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Woc (*without children*) gene control of ecdysone biosynthesis in *Drosophila melanogaster*

James T. Warren [a], Jasmine Wismar [b], Bhattiprolu Subrahmanyam [a], Lawrence I. Gilbert [a,*]

[a] *Department of Biology, Campus Box # 3280, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280, USA*
[b] *Institut für Genetik, Johannes Gutenberg Universität, Saarstraße 21a, 55099 Mainz, Germany*

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Abstract

The first step in ecdysteroidogenesis, i.e. the 7,8-dehydrogenation of dietary cholesterol (C) to 7-dehydrocholesterol (7dC), is blocked in *Drosophila melanogaster* homozygous *woc* (without children) third instar larval ring glands (source of ecdysone). Unlike ring glands from wild-type *D. melanogaster* larvae, glands from *woc* mutants cannot convert radiolabelled C or 25-hydroxycholesterol (25C) to 7dC or 7-dehydro-25-hydroxycholesterol (7d25C) in vitro, nor to ecdysone (E). Yet, when these same glands are incubated with synthetic tracer 7d25C, the rate of metabolism of this polar $\Delta^{5,7}$-sterol into E is identical to that observed with glands from comparably staged wild-type larvae. The absence of this enzymatic activity in vivo is probably the direct cause of the observed low whole-body ecdysteroid titers in late third instar homozygous mutant larvae, the low ecdysteroid secretory activity in vitro of brain–ring gland complexes from these animals, and the failure of the larvae to pupariate (undergo metamorphosis). Oral administration of 7dC, but not C, results in a dramatic increase in ecdysteroid production both in vivo and in vitro by the *woc* mutant brain–ring gland complexes and affects a partial rescue to the beginning of pupal-adult development, but no further, despite elevated whole-body ecdysteroid titers. Data previously reported (Wismar et al., 2000) indicate that the *woc* gene encodes a zinc-finger protein that apparently modulates the activity of the 7,8-dehydrogenase.

Keywords: Cholesterol; 7-Dehydrocholesterol; 25-Hydroxycholesterol; Ecdysone biosynthesis; Cholesterol-7,8-dehydrogenase; Cytochrome P450; Zinc-finger transcription factor

1. Introduction

Unlike vertebrates, insects and other arthropods cannot synthesize cholesterol (C) from acetate (see reviews by Rees, 1985, 1995; Warren and Hetru, 1990; Grieneisen, 1994; Lafont, 1997, 2000), and with few exceptions must obtain C or similar sterols from their diet. During ecdysteroidogenesis by *Manduca sexta* larval prothoracic glands or *Drosophila melanogaster* larval ring glands and adult ovaries, dietary C must first be dehydrogenated at carbons 7 and 8 to 7-dehydrocholesterol (7dC) prior to the downstream formation of an ecdysteroid that is the principal molting hormone of insects, 20-hydroxyecdysone (20E) (Warren and Gilbert, 1996; Warren et al., 1988a, 1995, 1996; Grieneisen et al., 1991, 1993; see Fig. 1).

As is the case with the temperature-sensitive mutant *ecdysoneless*, *ecd*^{1ts}, (Garen et al., 1977) the late third larval instar *woc* (without children) homozygous *D. melanogaster* mutant is ecdysteroid deficient (Wismar et al., 2000). The whole body ecdysteroid titer and the ecdysteroid secretory activity of its brain–ring gland complexes in vitro are less than 37 and 16%, respectively, that of comparable wild-type larvae. The result

Abbreviations: C, cholesterol; 7dC, 7-dehydrocholesterol; 25C, 25-hydroxycholesterol; 7d25C,7-dehydro-25-hydroxycholesterol; Diketol, 5βH-cholesta-7-ene-3,6-dione-14α-ol; E, ecdysone; 20E, 20-hydroxyecdysone; Ketodiol, 5βH-cholesta-7-ene-6-one-3β,14α-diol; Ketotriol, 5βH-cholesta-7-ene-6-one-3β,14α,25-triol; NP-HPTLC, normal phase, high-performance thin layer chromatography; RP-HPLC, reversed phase, high-performance liquid chromatography; RIA, radioimmunoassay.

* Corresponding author. Tel.: +1-919-9662055; fax: +1-919-9621344.

E-mail address: lgilbert@unc.edu (L.I. Gilbert).

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of this lesion is an inability to pupariate, i.e. dauer larvae, although these larvae can be rescued partially to a stage at the beginning of pupal-adult development by the inclusion of 20E in the diet. The *woc* gene encodes a protein of 187 kDa containing eight zinc fingers of the Cys2-Cys2 type (Wismar et al., 2000). As such, it may function as a transcription factor. Portions of the woc protein show considerable homology (up to 65%) to human zinc-finger proteins that have been implicated in both mental retardation and a leukemia/lymphoma syndrome (van der Maarel et al., 1996; Xiao et al., 1998). In a manner akin to a previous study involving the *ecd*^{1ts} mutant (Warren et al., 1996), we herein report the use of radiotracer precursor sterols in experiments in vitro to identify the specific biochemical step in the ecdysteroidogenic pathway that is defective in the *woc* mutant.

2. Materials and methods

2.1. Chemicals

Chemicals, radiochemicals and tissue culture medium are identical to those described previously (Grieneisen et al., 1991, 1993; Warren et al., 1995, 1996, 1999; Warren and Gilbert, 1996).

