* decreases significantly in older embryos but remains primarily epidermal (data not shown). *In situ* hybridization of third instar larvae reveals expression of the *shd* transcript in the midgut copper cells, Malpighian tubules, and fat body (Fig. 3 b–e). There is no *shd* expression in the brain-ring gland complex (Fig. 3b). *Shd* expression is also very low or absent in salivary glands and muscle tissue. In the adult ovaries, *shd* is expressed in both follicle and nurse cells (Fig. 3f), and prominent staining occurs in the centripetally migrating follicle cells (Fig. 3g). At present, we cannot exclude the possibility of a maternal contribution. Semiquantitative RT-PCR analysis of



**Fig. 4.** Immunolocalization of Halloween proteins. Confocal sections of S2 cells transfected with HA-tagged Sad (a–c), Dib (d–f), and Shd (g–i). HA immunoreactivity is shown in a, d, and g (green), MitoTracker Red signal is shown in b, e, and h (red), and the merge of HA and MitoTracker staining is shown in c, f, and i.

both larval and adult tissues is consistent with the *in situ* results (Fig. 3h).

**Subcellular Localization of Dib, Sad, and Shd Proteins.** Numerous charged residues and other conserved motifs characteristic of mitochondrial P450 localization signals (23, 24) were found at the N terminus of Sad and Dib, suggesting that these gene products are likely mitochondrial (9, 16), in agreement with the previously reported subcellular localization of the 2- and 22-monoxygenase activities (25). To test this prediction experimentally, epitope-tagged versions of these proteins were cotransfected into S2 cells with a GFP-tagged marker for the endoplasmic reticulum, and mitochondria were labeled with MitoTracker Red. As shown in Fig. 4, both Sad (a–c) and Dib (d–f) colocalized with the mitochondrial marker, without a detectable signal in the endoplasmic reticulum (not shown), thus confirming their predicted localization. Analogous studies employing an epitope-tagged version of Shd revealed its colocalization with mitochondria as well (Fig. 4g–i).

**Transgenic Rescue of *shade* Mutants.** Because *shd* shows strong expression in both the embryonic and larval midgut (Fig. 3a–e), particularly in the copper cells, we expressed *shd* by using the Gal4-UAS expression system with *twist* or *armadillo* promoters. *Twist* is an embryonic mesodermal driver that is also expressed in the larval midgut in both copper cells and muscle nuclei, but not in fat body, Malpighian tubules, salivary glands, or epidermis (data not shown), and *armadillo* is a ubiquitous driver that is expressed throughout development (15). When *shd* was expressed by using either the *twist* or *armadillo* drivers, all *shd* mutant embryos developed into normal larvae and these larvae eclosed into adults [for *twist*>*Gal4*, *shd*<sup>329</sup>/*TM6B* × *UAS-shd*, *shd*<sup>Z320</sup>, 248 total progeny were recovered, of which 88 were rescued animals (102% of expected); for *arm*>*Gal4*, *shd*<sup>329</sup>/*TM6B* × *UAS-shd*, *shd*<sup>Z320</sup>, 145 total progeny were recovered, of which 38 were rescued animals (80% of expected)]. However, with the *armadillo* driver, the adults were fertile, whereas with the *twist* driver all of the females were sterile and ovaries stopped development at stages 8–9 (Fig. 2k vs. l).

**Embryonic Ecdysteroid Titers and Composition.** Previously, very low ecdysteroid titers had been measured by RIA in sorted homozygous mutant *dib* and *sad* embryos relative to the titers in either sorted mutant heterozygous *dib* and *sad* or wild-type embryos of the same

