Eur. J. Entomol. **94**: 57–65, 1997
ISSN 1210–5759

Ecdysone 20-hydroxylation in *Manduca sexta* (Lepidoptera: Sphingidae) midgut: Development-related changes of mitochondrial and microsomal ecdysone 20-monooxygenase activities in the fifth larval instar

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Tobacco hornworm, *Manduca sexta*, midgut, 20-hydroxylation, ecdysone 20-monooxygenase, ecdysone 26-monooxygenase, microsomes, mitochondria, cytochrome P-450

Abstract. In the mid fifth-larval-instar tobacco hornworm, *Manduca sexta*, the midgut has high ecdysone 20-monooxygenase (E20MO) activity, with a peak at the onset of wandering on day five. Activities of E20MO, present both in mitochondria and in microsomes, were determined around the time of the peak. During the day preceding the peak the microsomal E20MO increased 60-fold (total activity) or 115-fold (specific activity), and it decreased to one half or less of the peak activities within two days after the peak. The mitochondrial E20MO increased only 1.3 to 2.4-fold (total and specific activities, respectively) before the peak, but declined more rapidly than the microsomal E20MO after the peak. It is concluded that mitochondrial and microsomal E20MO activities are controlled independently and that changes in the physiological rate of ecdysone 20-hydroxylation are effected primarily by changes of the microsomal E20MO activities.

INTRODUCTION

Ecdysone 20-monooxygenase [ecdysone, hydrogen donor: oxygen oxidoreductase (20-hydroxylating), EC 1.14.99.22] catalyzes the hydroxylation of ecdysone to 20-hydroxyecdysone, the last and probably rate-limiting step in the biosynthesis of the insect molting hormone (Weirich et al., 1984; Smith, 1985). Ecdysone 20-monooxygenase (E20MO) is a cytochrome P-450 enzyme found in mitochondria and/or in microsomes (endoplasmic reticulum) of various insect and crustacean tissues (for refs., see Weirich et al., 1984; Smith, 1985; Weirich, 1989; Thompson et al., 1990; Svoboda et al., 1991).

The activity of E20MO has been shown to undergo changes related to postembryonic development, oocyte maturation, and adult age, and these changes are believed to be essential for the regulation of the 20-hydroxyecdysone titer and thereby indirectly for the control of development (Nigg et al., 1976; Smith et al., 1983; Zhu et al., 1983; Beckage & Templeton, 1986; Halliday et al., 1986; Smith & Mitchell, 1986; Liebrich et al., 1991; Darvas et al., 1993). In some cases these changes in E20MO activities reflect similar changes in hemolymph ecdysteroid titers (Johnson & Rees, 1977; Feyereisen & Durst, 1980a,b; Mitchell & Smith, 1988) and may in fact be controlled by the concentration of circulating ecdysteroids. Increased E20MO activity has been elicited in *Locusta migratoria* (Feyereisen & Durst, 1980b) and *Manduca sexta* (Keogh et al., 1989) by injection of

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ecdysone, and in *Musca domestica* by feeding an ecdysone-supplemented diet (Srivatsan et al., 1987). Removal of prothoracic glands or ligation of the larvae, on the other hand, reduced the E20MO activity in *L. migratoria* and *M. sexta* (Feyereisen & Durst, 1980a,b; Keogh et al., 1989).

Smith et al. (1983) traced the changes in E20MO activities of *M. sexta* midgut and fat body to changes in the maximal velocities but found no indications for significant changes in the Michaelis constants. In mitochondria of ecdysone-fed *Musca domestica* larvae increased E20MO activity involved both an increase in the V_{max} and a decrease in the K_m (Srivatsan et al., 1987). Changes of the E20MO activity in fat body mitochondria of *Spodoptera littoralis* were correlated with the abundance of polypeptides immunologically related to components of steroidogenic enzyme systems in vertebrate mitochondria. Antibodies to bovine adrenal cytochrome P-450_{scc}, cytochrome P-$450_{11\beta}$, adrenodoxin and adrenodoxin reductase were also shown to inhibit the E20MO of *S. littoralis* mitochondria (Chen et al., 1994a). The possibility of a short-term control of E20MO activity by reversible phosphorylation-dephosphorylation of some components of the enzyme system was demonstrated in mitochondria and microsomes of *S. littoralis* fat body (Hoggard & Rees, 1988; Hoggard et al., 1989).