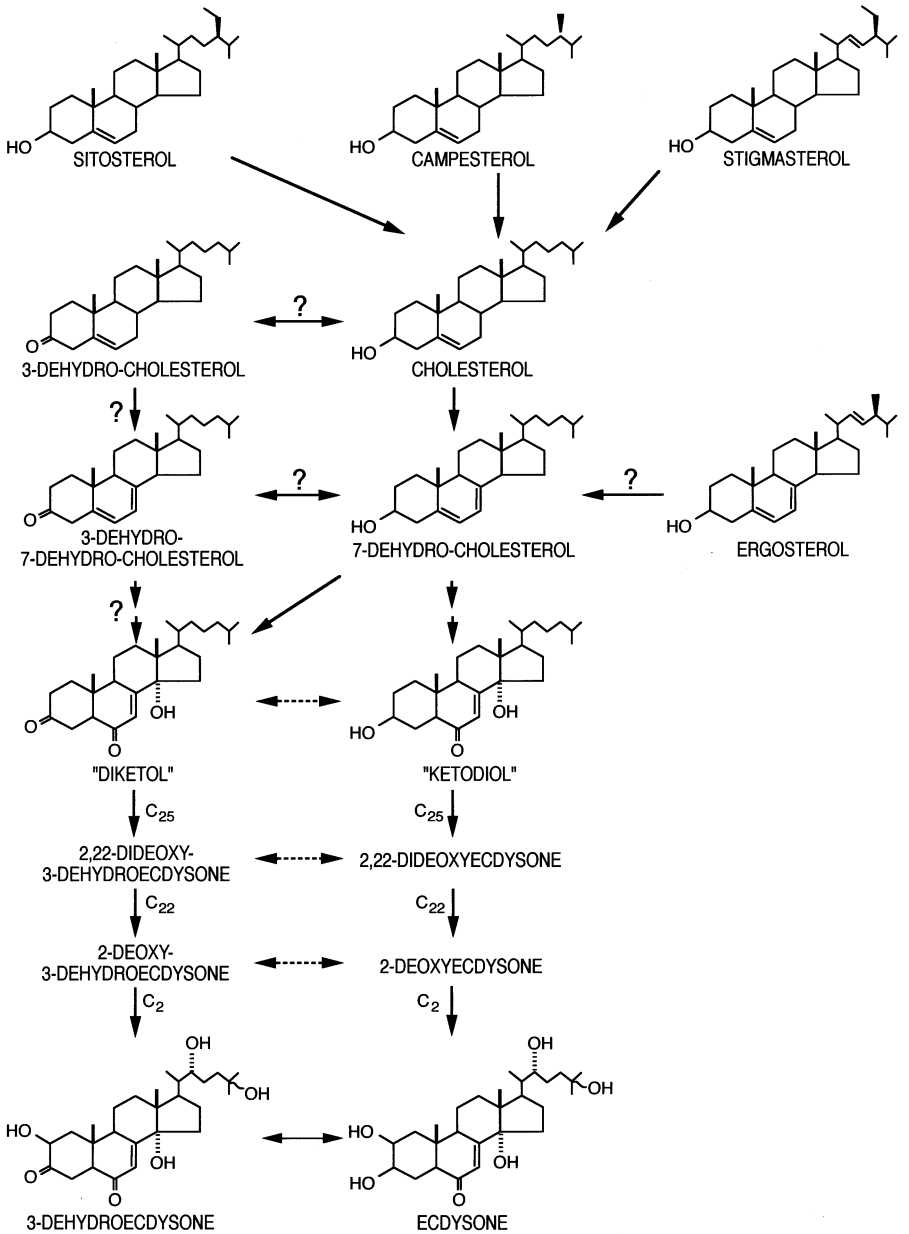

Fig. 1. Scheme of ecdysteroid biosynthesis from dietary sterols. Dotted lines denote reactions that are known to occur but have not been fully characterized. Question marks refer to hypothetical reactions thought to occur, but that have not been demonstrated directly. (Revised from Grieneisen et al., 1993).

2.2. Insect propagation and dissection

Wild-type stock (Oregon R), heterozygous and homozygous *woc* mutant *D. melanogaster* and *Drosophila pachea* were all maintained at 22°C. The result of EMS-mutagenesis, homozygous *woc* animals were the selected progeny of crosses between heterozygous *woc* adults containing the third chromosome balancer *TM6b* and exhibiting the *ss*-, bx^{340}, *e* (ebony), *Hu* (humoral), *ca* (claret), *Tb* (tubby) genotype (Wismar et al., 2000). The unusual *Senita* cactus-specific and dependent species *D. pachea* (Heed and Kircher, 1965; Goodnight and Kircher, 1971) (a kind gift from Dr Heed) was raised on *Drosophila* food containing 7dC (0.5% wet weight), a $\Delta^{5,7}$-sterol that meets the absolute Δ^{7}-sterol requirement of this novel desert fruit fly species. In addition to the regular prepared *Drosophila* diet containing corn meal, yeast and sugar, cultures of wild-type and *woc D. melanogaster* flies, as with *D. pachea*, were also fed diets augmented with 7dC or, in some cases, with C (also at 0.5%). A sonicated suspension of 200 mg finely ground 7dC (Aldrich, 98%) or C (Sigma, 96%) in water (2 ml) containing Tween-80 (0.01%) was mixed thoroughly into 40 g re-heated *Drosophila* food.

Adult flies (40–60) were transferred daily onto new food to prevent overcrowding and to help synchronize the various cultures. Newly eclosed flies were added weekly. Third instar larvae were harvested as soon as they left the food and began to wander. Wild-type and *woc* mutant *D. melanogaster* or wild-type *D. pachea* larval ring glands (15 each), brain–ring gland complexes (Sliter and Gilbert, 1992) with imaginal disks intact (30–100 each), or brains (15 each) alone, were dissected under ice-cold *Drosophila* saline solution. They, and the remaining carcasses, were either frozen immediately on dry ice for later sterol analysis or pooled in ice-cold Grace's medium prior to incubation with radiolabelled substrates. Alternatively, in order to measure ecdysteroid secretory activity in vitro, similar individual wild-type, heterozygous or homozygous mutant *D. melanogaster* brain–ring gland complexes were incubated in drops of Grace's medium (see later).

2.3. Incubations and sample preparation

For radiolabel incorporation studies, freshly dissected intact third-instar larval tissues (brains, ring glands or brain–ring gland complexes) were incubated at 26°C for 4–6 h under constant agitation with 1.0×10^6 DPM ^{14}C-C (60 mCi/mmol), or $(1.0–4.0) \times 10^7$ DPM ^{3}H-C, ^{3}H-25-hydroxycholesterol (25C), ^{3}H-7-dehydro-25-hydroxycholesterol (7d25C) (made from ^{3}H-25C by *M. sexta* prothoracic glands in vitro), or ^{3}H-5βH-cholesta-7-ene-3,6-dione-14α-ol (diketol) (a kind gift from Dr C. Hetru, Strasbourg, France), all at 60 Ci/mmol, or with a mixture of ^{14}C-C and ^{3}H-7d25C (77:1 molar ratio). Immediately prior to incubation with tissues, the evaporated radiolabelled compounds were emulsified in Grace's medium (0.5 ml) containing 0.001% Tween-80 and 1 mM NADPH. Following incubation, the reaction mixtures were first diluted with methanol to 80%, and standard unlabeled ecdysteroids and (in some cases) unlabeled sterols were added (1 μg each). The mixtures were centrifuged, the pellets were extracted repeatedly with ethanol, and the solvent extracts were pooled and evaporated under low pressure, low temperature and low light conditions. The residues were taken up immediately into 80% methanol and stored at −70°C prior to reversed phase, high-performance liquid chromatography (RP-HPLC).

The endogenous tissue $\Delta^{5,7}$-sterol contents (i.e. ergosterol and 7dC) were measured by RP-HPLC (UV detection) in similarly extracted wild-type, heterozygous and homozygous *woc* mutant third instar *D. melanogaster* and *D. pachea* brain–ring gland complexes, and in the remaining carcasses lacking these brain–ring gland complexes (100 each), and also in whole wild-type and heterozygous mutant *woc D. melanogaster* and *D. pachea* adult flies, all from animals raised on food containing 7dC (Warren et al., 1999).

Whole body ecdysteroid titers of wild-type and mutant *D. melanogaster* larvae and pupae, either raised on regular, C-augmented or 7dC-augmented food, were determined by radioimmunoassay (RIA) (Warren et al., 1984; Warren and Gilbert, 1986, 1988) employing the SHO-3 antibody (a kind gift from Dr Sho Sakurai, Kanazawa, Japan) (Kiriishi et al., 1990). Wild-type and heterozygous or homozygous *woc* mutant animals (10–100 each) were homogenized thoroughly into methanol (0.5–2 ml) and the homogenates then centrifuged. The precipitated material was re-extracted one or more times with ethanol as necessary for the complete (> 95%) extraction of free ecdysteroids. Solvent extracts were then pooled and suitable aliquots, in triplicate, were evaporated prior to assay.