**Table 1. Endogenous embryonic ecdysteroid composition: Percent of total Ecdysteroids (average of two replicates)**

| Age, h | Wild type |     |    |     |     | Homozygous <i>shd</i> <sup>Z320</sup> |     |    |     |     | Heterozygous <i>shd</i> <sup>Z320</sup> |     |   |     |     |
|--------|-----------|-----|----|-----|-----|---------------------------------------|-----|----|-----|-----|---|-----|---|-----|-----|
|        | VPP       | 20E | E  | 2dE | LPP | VPP                                   | 20E | E  | 2dE | LPP | VPP                                     | 20E | E | 2dE | LPP |
| 0–2    | 0         | 0   | 3  | 97  | 0   |                                       |     |    |     |     |   |     |   |     |     |
| 2–4    | 0         | 0   | 20 | 80  | 0   |                                       |     |    |     |     |   |     |   |     |     |
| 4–6    | 0         | 0   | 50 | 50  | 0   |                                       |     |    |     |     |   |     |   |     |     |
| 6–8    | 5         | 10  | 72 | 10  | 3   |                                       |     |    |     |     |   |     |   |     |     |
| 10–12  | 85        | 5   | 0  | 0   | 10  |                                       |     |    |     |     |   |     |   |     |     |
| 12–14  | 87        | 3   | 0  | 0   | 10  | 4                                     | 0   | 45 | 50  | 1*  | 85                                      | 8   | 0 | 0   | 7*  |

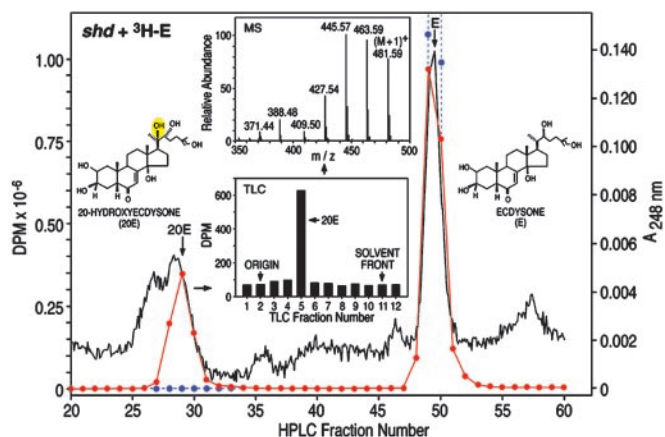
Values were corrected for antibody cross-reactivity. In order of decreasing polarity: VPP, very polar products; 20E, E; 2dE, LPP, low-polarity products. \*Ten to 14 h

age (16). However, analysis of ecdysteroid titers in extracts from machine-sorted 10- to 14-h-old homozygous or heterozygous mutant *shd*<sup>Z320</sup> embryos were identical to those of 10- to 14-h-old wild-type embryos, i.e., ≈50 pg/mg of wet weight. Nevertheless, subsequent chromatographic separation (RP-HPLC and TLC) and differential RIA analysis of the ecdysteroids in these sorted homozygous *shd*<sup>Z320</sup> embryos revealed that E, together with its immediate precursor 2dE (26), were the major ecdysteroids present. No 20E was detected (Table 1). In contrast, in both sorted heterozygous *shd*<sup>Z320</sup> and wild-type embryos of the same age, 20E and more polar products predominated and E and 2dE were low or absent (27). In fact, the ecdysteroid profile in these 10- to 14-h-old homozygous mutant *shd*<sup>Z320</sup> embryos was very similar to that of early (4–6 h old) wild-type embryos (27), i.e., before the appearance at 6–8 h of either detectable endogenous 20E or significant E20MO activity (28) (see below).

**Characterization of E20MO Enzymatic Activity in S2 Cells Transfected with *shd*.** Because the above RIA data suggested that *shd* encodes the E20MO, S2 cells transfected with *shd* or the GFP-control construct were homogenized into media containing both [<sup>3</sup>H]E and nonradiolabeled E for 8 h. The medium and cells were then extracted and analyzed by RP-HPLC/TLC/ESI-MS. The data revealed that these *shd*-transfected S2 cells hydroxylate E solely to 20E, whereas no such activity was observed in GFP-transfected cells. As shown in Fig. 5, 0.23 μg of 20E was recovered from 1 μg of E substrate, a yield of 23% at a substrate concentration of 2 μM. The characterization of this product as 20E was not only consistent with its UV spectrum (UV<sub>max</sub> 248 nm; data not shown) but was also consistent with its specific UV absorption (E 12,400) and radiochemical analysis (Fig. 5). The identity of this product was confirmed by RP-HPLC (20% acetonitrile)/ESI-MS after additional normal-phase TLC purification (Fig. 5 *Insets*). Note the molecular ion (M + 1)<sup>+</sup> at 481 and the sequential loss of four molecules of water at (M + 1)<sup>+</sup>-H<sub>2</sub>O<sub>(1–4)</sub>, characteristic of 20E (29). Additional kinetic analysis with intact S2 cell cultures revealed a K<sub>m</sub> of 1.0 μM and a V<sub>max</sub> of 1 nmol/8 h for this enzyme (data not shown). We conclude from these data that *shd* codes for the E20MO.