In the first study of E20MO, the activity in *M. sexta* midgut was found to be limited to the late fifth larval and early prepupal stages (Nigg et al., 1976). A detailed analysis of E20MO in midgut and fat body tissues (slices) and homogenates for the period between the last (fifth) larval and the pupal ecdysis revealed a particularly large E20MO activity peak in homogenates of the mid fifth-instar midgut (Smith et al., 1983). However, *M. sexta* midgut contains two E20MOs, one located in the mitochondria and one in the microsomes (Mayer et al., 1978; Kaplanis et al., 1980; Weirich et al., 1985), and the enzyme assays with whole tissues or homogenates did not allow the distinction between mitochondrial and microsomal E20MO activities. Based on a recent kinetic analysis which showed the microsomal E20MO to have a 44 times lower K_m (i.e., higher affinity) for ecdysone than the mitochondrial E20MO, we concluded that the microsomal E20MO is the primary midgut enzyme involved in ecdysone 20-hydroxylation (Weirich et al., 1996). The present study was undertaken to further clarify the function of the two enzyme systems. Activity changes of mitochondrial and microsomal E20MOs have been determined separately and compared to total midgut E20MO activities (Smith et al., 1983) around the time of the large fifth instar peak.

MATERIAL AND METHODS*

Chemicals

Ecdysone was obtained from Simes Pharmaceuticals (Milan, Italy). All other reagents were as specified previously (Weirich et al., 1985).

Preparation of mitochondria and microsomes

Tobacco hornworms (*M. sexta* L.; Lepidoptera: Sphingidae) were reared as described previously (Weirich et al., 1993). Groups of insects were collected on the fourth day of the fifth instar (V-4, one day before the E20MO activity peak), on the fifth day of the fifth instar ("wandering larvae", WL, peak of E20MO activity), and two days after the onset of wandering (WL+2, E20MO activity in the post-peak

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downslope; Smith et al. 1983). Midguts were dissected, cleaned and homogenized (Weirich & Adams, 1984; Weirich, 1985) in 30 mM HEPES buffer, pH 7.6, containing 0.3 M sucrose and 0.1 mM EDTA (buffer A). Mitochondria and microsomes were obtained by differential centrifugation (Weirich & Adams, 1984; Weirich, 1985). Mitochondria were washed twice by resuspension in buffer A and recentrifugation. Microsomal pellets were rinsed with buffer A.

Ecdysone 20-monooxygenase assay

Suspensions of mitochondria and microsomes in buffer A were used for the assays. In addition to the enzyme preparation (0.74–3.0 mg protein per assay) and ecdysone (30–32 μM), the hypotonic reaction mixtures contained 30 mM potassium phosphate, 10 mM HEPES, pH 7.4, 0.1 M sucrose, 0.1 mM EDTA, and an NADPH-generating system, consisting of 0.6 mM $NADP^+$, 6 mM Glc-6-P, and 0.6 U Glc-6-P dehydrogenase, in a total volume of 250 μl. Assays of mitochondrial E20MO also contained 2 mg bovine serum albumin per ml which has been shown to stabilize the mitochondrial E20MO (Weirich et al., 1985; Weirich, 1985). The incubation mixtures were shaken continuously at 30°C in 2-ml polypropylene microcentrifuge tubes (nonconical) providing a large surface area for efficient gas exchange. After a 5-min preincubation of all other components, the reactions were started by the addition of ecdysone dissolved in 10 mM HEPES, pH 7.6. Each preparation was tested at two different protein concentrations and/or incubation times with duplicate assays for each combination of parameters. Incubation times were 15 or 30 min, after which the reactions were stopped by addition of 250 μl methanol, and samples were kept at –88°C until analyzed.

Prior to analysis samples were centrifuged for 15 min at 16,000 g and 5°C. All samples were analyzed twice by reversed-phase HPLC on Resolve Radial Pak C_{18} cartridges (10 cm × 5 mm, 10 μm particle size; Waters) at 35°C and a flow rate of 1.1 ml/min (Weirich & Svoboda, 1992). The cartridge was first washed with water for 5 min, then developed with 15–17% acetonitrile in water for 35 min, followed by a 10-min rinse with 50% acetonitrile in water and 5 min reequilibration with water. This HPLC system was capable of resolving 20-hydroxyecdysone and 26-hydroxyecdysone. The ecdysteroids were quantified by integration of the $A_{254\ nm}$ peaks (Waters Model 810 Baseline Workstation). Combined recoveries from enzyme assays were between 97 and 100% of the amount of ecdysone recovered from control incubations without (live) enzyme.

Protein determination

Protein concentrations were determined by a Folin microassay (Peterson, 1977), with bovine serum albumin as the standard.

RESULTS

Figure 1 shows the specific and total E20MO activities of microsomes and of mitochondria. The specific activity of the microsomal E20MO increased 115-fold between days four and five (from 0.4 to 45.9 pmol/min/mg protein) and declined to about half the peak activity (21.5 pmol/min/mg protein) two days later. The total E20MO activity in microsomes showed a smaller increase than the specific activity before the peak (65×; from 1.1 to 71.4 pmol/min/midgut) and a larger decline (to about 1/4 or 16.2 pmol/min/midgut) after the peak. These different proportional changes are the consequence of a continuous decrease in the amount of microsomal protein per midgut during the period from V-4 to WL+2 (Table 1).