The in vitro ecdysteroid synthetic and secretory activity of wild-type, heterozygous or homozygous *woc* mutant *D. melanogaster* third instar larval brain–ring gland complexes from animals raised on regular, C- or 7dC-augmented food were similarly determined by RIA. Individual complexes were incubated for 2 h with gentle agitation at 26°C in 70 μl Grace's medium in a 96-well plate. The medium minus the tissue was assayed.

2.4. Chromatography

Conditions for RP-HPLC and normal phase, high-performance thin layer chromatography (NP-HPTLC) have been detailed previously (Warren et al., 1986, 1995, 1999; Grieneisen et al., 1991, 1993; Warren and

Gilbert, 1996). Either the radiolabelled or unlabeled tissue extracts in 80% methanol were eluted initially at 1 ml/min from a C18-silica based column (Nova-Pak, 3.9 × 150 mm; Waters) with 80% isocratic methanol for 60 min in order to separate ecdysteroids from the relatively non-polar sterols ^{3}H-7d25C and ^{3}H-25C. This initial isocratic elution was then followed by a slow gradient to 95% methanol over 60 min in order to separate the very non-polar endogenous $\Delta^{5,7}$-sterols ergosterol and 7dC from C. The residue from the original sample that was not soluble in 80% methanol following the first injection was subsequently taken up in 95% methanol, re-injected and eluted with 95% methanol in order to quantify completely all endogenous ergosterol and/or radiotracer 7dC and C.

For the analysis of radiolabelled sterol incorporation into ecdysteroids, the polar material eluting prior to the diketol, 7d25C, 7dC, or C in the initial 80% methanol RP-HPLC of the injected sample was pooled and evaporated as already described. The residues were then dissolved in 30% methanol, re-injected onto the RP-HPLC column and eluted isocratically with 30% methanol (20 min), followed by a slow gradient elution to 100% methanol over 120 min. Ecdysteroid standards were detected at 248 nm, while standard or endogenous $\Delta^{5,7}$-sterols were quantified at 282 nm. Identified radiolabelled products were further characterized by NP-HPTLC (Warren et al., 1999).

3. Results

3.1. Radiolabel incorporation studies in vitro

3.1.1. Heterozygous or homozygous woc mutant or wild-type D. melanogaster or D. pachea brain–ring gland complexes incubated with ^{3}H-25C

Intact brain–ring gland complexes (30 each) from heterozygous *woc* larvae were incubated for 4 h with the polar sterol precursor ^{3}H-25C. The products extracted from the incubation medium were subjected to RP-HPLC, which revealed significant metabolism of this radiolabelled polar C analog to ^{3}H-7d25C (0.36% of total radiolabel) (Table 1). This was presumably the result of cholesterol 7,8-dehydrogenase activity. When the polar material eluting prior to ^{3}H-7d25C was rechromatographed using 30–100% methanol, a significant overall conversion of ^{3}H-25C to ^{3}H-ecdysone (E) (0.16%), presumably via ^{3}H-7d25C, was also observed. In stark contrast, no conversion of ^{3}H-25C to ^{3}H-7d25C ($< 0.001\%$) was detected after an otherwise identical incubation and analysis of brain–ring gland complex preparations from comparably staged intact homozygous *woc* mutants. Further, RP-HPLC analysis of the more polar eluting material revealed no ecdysteroid formation (^{3}H-E $< 0.001\%$) in this case. This analysis was repeated twice, each time by incubating 100 brain–ring gland complexes from either wild-type or homozygous *woc D. melanogaster* larvae and the results were essentially the same as already noted, i.e. wild-type and heterozygous *woc* were identical (data not shown).

In an otherwise absolutely identical manner, the ecdysteroidogenic organ from a desert fruit fly that requires a Δ^{7}-sterol from an exogenous source in order to survive (Heed and Kircher, 1965), i.e. brain–ring gland complexes from third instar *D. pachea* larvae that were propagated on food containing 7dC, were also unable to convert ^{3}H-25C to ^{3}H-7d25C ($< 0.001\%$) in vitro (Table 1), nor to ^{3}H-E ($< 0.001\%$). (*D. pachea* were studied since their biochemical phenotype was similar to that of the *woc* mutant and we believed that such analyses would provide insights to the biochemical basis of the *woc* mutation.) In contrast to the earlier *D. pachea* organs, complexes from identically staged wild-type *D. melanogaster* larvae raised on identical food containing 7dC were able to accomplish these reactions (Table 1), just as brain–ring gland complexes from wild-type animals raised on regular food without supplemental 7dC did (data not shown).

3.1.2. Heterozygous or homozygous woc mutant or wild-type D. melanogaster brain–ring gland complexes incubated with ^{3}H-C

Similar incubations of intact brain–ring gland complexes (30) from *woc* heterozygotes were conducted with ^{3}H-C, the basic endogenous precursor sterol of insect ecdysteroidogenesis. Significant conversion of ^{3}H-C to ^{3}H-7dC (0.051%) was observed (Table 1). Further analysis of the polar material revealed a small but significant (0.005%) conversion to ^{3}H-E. Similar results were obtained using complexes from wild-type *D. melanogaster* larvae. However, the metabolism of ^{3}H-C to ^{3}H-7dC was not detected in the incubation medium ($< 0.001\%$) when homozygous mutant brain–ring gland complexes were incubated with ^{3}H-C, nor was there significant conversion to ^{3}H-E ($< 0.0005\%$). This analysis was repeated with 100 wild-type complexes with results similar to those with heterozygous *woc* glands or with 100 homozygous *woc* mutant brain–ring gland complexes (data not shown). In order to insure that it was the ring gland that was responsible for these results, isolated ring glands were also analyzed for their capacity to synthesize E, i.e. in the absence of the brain, ventral ganglia and imaginal disks.

3.1.3. Homozygous woc mutant or wild-type D. melanogaster ring glands (without brains) incubated with ^{3}H-25C

As was observed with the much larger brain–ring gland complexes, the incubation of 15 wild-type ring glands alone with ^{3}H-25C also resulted in significant

Table 1
In Vitro Metabolism of Radiolabelled Sterols by Intact Tissues[a]

Tissue	Source	Reactions[b]					
		C → 7dC → → → E		25C → 7d25C → → → E		7d25C → → → E	Diketol[g] → 3-Oxo-ketotriol[h] → → → E	
Third instar larval brain–ring gland complexes	Het[c] *woc*	0.051	0.005	0.36	0.16	0.27	30	0.9
	Hom[d] *woc*	<0.001	<0.0005	<0.001	<0.001	0.31	46	1.5
	Wild type[e]	NA		0.25	0.13	0.25	32	0.8
	D. pachea[e]	NA		<0.001	<0.001	0.20	35	1.1
Third instar larval ring glands	Wild type[f]	0.11 (^{14}C)	0.021 (^{14}C)	-		0.016 (^{3}H)	NA	
	Wild type	NA		0.38	0.15	NA	NA	
	Hom[d] *woc*[f]	<0.001 (^{14}C)	<0.001 (^{14}C)	-		0.037 (^{3}H)	NA	
	Hom[d] *woc*	NA		<0.001	<0.0005	NA	NA	