**Embryonic, Larval, and Adult *in Vitro* E20MO Enzymatic Activity.** In a similar fashion, analysis of embryonic tissue homogenates (Table 2) revealed that both wild-type (10–12 and 12–14 h old) and heterozygous mutant *shd*<sup>Z320</sup> embryos (10–14 h old) exhibited definitive conversion of the [<sup>3</sup>H]E substrate to [<sup>3</sup>H]20E (averages of 1.8% and 1.7%, respectively). In contrast, E20MO activity was undetectable in both 10- to 14-h-old homozygous *shd*<sup>Z320</sup> embryos and in wild-type embryos before 6–8 h, i.e., the stage when significant E20MO activity (0.5%) is first observed (28) and *shd* expression first becomes apparent (Fig. 3)

Later, during the third instar (28), considerable E20MO activity was observed in wandering wild-type, *yw*, or heterozygous UAS-



**Fig. 5.** RP-HPLC/TLC/ESI-MS analysis of ecdysteroids after *shd*- or GFP-transfected S2 cell homogenate incubation (8 h) with [<sup>3</sup>H]E containing E (1 μg) and NADPH (30–100% methanol gradient). Radioactivity was measured after incubations with *shd*-transfected (red circles) or GFP-transfected (blue circles) cell homogenates with substrate. UV absorption was measured at 248 nm (solid line). (Upper Inset) TLC (chloroform/ethanol) of RP-HPLC-purified 20-hydroxyecdysone (20E) product (1/1,000th of total sample). (Lower Inset) RP-HPLC/ESI-MS on a TSQ Quantum (Thermo Finnigan, San Jose, CA) of the TLC-purified 20E product.

*shd*/*twistGal4* larval homogenates (42.2%, 31.0%, and 19.2%, respectively), whereas very low activity (1.3%) was present in homozygous UAS-*shd*/*twistGal4* (rescued) larvae (Table 3). Within individual larval tissues, the majority of activity was found in the fat body, i.e., wild-type, *yw*, and heterozygous UAS-*shd*/*twistGal4* (26.7%, 12.8%, and 7.3%, respectively), whereas E20MO activity was undetectable in homozygous UAS-*shd*/*twistGal4* (rescued) larval fat body. Significant E20MO activity was also observed in wild-type, *yw*, and heterozygous UAS-*shd*/*twistGal4* Malpighian tubules and salivary glands, yet none was detected in these same tissues from homozygous UAS-*shd*/*twistGal4* (rescued) larvae. In contrast, homogenates of the remaining carcass (mostly muscle and epidermis) and midgut tissues exhibited moderate E20MO activity in all types of larvae, whether wild-type or transgenic. However, no E20MO activity was found in larval brain ring-gland complexes.

E20MO activity could also be detected in whole fly homogenates of all 3-day-old females (Table 3), i.e., wild-type, *yw*, and heterozygous UAS-*shd*/*twistGal4* (1.8%, 1.6%, and 1.1%, respectively), but it was much lower in homozygous UAS-*shd*/*twistGal4* (rescued) adults (0.09%). Although the majority of this whole-body activity was found to be localized in the ovarian tissues of wild-type, *yw*, and heterozygous UAS-*shd*/*twistGal4* (1.7%, 2.1%, and 1.0%, respectively) it was not present in the homozygous UAS-*shd*/*twistGal4* (rescued) ovaries. Nevertheless, low but detectable E20MO activity was found in the carcass (comprising all of the remaining tissues) of all four fly lines.