[a] Tissues were incubated in Grace's medium for 4–6 h at 26° C and then analyzed for metabolites of the radiolabelled sterols by RP-HPLC.
[b] Percenatge of total administered radiolabelled sterol subsequently recovered and identified.
[c] Heterozygous
[d] Homozygous
[e] Brain-ring gland complexes are from wild type *D. melanogaster* or *D. pachea* raised on food containing 7dC.
All other ring glands and complexes are from wild type and *woc* mutant *D. melanogaster* larvae raised on regular food.
[f] Dual label experiment (^{14}C-C and ^{3}H-7d25C).
[g] 5βH-Cholesta-7ene-3,6-dione-14α-ol
[h] 5βH-Cholesta-7ene-3,6-dione-14α, 25-diol
NA: not analyzed.

conversion to ^{3}H-7d25C (0.38%) (Table 1). Analysis of the polar material from this initial RP-HPLC run further revealed significant (0.15%) conversion to ^{3}H-E. Neither initial conversion to ^{3}H-7d25C ($<0.001\%$) nor subsequent conversion to ^{3}H-E ($<0.0005\%$) was detected following the incubation of the larger ring glands from homozygous *woc* mutants (Wismar et al., 2000) with ^{3}H-25C. This latter experiment was repeated with another 15 ring glands from homozygous mutant larvae with an identical negative result (data not shown).

3.1.4. Heterozygous or homozygous woc mutant or wild-type D. melanogaster or D. pachea brain–ring gland complexes incubated with ^{3}H-7d25C

In contrast to the already presented results employing the Δ^{5}-sterol precursors C and 25C, were the data obtained when *woc* brain–ring gland complexes from either heterozygous or homozygous mutants (30 each) were incubated with the biosynthetic metabolite ^{3}H-7d25C, the sterol that the homozygous *woc* mutants are unable to synthesize, and the medium then analyzed as already described. The data revealed a significant conversion of ^{3}H-7d25C to ^{3}H-E in both gland incubations, i.e. 0.27% for heterozygous *woc* and slightly more (0.31%) for the homozygous mutant (Table 1). This analysis was repeated twice with 100 brain–ring gland complexes from wild-type or homozygous *woc D. melanogaster* animals with similar results (data not shown). In addition, essentially the same results were obtained with both wild-type *D. melanogaster* and *D. pachea* brain–ring gland complexes from animals raised on food containing 7dC, i.e. ^{3}H-7d25C was clearly metabolized in vitro to ^{3}H-E, 0.25% by wild-type *D. melanogaster* complexes and 0.20% by *D. pachea* complexes (Table 1).

3.1.5. Homozygous woc mutant or wild-type D. melanogaster ring glands (without brains) incubated with both ^{3}H-7d25C and ^{14}C-C

With respect to ring gland-mediated ecdysteroidogenesis, further confirmation that only cholesterol 7,8-dehydrogenase activity is lacking in the homozygous *woc* mutant was obtained when ring glands (15 each) from wild-type or homozygous *woc* mutants were incubated with both ^{3}H-7d25C and ^{14}C-C (1:77 molar ratio), and analyzed as already described. Significant conversion of ^{14}C-C to ^{14}C-7dC (0.11%) was observed with wild-type ring glands (Table 1). In addition, when the initial eluting polar material from this RP-HPLC analysis was re-chromatographed in 30–100% methanol, it was determined that both ^{3}H-E (0.016%) and ^{14}C-E (0.021%) were produced, presumably derived from the exogenous ^{3}H-7d25C and the ^{14}C-7dC formed in situ, respectively.

Unlike the situation for ring glands from wild-type larvae, following the identical dual-label incubation of homozygous *woc* ring glands with ^{3}H-7d25C and ^{14}C-C, no initial conversion of ^{14}C-C to ^{14}C-7dC ($<0.001\%$) was detected. However, when the polar material from this RP-HPLC elution was re-chromatographed, significant production of ^{3}H-E (0.037%) from ^{3}H-7d25C was observed, i.e. more than twice that of wild-type glands. However, in this case, no ^{14}C-E ($<0.001\%$) was detected. That is, while the homozygous *woc* ring glands were able to convert ^{3}H-7d25C to ^{3}H-E as in previous experiments, they were, at the same time, unable to convert ^{14}C-C to ^{14}C-7dC, and so could not produce ^{14}C-E.

3.1.6. Homozygous woc mutant or wild-type D. melanogaster larval brains incubated with ^{3}H-25C or a mixture of ^{3}H-7d25C and ^{14}C-C

As tissue controls, either wild-type or homozygous *woc* mutant brains (15 each), dissected free of the ring glands used in the earlier experiments, were incubated with either ^{3}H-25C or a mixture of ^{3}H-7d25C and ^{14}C-C (as earlier). Analysis of the extracts by RP-HPLC revealed neither initial conversion of the radiotracers to ^{3}H-7d25C or ^{14}C-7dC nor subsequent production of ^{14}C/^{3}H-E by either brain preparation (data not shown; see Warren et al., 1999).

3.1.7. Heterozygous or homozygous woc mutant or wild-type D. melanogaster or D. pachea brain–ring gland complexes incubated with the ^{3}H-diketol

To obtain information directly about the activity of some of the other better-characterized enzymes in the ecdysteroid biosynthetic pathway downstream from the first enzyme, i.e. downstream from cholesterol 7,8-dehydrogenase, both heterozygous and homozygous *woc* brain–ring gland complexes (30 each) from animals raised on regular food were incubated with the ^{3}H-diketol. Similarly, gland complexes from wild-type *D. melanogaster* and *D. pachea* animals raised on food containing 7dC were incubated with the ^{3}H-diketol. Complexes from all animals were capable of significant metabolism of this radiolabelled precursor (Table 1). Initially, the ^{3}H-diketol was converted extensively to ^{3}H-5βH-cholesta-7-ene-3,6-dione-14α, 25-diol (3-oxo-ketotriol), i.e. heterozygous *woc* (30% conversion), homozygous *woc* (46%), wild-type *D. melanogaster* (32%) and *D. pachea* (35%), apparently via facile terminal ecdysteroid 25-hydroxylation (Fig. 1). To a lesser degree, this intermediate then became a substrate for subsequent terminal ecdysteroid 2- and 22-hydroxylation reactions resulting in the observed production of ^{3}H-E, i.e. heterozygous mutant (0.9%), homozygous mutant (1.5%), wild-type *D. melanogaster* (0.8%) and *D. pachea* (1.1%).

3.2. 7dC Feeding (rescue) studies

To test our hypothesis that ring glands from homozygous *woc* larvae do not synthesize normal amounts of ecdysone only because they lack the ability to synthesize 7dC from endogenous C, this critical metabolic intermediate (7dC) was supplied in their diet, using culture conditions identical to those required for the successful propagation of *D. pachea* (Heed and Kircher, 1965), which presumably has the same biochemical defect. When raised on regular *Drosophila* food, less than 5% of homozygous, wandering *woc D. melanogaster* third instar larvae ever develop to the white puparium stage or beyond, similar to *D. pachea* (Heed and Kircher, 1965). When 7dC was added to the food (0.5%), at least 90% of the resulting homozygous (F1) *woc* mutant third instar larvae were partially rescued, i.e. they pupariated and initiated pupal-adult development about 12 h later. That is, newly wandering homozygous larvae became immobile after 6–8 h, shortened and formed a white puparium (stage P1; Bownes, 1990) that subsequently darkened to the brown puparium (stage P2). Development of this prepupa continued with apolysis and the formation of an abdominal bubble, i.e. the bubble puparium (stage P3), which subsequently could be conveniently staged by its ability to float as posterior bubble formation displaced the pupa forward and the abdominal bubble disappeared, i.e. the cryptocephalic pupa or buoyant pupa (stage P4*i*). Further development was indicated by the subsequent anterior movement of this bubble as the pupa withdrew to the posterior end of the pupal case (moving bubble stage P4*ii*). Gently pulling the pupa out of the old larval cuticle and demonstrating its ability to exclude trypan blue confirmed the formation of a truly intact pupal cuticle. However, no further development occurred in these animals and they died of dessication after 1–2 weeks, although partial head eversion was occasionally observed. Even with the inclusion of 7dC in the diet, some third instar homozygous *woc* larvae (5–10%) showed no signs of pupariation. Unlike the effect of feeding 7dC, raising *woc* animals on an otherwise normal diet, but containing large amounts of C (0.5%), had no observable effect on homozygous *woc* larval development, i.e. it was identical to raising the larvae on regular diet.

In contrast to the homozygous *woc D. melanogaster* mutants receiving 7dC that were unable to complete normal pupal-adult development, *D. pachea* raised on the 7dC-fortified diet do complete their development to the eclosion of fertile adults. This desert species is otherwise totally dependent on a Δ^7-sterol precursor (Δ^7-campesterol) found only in the *Senita* cactus. Without access to this cactus with its unusual sterol component, or the inclusion of 7dC or similar sterols in the diet, *D. pachea* larvae fail to develop normally and the adults become sterile (Heed and Kircher, 1965; Goodnight and Kircher, 1971; Fogleman et al., 1986).

This regimen of feeding 7dC did not elicit any observable toxicity in *D. pachea* or wild-type and heterozygous *woc D. melanogaster* animals. Their survival and overall rate of development were indistinguishable from animals raised on regular food, even after many generations. The same results were obtained with food containing lesser concentrations of 7dC, as low as 0.025% by weight, but still well above the aqueous solubility of 7dC. Similarly, no apparent toxicity was observed with the C-feeding protocol.

3.3. Endogenous sterols in wild-type and woc mutant *D. melanogaster* and *D. pachea* larval tissues and adult flies raised on a diet containing 7dC.

In adult flies and in late third instar brain–ring gland complexes from wild-type and *woc* mutant *D. melanogaster* and *D. pachea* animals raised on food containing 7dC, and likewise in their remaining carcasses, the content of 7dC in these compartments was consistently twice that of dietary-derived ergosterol. Thus, the average concentration of ergosterol and 7dC in these brain–ring gland complexes was 33 and 66 ng/complex, respectively. Both concentrations were about tenfold higher in the remaining carcasses and adult flies.

3.4. Whole body ecdysteroid titers

To better characterize the hormonal deficiency of the *woc* larvae and to further test our hypothesis concerning the nature of its rescue by the oral administration of 7dC, the whole body ecdysteroid titer of larvae and pupae raised on either regular food or food containing C or 7dC was determined by RIA (Fig. 2; see also Wismar et al., 2000). Wild-type *D. melanogaster* third instar larvae, harvested as soon as they leave the (regular) food and begin wandering, contained about 27 pg ecdysteroid/animal, a value similar to published data (Garen et al., 1977; Hodgetts et al., 1977; Richards, 1981; Berreur et al., 1984). Heterozygous *woc* mutant early wandering larvae contained about 66% as much ecdysteroid (18 pg/animal), while early wandering homozygous *woc* larvae yielded only about 37% of the amount of ecdysteroid in wild-type animals (10 pg/animal). Two to 3 days later, the wild-type and heterozygous *woc* larvae had all undergone pupariation and subsequent pupation. The still wandering homozygous *woc* dauer larvae continued feeding and by then contained one or more small black inclusion bodies (Wismar et al., 2000), but these 'late' larvae still contained 10 pg ecdysteroid/animal. However, after 1–2 weeks, i.e. shortly before death, these old large yellowing larvae were full of masses of dark inclusion

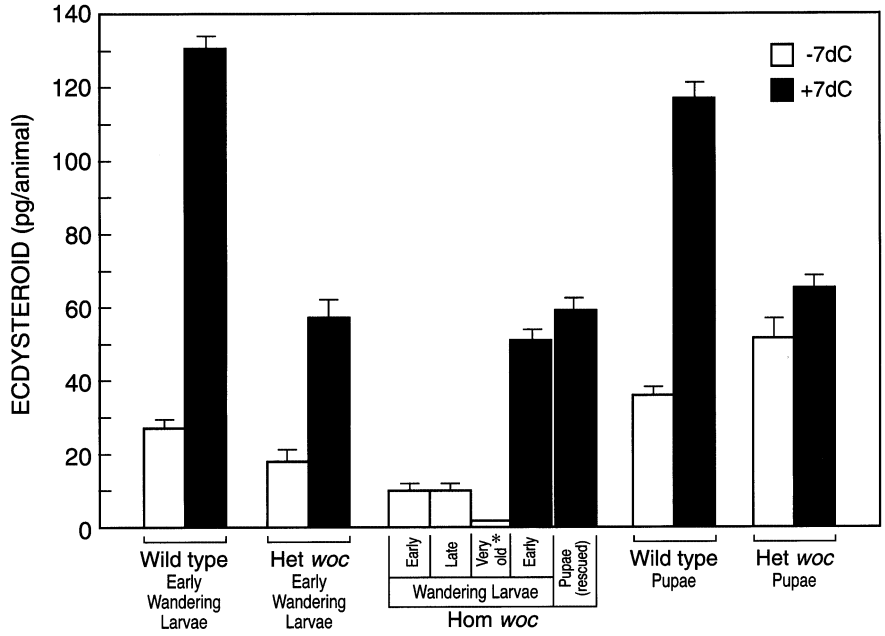

Fig. 2. Effect of 7dC in the food on the whole-body ecdysteroid titers of wild-type and *woc* mutant *D. melanogaster*. Het and Hom, heterozygous and homozygous *woc*, respectively. * Data represent the lower limit of assay sensitivity.

crystals and had a whole body titer below 1.6 pg/animal (Fig. 2), the lower limit of the resolving power of the RIA (16 pg/assay tube).

Consistent both with our hypothesis that the phenotype of the *woc* mutation (i.e. low ecdysteroid titer) results from the inability of the ring glands to synthesize 7dC from dietary C and with the observed partial rescue of *woc* homozygotes by oral administration of 7dC, the addition of 7dC to the food dramatically affected the whole body titers of both mutant larvae and pupae (Fig. 2). Early wandering, apparently rescued, homozygous *woc* third instar larval ecdysteroid titers were increased fivefold over those of comparable homozygous *woc* animals raised on the regular diet. In addition, titers of wild-type larvae were increased fivefold, and heterozygous *woc* larvae threefold, over those of comparable animals raised on regular food. The ecdysteroid titers of newly pupated, i.e. clearly rescued, homozygous *woc* animals were increased sixfold over the titers of homozygous *woc* larvae that were not rescued. That is, both those larvae raised on regular food and 5–10% of the larvae that did not respond to the 7dC-feeding regimen behaved as homozygous mutants, i.e. did not pupariate nor show increased titers. Analysis of wild-type pupae that had been raised on the 7dC diet revealed a threefold increase in ecdysteroid titer over those raised on regular food, but a similar increase was not observed in the heterozygous *woc* pupae fed 7dC. Unlike the case of homozygous *woc* rescue by including 7dC in the food, the addition of C to the diet did not rescue, nor did it increase the whole body ecdysteroid titers of homozygous *woc* larvae (data not shown).