**Table 2. E20-MO activity *in vitro*: Percent conversion of [<sup>3</sup>H]E to [<sup>3</sup>H]20E**

| Age   | Embryo (average of two or more replicates) |                                       |   |
|-------|--|---------------------------------------|---|
|       | Wild type                                  | Homozygous <i>shd</i> <sup>Z320</sup> | Heterozygous <i>shd</i> <sup>Z320</sup> |
| 0–2   | 0.0  |                                       |   |
| 2–4   | 0.0  |                                       |   |
| 4–6   | 0.0  |                                       |   |
| 6–8   | 0.5  |                                       |   |
| 10–12 | 1.5  |                                       |   |
| 12–14 | 2.0  | 0.0*                                  | 1.7*                                    |

\*Ten to 14 h.

**Table 3. E20-MO activity *in vitro*: Percent conversion of [<sup>3</sup>H]E to [<sup>3</sup>H]20E**

|                          | Wild type | Homozygous                            | Heterozygous                          |
|--------------------------|-----------|---------------------------------------|---------------------------------------|
|                          | <i>yw</i> | UAS- <i>shd</i> /<br><i>twistGal4</i> | UAS- <i>shd</i> /<br><i>twistGal4</i> |
| <b>Larvae*</b>           |           |                                       |                                       |
| Whole larvae             | 42.2      | 31.0                                  | 19.2                                  |
| Fat body                 | 26.7      | 12.8                                  | 7.3                                   |
| Malpighian tubules       | 5.6       | 4.6                                   | 3.6                                   |
| Salivary glands          | 0.5       | 0.6                                   | 0.3                                   |
| Brain-ring gland complex | 0.0       | —                                     | —                                     |
| Midgut                   | 3.2       | 3.0                                   | 1.6                                   |
| Carcass                  | 1.2       | 3.1                                   | 2.3                                   |
| <b>Adult*</b>            |           |                                       |                                       |
| Whole fly                | 1.8       | 1.6                                   | 1.1                                   |
| Ovaries                  | 1.7       | 2.1                                   | 1.0                                   |
| Carcass                  | 0.4       | 0.3                                   | 0.2                                   |

\*Fifteen larvae (wandering late third instar) or 15 adults (3-day-old females) or tissues from 15 larvae or adults.

## Discussion

The identification of *Dib* as the ecdysteroid C<sub>22</sub>-monooxygenase and *Sad* as the ecdysteroid C<sub>2</sub>-monooxygenase demonstrated the advantage of using a combination of *Drosophila* molecular genetics and biochemistry to elucidate the details of the ecdysteroid biosynthetic pathway (16). Herein, this paradigm was used to identify the *shd* gene product (CYP314a1) as the ecdysone 20-monooxygenase responsible for mediating the conversion of E to the active molting hormone, 20E. The capacity of S2 cells transfected with *shd* to convert E to 20E in addition to the absence of further metabolism of E to 20E in machine-sorted homozygous *shd* mutant embryos both *in vitro* (Table 2) and *in vivo* (Table 1), in contrast to wild-type or heterozygous *shd* mutant embryos, not only proves that *Shd* has E20MO activity but also that *Shd* is responsible for all of this activity during embryogenesis. Thus, unlike *dib* and *sad* homozygous mutants, which are “low E” embryos (9, 16), the homozygous *shd* embryos are “low 20E” mutants. This results in reduced IMP-E1 expression and embryonic developmental abnormalities similar to those observed in the “low E” Halloween mutants. The similarity in phenotype between these two types of mutants suggests that E, which is present in high levels in *shd* mutant embryos, has little functional activity at this stage.

The data in Fig. 3 and Tables 2 and 3 also indicate that *Shd* is responsible for all E20MO activity during larval and adult stages, because it is expressed in tissues (fat body, midgut, Malpighian tubules, epidermis, salivary glands, and ovaries) known to possess this enzyme activity *in vitro* during these stages, not only in *Drosophila* (30–34) but also in other Dipteran insects (35). In addition, *shd* is not expressed in tissues where such activity has been reported to be absent, such as in the ring glands, brain, ventral ganglion, and muscle of Diptera and other insects including *Drosophila* (5, 36–42). The complete absence of *in vitro* E20MO activity in the larval fat body, Malpighian tubules, salivary glands, and adult ovaries of rescued homozygous UAS-*shd*/*twistGal4* flies relative to that observed in corresponding heterozygous UAS-*shd*/*twistGal4*, *yw*, or wild-type tissues is presented as proof that *Shd* is solely responsible for E20MO activity in these tissues.