3.5. Ecdysteroid production in vitro by wild-type and woc mutant D. melanogaster brain–ring gland complexes

The ability of brain–ring gland complexes from early wandering third instar larvae to synthesize and secrete ecdysteroids into the medium was measured by RIA (Fig. 3; see also Wismar et al., 2000). When wild-type larvae are raised on regular food, their brain–ring gland complexes synthesize 60 pg/complex/h in vitro, similar to published values (Redfern, 1983; Henrich et al., 1987a,b). Complexes from heterozygous *woc* larvae were about 50% as active as complexes from wild-type larvae, but early wandering larval homozygous *woc* brain–ring gland complexes secreted only 16% of the ecdysteroid secreted by wild-type complexes. After wandering and feeding for 2–3 days, the in vitro activity of complexes from these late dauer mutant larvae remained constant, but fell below the level of detectability (< 5 pg/complex/h) in very old dauer larvae 1–2 weeks after the initiation of wandering.

Consistent with its ability to rescue and increase whole body ecdysteroid titers, the inclusion of 7dC in the food also resulted in a dramatic increase in the rate of ecdysteroid production by brain–ring gland complexes in vitro when compared with complexes from animals raised on regular food (Fig. 3). The rate of ecdysteroid synthesis by complexes from homozygous *woc* third instar larvae grown on 7dC-supplemented food showed fivefold the synthetic activity of complexes from non-rescued larvae. Wild-type and heterozygous *woc* mutant *D. melanogaster* complexes from larvae

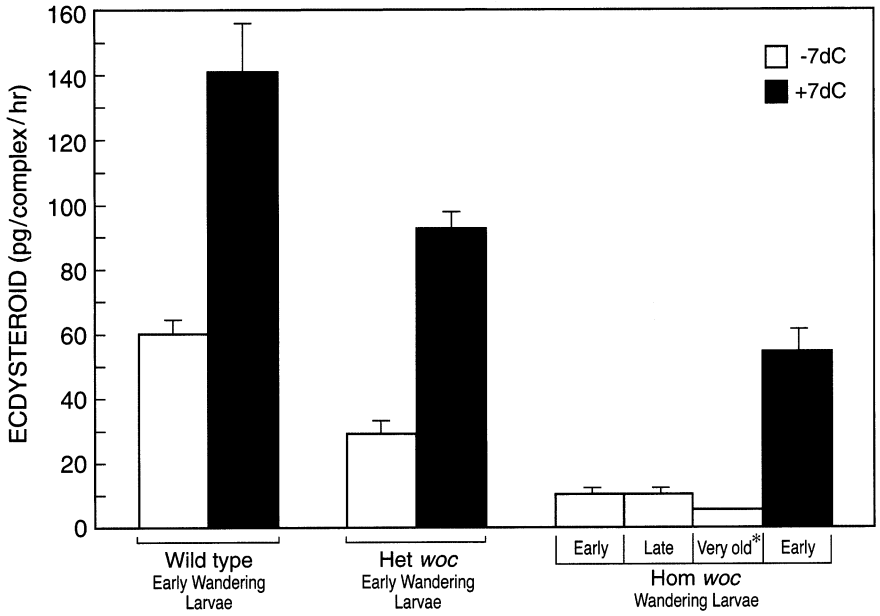

Fig. 3. Effect of 7dC in the food on the ability of wild-type and *woc* mutant *D. melanogaster* brain–ring gland complexes to synthesize ecdysteroids in vitro. Het and Hom, heterozygous and homozygous *woc*, respectively. * Data represent the lower limit of assay sensitivity.

grown on the 7dC diet showed similar two- and three-fold increases in the synthesis of ecdysteroid, respectively, over their siblings raised on normal diet. In the 5–10% homozygous *woc* larvae that were not rescued by 7dC feeding and that remain dauer larvae for 1–2 weeks, brain–ring gland ecdysteroid production was below the level of reliable detection, i.e. similar to that of the non-rescued very old mutant larvae. As was observed with the whole body titer, homozygous *woc* brain–ring gland complex activity was not increased when the diet was supplemented with C (data not shown).

4. Discussion

The conversion of a Δ^5-sterol to a $\Delta^{5,7}$-conjugated sterol mediated by a microsomal P450 monooxygenase in *M. sexta* (Grieneisen et al., 1993; Warren et al., 1995; Warren and Gilbert, 1996) is probably arthropod specific (Fig. 1). That is, during the biosynthesis of C by mammals, only the reverse reaction is observed, namely the 5,6-dehydrogenation of a Δ^7-sterol (lathosterol) to this same $\Delta^{5,7}$-sterol (7dC). Apparently, this ubiquitous vertebrate sterol 5,6-dehydrogenase enzyme is not a typical P450 monooxygenase, as it requires the ferric ion and is inhibited by cyanide but not by carbon monoxide (Kawata et al., 1985). Surprisingly, however, this same activity has been detected in at least one insect species. *D. pachea* can also convert lathosterol to 7dC (Goodnight and Kircher, 1971). Whether this activity occurs in other insects is not known.

In insects, except for the hypothesized, but as yet unidentified sterol intermediate 3-dehydro-7dC, or its facile rearrangement product, the 3-dehydro-$\Delta^{4,7}$-sterol diene (Lakeman et al., 1967; Grieneisen et al., 1991, 1993; Blais et al., 1996; Dauphin-Villemant et al., 1997; Lafont, 1997), the immediately subsequent oxidations of this endogenous endoplasmic reticulum-localized 7dC are believed to occur within the mitochondria of the prothoracic gland cells (Warren and Gilbert, 1996). This postulated process of 7dC translocation from the endoplasmic reticulum, first to the outer membrane of the mitochondria followed by movement to, and subsequent oxidation of 7dC by, enzymes located in the inner membrane/matrix of the mitochondria, may be analogous to the rate-limiting delivery step of C in the cytochrome P450-mediated side chain cleavage of C within the inner mitochondrial matrix of the mammalian adrenal cortex cells (see Warren and Gilbert, 1996). However, for invertebrates, there is a dearth of information on the mechanism(s) of this proposed translocation of 7dC to, and within, the mitochondria, the intermediates resulting from its metabolism therein, and any of the enzymes involved in mediating the resulting very efficient conversion of 7dC to ecdysteroid. As a result, this series of reactions has been appropriately termed the 'black box' (Lafont, 1997, 2000).