In embryos, it seems probable that both E and 20E are highly mobile and that 20E is able to reach all target tissues irrespective of where it is made. This follows because expression of *shd* in embryos is normally confined to epidermal cells. Nevertheless, ectopic expression of *shd* in the embryonic mesoderm as well as in larval and (presumably) pupal gut and muscle by using the UAS-*shd*/*twistGal4* system completely rescued embryonic, larval, and pupal development to the adult. That is, although the fat body is normally by far the most active tissue in the conversion of E to 20E

during larval stages in wild-type, *yw*, and heterozygous *UAS-shd/twistGal4* individuals (Tables 2 and 3), in the rescued homozygous *UAS-shd/twistGal4* individuals, fat body activity is nonexistent. Instead, relatively low E20MO activity expressed under *twist* promoter control is only observed in the midgut and muscle tissues of the carcass. Yet, this activity is nevertheless sufficient for normal development to the adult. In addition, when *shd* expression is placed under the control of the ubiquitous *armadillo* promoter and so is expressed in all tissues at all times, the same result is observed. Thus, it follows that the appearance of active hormone (20E) is not the result of the precise developmental control of *shd* transcription but rather of the biosynthesis and release of E from the prothoracic gland cells of the ring gland. Circulating E is efficiently distributed to sites of Shd activity for conversion to 20E, which subsequently equilibrates throughout the embryo, larva, pupa, and developing adult.

However, in the homozygous *UAS-shd/twistGal4* adult, no E20MO activity was detected in the ovaries (Tables 2 and 3) and severe degeneration of the egg chambers after stage 9 was observed (Fig. 2), even though some E20MO activity was found in the adult carcass. Apparently, in the adult, the ovary may be a closed system. That is, the ovary must not only synthesize ecdysone (43) but also must convert it to the active hormone 20E for normal ovarian development and fertility. Thus, *armadillo*, but not *twist*, is a suitable driver for necessary adult *shd* expression leading to fertility.

Another interesting aspect of the *shd* embryonic expression pattern is its delayed appearance relative to *dib* and *sad*, which are both first expressed at the blastoderm stage. The lag in *shd* expression likely accounts for the observations first made by Maróy *et al.* (27) and Mitchell and Smith (28), and confirmed here, that *Drosophila* embryos initially synthesize E from less-polar components (i.e., 2dE) during the first 6 h of development and only later (6–14 h) convert E to 20E and more-polar compounds. Indeed, it is only after 6 h that the 20E-inducible cascade of early transcription factor genes *E75A&B*, *DHR3*, *DHR4*, *DHR39*, and *βFTZ-F1* becomes apparent (April A. Sullivan and Carl S. Thummel, personal communication). Additional support comes from recent findings that E20MO activity, both *in vivo* and *in vitro*, increases dramatically

after gastrulation in developing nondiapause eggs of the silk moth, *Bombyx mori*, relative to the very low activity measured in diapause-destined eggs, which cease further embryonic development at this time (44, 45).

Finally, in S2 cells, the Dib, Sad, and Shd enzymes are all localized to the mitochondria. For Dib and Sad, this is consistent with prior biochemical fractionation data showing that both the 22- and 2-monooxygenase activities are mitochondrial (29). Prior cell fractionation data for the E20MO is more ambiguous, with a microsomal and/or mitochondrial localization identified, depending on the insect, tissue, and developmental stage being examined (4, 5, 7, 46, 47). The N terminus of the Shd protein contains both hydrophobic signal-type sequences typical of microsomal P450s (48) as well as a charged segment containing sequences characteristic of typical mitochondrial enzymes (49). Thus, as a result of differential post translational modification, it is possible that Shd resides in either location depending on tissue type and developmental stage. However, the cofactors for supplying reducing equivalents are quite different for mitochondrial and microsomal P450 enzymes, as are the sequences that are thought to interact with these cofactors. Thus, it remains a possibility that E20MO activity has evolved independently in different insect species and so a microsomal location in other species might reflect a divergent P450 not related to Shd, as is suggested by recent data concerning the tentative identification of CYP6H1 as a E20MO in the Malpighian tubules of the locust (50, 51). The future unequivocal identification of ecdysone 20-monooxygenases from a variety of insects may reveal whether there was evolution of the enzyme itself or of subcellular localization signals for a common enzyme.

We thank Susan Whitfield for graphics, Beni Shilo for ER GFP marker, C. Nüsslein-Volhard for *shd* alleles, and Carl Thummel, Rene Feyereisen, and Stan Smith for constructive criticism of the manuscript. The University of North Carolina MS facility is supported in part by National Institutes of Health Grants P30 CA16086 and P30 ES10126. This work was supported by National Institutes of Health Training Grant HD33692 (to A.P.) and National Science Foundation Grant IBN0130825 (to L.I.G. and J.T.W.). M.B.O. is an Investigator of the Howard Hughes Medical Institute. C.D.-V. and J.-P.P. are supported by the Université Pierre et Marie Curie and the Ministère de la Recherche Scientifique.

- Henrich, V. C., Rybczynski, R. & Gilbert, L. I. (1999) *Vitam. Horm.* **55**, 73–125.
- Warren, J. T. & Gilbert, L. I. (1986) *Insect Biochem.* **16**, 65–82.
- Hiruma, K., Böcking, D., Lafont, R. & Riddiford, L. M. (1997) *Gen. Comp. Endocrinol.* **107**, 84–97.
- Feyereisen, R. & Durst, F. (1978) *Eur. J. Biochem.* **88**, 37–47.
- Bollenbacher, W. E., Smith, S., Wielgus, J. & Gilbert, L. I. (1977) *Nature* **268**, 660–663.
- Blais, C. & Lafont, R. (1986) *Arch. Insect Biochem. Physiol.* **3**, 501–512.
- Smith, S., Bollenbacher, W., Cooper, D., Schleyer, H., Wielgus, J. & Gilbert, L. I. (1979) *Mol. Cell. Endocrinol.* **15**, 111–133.
- Smith, S. L., Bollenbacher, W. E. & Gilbert, L. I. (1983) *Mol. Cell. Endocrinol.* **31**, 227–251.
- Chávez, V. M., Marqués, G., Delbecq, J. P., Kobayashi, K., Hollingsworth, M., Burr, J., Natzle, J. E. & O'Connor, M. B. (2000) *Development (Cambridge, U.K.)* **127**, 4115–4126.
- Jürgens, G., Wieschaus, E., Nüsslein-Volhard, C. & Kluding, H. (1984) *Roux's Arch. Dev. Biol.* **193**, 283–295.
- Brown, N. & Kafatos, F. (1988) *J. Mol. Biol.* **203**, 425–437.
- Haerry, T. E. & O'Connor, M. B. (2002) *Gene* **291**, 85–93.
- Brand, A. H. & Perrimon, N. (1993) *Development (Cambridge, U.K.)* **118**, 401–415.
- Furlong, E. E. M., Anderson, E., Null, B., White, K. & Scott, M. (2001) *Science* **293**, 1629–1633.
- Sanson, B., White, P. & Vincent, J. P. (1996) *Nature* **383**, 627–630.
- Warren, J. T., Petryk, A., Marqués, G., Jarcho, M., Parvy, J.-P., Dauphin-Villemant, C., O'Connor, M. B. & Gilbert, L. I. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 11043–11048.
- Warren, J. T., Steiner, B., Dorn, A., Pak, M. & Gilbert, L. I. (1986) *J. Liquid Chromatogr.* **9**, 1759–1782.
- Han, K. (1996) *Nucleic Acids Res.* **24**, 4362–4363.
- Cherbas, L., Moss, R. & Cherbas, P. (1994) *Methods Cell Biol.* **44**, 161–179.
- Tsruya, R., Schlesinger, A., Reich, A., Gabay L., Sapir, A. & Shilo, B.-Z. (2002) *Genes Dev.* **16**, 222–234.
- Rogers, S. L., Rogers G. C., Sharp, D. J. & Vale, R. D. (2002) *J. Cell Biol.* **158**, 873–884.