The earliest identified ecdysteroid-like entities in the ecdysteroid biosynthetic pathway are the 'diketol' and 'ketodiol' (Dolle et al., 1990, 1991; Blais and Lafont, 1991). More is known about the subsequent (terminal) hydroxylations of these ecdysteroids to 3-dehydroecdysone (3dE) and E, i.e. first at C25 to the ke-

totriol (catalyzed by a microsomal P450), then at C22 (mitochondrial P450), and finally C2 (perhaps also a mitochondrial P450; Kappler et al., 1988). E and 3dE are the ultimate secretory products of the prothoracic glands of many insects (Warren et al., 1988b; Kiriishi et al., 1990; Böcking et al., 1994; Lafont, 1997; Dauphin-Villemant et al., 1998) and both are eventually converted to the molting hormone (20E) by ecdysone-20-hydroxylase, a P450 monooxygenase present in the mitochondria and microsomal fractions of various insect tissues, but especially fat body and mid gut (Bollenbacher et al., 1977; Feyereisen, 1977; Smith et al., 1979; Weirich, 1997; Mitchell et al., 1999).

Low ecdysteroid *Drosophila* mutants have been important for studying the effect of 20E on larval growth and development. In general, about a 66% reduction in the synthesis of E (and 20E) appears sufficient to prevent normal pupariation and pupation in these mutants (Garen et al., 1977; Klose et al., 1980; Audit-Lamour and Busson, 1981; Redfern and Bownes, 1983; Berreur et al., 1984; Holden et al., 1986; Walker et al., 1987; Henrich et al., 1987b; Sommé-Martin et al., 1988; Sliter and Gilbert, 1992; Warren et al., 1996). However, a clear understanding of the underlying biochemical defects in ecdysteroidogenesis occurring in these low ecdysteroid mutant dauer larvae has been conjectural until the present research.

Expanding on early studies employing radiolabelled C in other dipterans (Monroe et al., 1967; Willig et al., 1971), it was shown recently that the relatively water soluble, polar sterol precursor ^{3}H-25-hydroxycholesterol (25C) is metabolized efficiently by both insect and crustacean ecdysteroidogenic organs in vitro; first into ^{3}H-7d25C and subsequently into ^{3}H-ecdysteroid (Böcking et al., 1994). This result was later confirmed with the *Manduca* prothoracic glands (Warren and Gilbert, 1996), and the larval ring glands and adult ovaries of both wild-type and mutant l(3)*ecd*^{1ts} *D. melanogaster* (Warren et al., 1996). The only observable biochemical defect of the *woc* mutation in ring glands and brain–ring gland complexes from wandering homozygous third instar larvae, but not from wild-type or heterozygous woc larvae, was inhibition of the first committed step in ecdysteroidogenesis, namely the 7,8-dehydrogenation of C and 25C to their respective $\Delta^{5,7}$-sterols (Fig. 1). This result was based on radiolabelled tracer sterol (C or 25C) incorporation into distal biosynthetic intermediates (7dC or 7d25C) and, ultimately, into E by the ecdysteroidogenic tissues of wild-type and *woc* mutant *D. melanogaster* in vitro (Table 1). All other observable and potentially identifiable biochemical activities, such as the well-characterized sequential ecdysteroid terminal-hydroxylation reactions undergone by the ^{3}H-diketol (Kappler et al., 1988; Blais and Lafont, 1991; Dolle et al., 1991), exhibited little difference between the wild-type, heterozygous and homozygous *woc D. melanogaster* brain–ring gland complexes or those from *D. pachea*. Most importantly, however, was the observation that no inhibition occurred in any of the reactions comprising the mitochondrial 'black box' oxidation(s) of ^{3}H-7dC/7d25C leading to ^{3}H-ecdysone, i.e. via the ^{3}H-diketol or ^{3}H-ketotriol (Table 1).

The data suggest strongly that the *woc* mutation ultimately prevents the formation of 7dC from dietary C, and therefore inhibits the biosynthesis of E from C, in third instar homozygous *woc* larvae but not in heterozygous *woc* larvae. The effect of this lesion in homozygotes is low biosynthetic ecdysteroidogenic activity by their ring glands in vitro (Fig. 3) and in vivo, resulting in low, apparently sub-threshold, whole-body ecdysteroid titers in these animals (Fig. 2), and their inability to pupariate and molt to the pupa. However, the whole-body ecdysteroid titer and in vitro brain–ring gland complex secretory activity of heterozygous *woc* mutant third instar larvae, as measured by RIA but not radiotracer incorporation, was only 66 and 50%, respectively, that of wild-type animals (Figs. 2 and 3); (Wismar et al., 2000). While resulting in no observable developmental abnormalities, these reductions may be the result of pleiotropic effects of the *woc* gene, a putative transcription factor, separate from its hypothesized control of cholesterol 7,8-dehydrogenase expression, as suggested by R. Feyereisen (personal communication).

It has been shown previously that these wandering third instar dauer homozygous *woc* larvae could be rescued to the beginning of pupal-adult development by the inclusion of 20E in their food (Wismar et al., 2000), similar to the data on 20E administration to other low ecdysteroid mutant species (Rayle, 1967; Garen et al., 1977; Klose et al., 1980; Berreur et al., 1984; Holden et al., 1986; Sliter et al., 1989). However, in a more specific manner, we have demonstrated the identical rescue of third instar homozygous *woc* larvae, first to the white puparium stage, then to the pupa and further to a well-characterized stage precisely at the beginning of pupal-adult development, by including only 7dC in the larval diet. C was completely ineffective. 7dC is therefore established as the critical intermediate not synthesized by these mutant larvae, at least not from ^{3}H-C, within the prothoracic cells of the ring gland. When fed 7dC in this manner, at least 90% of the mutant larvae progressed to the beginning of pupal-adult development, but no further.

In animals raised on the normal *Drosophila* diet, the endogenous 7dC tissue content was too low to be detected by UV absorption, i.e. < 0.5 ng/complex, carcass or fly (< 50 ng in the injected sample). Greatly increased concentrations of 7dC (at least 100-fold) were noted in the larval brain–ring gland complexes, remaining carcasses and also adult tissues of both wild-type

and *woc* mutant *D. melanogaster* and *D. pachea* flies raised on the modified diet. These results indicate that the 7dC added to the regular diet is both absorbed efficiently and distributed extensively throughout the tissues of these animals at all stages of development. In the prothoracic gland cells of the ring gland, this elevated 7dC content apparently acts to stimulate ecdysteroidogenesis significantly, presumably by dramatically increasing 7dC substrate availability. As a result, the whole-body ecdysteroid titers of wandering third instar homozygous *woc* larvae and newly molted (rescued) pupae raised on food containing 7dC, but not C, were several fold higher than those of comparable mutant animals raised on regular food (Fig. 2). These levels were apparently adequate for pupariation and the normal initiation of pupation in the homozygous *woc* mutant. Similarly, feeding 7dC to developing wild-type and heterozygous *woc D. melanogaster* larvae also markedly increased their whole-body ecdysteroid titers relative to those of comparably staged individuals fed on normal diet (Fig. 2), but had no apparent effect on their development.

Consistent with the presented whole-body titer data, the in vitro ecdysteroid secretory rates of brain–ring gland complexes from early wandering wild-type and *woc* mutant larvae raised on a 7dC-containing diet were also increased several fold above those of animals raised on regular food (Fig. 3). In contrast, after C administration, homozygous *woc* gland activity did not increase, consistent with observed low whole-body titers and the failure of C to rescue the dauer mutant *woc* larvae.