- Adams, M. D., Celniker, S., Holt, R., Evans, C., Gocayne, J., Amanatides, P., Scherer, S., Li, P., Hoskins, R., Galle, R., *et al.* (2000) *Science* **287**, 2185–2195.
- Feyereisen, R. (1999) *Annu. Rev. Entomol.* **44**, 507–533.
- Tijet, N., Helvig, C. & Feyereisen, R. (2001) *Gene* **262**, 189–198.
- Kappler, C., Kabbouh, M., Hetru, C., Durst, F. & Hoffmann, J. A. (1988) *J. Steroid Biochem.* **31**, 891–898.
- Gilbert, L. I., Rybczynski, R. & Warren, J. T. (2002) *Annu. Rev. Entomol.* **47**, 883–916.
- Maróy, P., Kaufmann, G. & Dübendorfer, A. (1988) *J. Insect Physiol.* **34**, 633–637.
- Mitchell, M. J. & Smith, S. L. (1988) *Gen. Comp. Endocrinol.* **72**, 467–470.
- Wainwright, G., Prescott, M., Lomas, L., Webster, S. & Rees, H. H. (1997) *Arch. Insect. Biochem. Physiol.* **35**, 21–31.
- Bownes, M., Dübendorfer, A. & Smith, T. (1984) *J. Insect Physiol.* **30**, 823–830.
- Dübendorfer, A. & Maróy, P. (1986) *Insect Biochem.* **16**, 109–113.
- Sommé-Martin, G., Colardeau, J. & Lafont, R. (1988) *Insect Biochem.* **18**, 729–734.
- Sommé-Martin, G., Colardeau, J. & Lafont, R. (1988) *Insect Biochem.* **18**, 735–742.
- Grau, V. & Lafont, R. (1994) *J. Insect Physiol.* **40**, 87–96.
- Smith, S. & Mitchell, M. (1986) *Insect Biochem.* **16**, 49–55.
- Bollenbacher, W. E., Goodman, W., Vedeckis, W. V. & Gilbert, L. I. (1976) *Steroids* **27**, 309–324.
- Redfern, C. P. F. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5643–5647.
- Pak, M. & Gilbert, L. I. (1987) *J. Liquid Chromatogr.* **10**, 2591–2611.
- Henrich, V., Pak, M. & Gilbert, L. I. (1987) *J. Comp. Physiol.* **157**, 543–549.
- Böcking, D., Dauphin-Villemant, C., Toulllec, J. Y., Blais, C. & Lafont, R. (1994) *C. R. Acad. Sci. Ser. III* **317**, 891–898.
- Warren, J. T., Bachman, J., Dai, J. D. & Gilbert, L. I. (1996) *Insect Biochem. Mol. Biol.* **26**, 931–943.
- Warren, J. T. & Gilbert, L. I. (1996) *Insect Biochem. Mol. Biol.* **26**, 917–929.
- Kelly, T. J. (1994) in *Perspectives in Comparative Endocrinology*, eds Davey, K. G., Peter, R. G. & Tobe, S. S. (Natl. Res. Council of Canada, Ottawa), pp. 282–290.
- Horike, N. & Sonobe, H. (1999) *Arch. Insect Biochem. Physiol.* **41**, 9–17.
- Sonobe, H., Tokushige, H., Makka, T., Tsutsumi, H., Hara, N. & Fujimoto, Y. (1999) *Zool. Sci.* **16**, 935–943.
- Mitchell, M. J. & Smith, S. L. (1986) *Insect Biochem.* **16**, 525–537.
- Smith, S. L. & Mitchell, M. J. (1986) *Insect Biochem.* **16**, 49–55.
- Van den Broek, P., Barroso, M. & Lechner, M. (1996) *Experientia* **52**, 851–855.
- Omura, T. & Ito, A. (1991) *Methods Enzymol.* **206**, 75–81.
- Winter, J., Bilbe, G., Richener, H., Sehringer, B. & Kayser, H. (1999) *Biochem. Biophys. Res. Commun.* **259**, 305–310.
- Winter, J., Eckerskorn, C., Waditschats, R. & Kayser, H. (2001) *Biol. Chem.* **382**, 1541–1549.