That the brain–ring gland complex of late third instar *D. pachea* larvae appears to be lacking the same cholesterol 7,8-dehydrogenase activity as the homozygous *woc* mutant (Table 1) is interesting from an evolutionary standpoint, but completely consistent with the known nutritional requirement for a Δ^7-sterol (like 7dC) by this novel desert fly species (Heed and Kircher, 1965). In this case, however, oral administration of 7dC to *D. pachea* larvae affected a total 'rescue' to the reproductively competent adult stage. This is in contrast to the complete arrest observed at a precise stage at the beginning of the pupal-adult development of the 7dC-fed 'rescued' homozygous *woc* larvae, despite the markedly elevated whole-body ecdysteroid titers of these latter animals (Fig. 2, see below). Of perhaps greater importance to the overall understanding of the control of insect metamorphosis, this observation raises the possibility that a 'factor' (or 'factors'), the synthesis of which is upregulated by the *woc* gene product, may be required for normal pupal-adult development in *Drosophila*. This 'factor' would then act in consort with 20E to stimulate metamorphosis. Apparently, this unknown substance is present in *D. pachea* during pupal-adult development. Alternatively, the woc protein could downregulate the production of a 'factor' whose continued presence in the 7dC-rescued homozygous *woc* mutants acts to inhibit normal (i.e. 20E-stimulated) pupal-adult development. The identification of any such 'factors' is an important goal to pursue.

It is nevertheless curious that homozygous *woc* larvae raised on regular food are able to develop as far as they do, i.e. to the third larval instar. If the homozygous *woc* mutant cannot synthesize significant amounts of 7dC from C, yet has no other source of this critical intermediate, then it should not have the capacity to synthesize E at any stage. Just as at the end of the third larval instar, a surge in the 20E titer controls larval molting from the first to the second instar, from the second to the third and possibly during the development of the embryo as well (see Henrich et al., 1999). Thus, the question arose as to how sufficient molting hormone could be synthesized during embryogenesis and the first and second instars, since significant levels of either free or conjugated E or 20E are absent from newly laid *Drosophila* eggs (Maroy et al., 1988; Warren et al., unpublished information). It is possible that eggs contain maternal 7dC, sufficient to fulfill the sterol requirements for the ecdysteroidogenesis needed to coordinate embryonic development and the molting of the first and second instar larvae. Wild-type and *ecdysoneless D. melanogaster* ovaries, and so probably heterozygous *woc* adult female ovaries as well, can synthesize 7dC from C (Warren et al., 1996), and this 7dC may be transferred to the homozygous *woc* embryos and then to their larvae. Such embryonic 7dC could be available for E biosynthesis analogous to our finding that large amounts of 7dC in the diet stimulate ecdysteroid biosynthesis in third instar larvae. Further research is needed to answer this question unequivocally.

Alternatively, or in addition, *D. melanogaster* may have the capacity to synthesize sufficient 7dC from a substrate other than cholesterol in order to fulfill molting hormone requirements of the homozygous *woc* mutant before the third larval instar. It has been reported that *Drosophila* is unable to dealkylate sitosterol 'significantly', a plant sterol analogous to C (Svoboda et al., 1989). Indeed, fruit flies can use campesterol, another alkylated C analog of plants, to ultimately synthesize an alternative alkylated active molting hormone, makisterone A (Redfern, 1986; Pak and Gilbert, 1987; Feldlaufer et al., 1995), but this campesterol must still first be 'activated' by sterol 7,8-dehydrogenation. However, dealkylation and reduction of these and other plant sterols to C (Fig. 1) is common among other insects (Svoboda and Feldlaufer, 1991; Svoboda, 1999). For example, ergosterol in the diet of *M. sexta* is most probably dealkylated and reduced to 7dC as it is absorbed from the midgut into the hemolymph (Warren et al., 1999), similar to the formation of C from campesterol, stigmasterol and sitosterol (Svoboda,

1999). From the hemolymph compartment, this ergosterol-derived 7dC is distributed to other tissues of the animal and was found to be concentrated within the nervous system (Warren et al., 1999). A similar transformation of yeast-derived ergosterol in the diet of fruit flies to 7dC in quantities sufficient to support minimum molting hormone biosynthesis early in development is also a possibility.

Nevertheless, with respect to dietary C-based ecdysteroidogenesis in third instar homozygous *woc* mutant larvae, it is apparent that there is insufficient 7,8-dehydrogenation of C to 7dC in the homozygous mutant ring gland. This is the reason that dietary C is unavailable for ecdysone biosynthesis in the mutant ring gland, even when its concentration in the food is dramatically increased. As a result, active molting hormone levels in this organism do not attain the threshold level necessary for the initiation of molting and metamorphosis to the pupal stage, regardless of the tissue load of C. Yet, when the homozygous *woc* brain–ring complex content of 7dC is dramatically elevated at least a 100-fold in feeding larvae by oral administration of this critical intermediate, brain–ring gland complex ecdysteroid production increases, whole-body titers increase and the larvae are rescued to the beginning of pupal-adult development. Apparently, this far greater tissue 'load' of 7dC, derived from dietary 7dC, is more than adequate to support levels of molting hormone biosynthesis necessary for normal molting and metamorphosis of the homozygous *woc* mutants to the beginning of pupal-adult development.

Initially, it was hypothesized that the *woc* gene mutation might occur within the coding region of the specific microsomal cytochrome P450 monooxygenase that catalyzes cholesterol 7,8-dehydrogenation in *Drosophila*. However, the deduced coding region for the wild-type *woc* gene (Wismar et al., 2000) does not contain the highly conserved consensus sequence that is normally associated with the binding of the heme group to a cytochrome P450, e.g. FXXGXXXCXG (Snyder et al., 1996) or F(G,S)XGXRXCXG (Holten and Lester, 1996). Instead, *woc* appears to be an important atypical zinc-finger protein, perhaps a transcription factor (Wismar et al., 2000) that controls the expression of cholesterol 7,8-dehydrogenase activity. Indeed, portions of the *woc* sequence are upwards of 65% homologous to two ubiquitous, but also atypical zinc-finger proteins that may function as orphan transcription factors in humans: ZNF198 (Xiao et al., 1998) and DXS6637E (van der Maarel et al., 1996). These genes, when mutated, result in a human leukemia/lymphoma syndrome and X-linked mental retardation, respectively.

If this putative *woc* transcription factor selectively upregulates a specific cholesterol 7,8-dehydrogenase enzyme that mediates the first step in the ecdysteroidogenic pathway, then it may act similarly to SF1 and related orphan transcription factors in mammals (Morohashi and Omura, 1996; Parker and Schimmer, 1997; Ito et al., 1998). However, in the insect, the distal enzymatic activities involved in ecdysteroidogenesis do not appear to be so regulated by *woc*, although the presence of an additional 'factor' (or its absence) under the control of *woc* does seem to be necessary, along with 20E, for normal pupal-adult development. As such, *woc* and this additional factor or factors, along with cholesterol 7,8-dehydrogenase, may be suitable targets for selective insect control. We are presently investigating whether *woc* is such a transcription factor and if so, whether it in fact regulates cholesterol-7,8-dehydrogenase P450 transcriptional activity in the ring glands of *Drosophila* and the prothoracic gland cells of *M. sexta*.

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