The profile of the microsomal E20MO activities was very similar to that reported for midgut homogenate (Smith et al., 1983), which showed a 60-fold increase over a period of one to two days, followed by a decline to half the peak activity about two days later.

The E20MO activities in the mitochondria followed a different pattern from that of the homogenate and the microsomes. The mitochondria already contained substantial E20MO activity on day four (17.6 pmol/min/mg protein; 91.6 pmol/min/midgut). The activities increased only moderately (2.4 and 1.3-fold for specific and total activities, respectively)

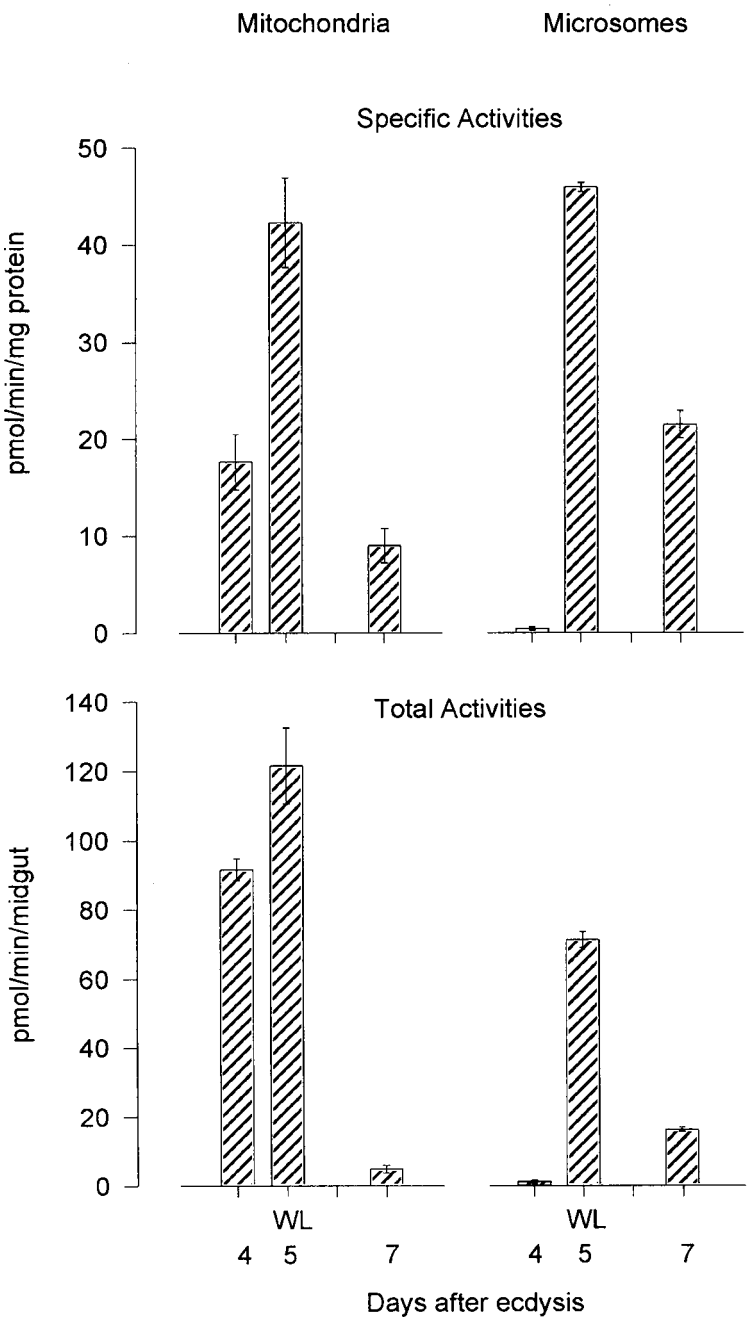

Fig. 1. Ecdysone 20-monooxygenase activities in mitochondria and microsomes of fifth instar *M. sexta* larvae at three different ages (one day before peak, at peak, two days after peak activity). Mean ± S.D. of two preparations per age, each assayed in duplicate, two HPLC analyses per assay. WL, wandering larvae.

between days four and five and declined more precipitously than the microsomal activities during the two days following the peak (to 1/5 and 1/25 of the peak for specific and total activities, respectively). Again, the total activity increased less during the day before the peak and declined more strongly after the peak than the specific activity did because of decreasing amounts of mitochondrial protein per midgut (Table 1).

TABLE 1. Mitochondrial and microsomal protein yields from fifth instar *M. sexta* midgut.

Age[a] (days after ecdysis)	Protein yield (mg protein/midgut[b])	
	mitochondria	microsomes
4	5.33 ± 1.17	2.60 ± 0.11
5 (WL)	2.88 ± 0.30	1.59 ± 0.01
7 (WL+2)	0.54 ± 0.02	0.76 ± 0.09

[a] WL – wandering larvae; WL+2 – larvae two days after onset of wandering.
[b] Means ± S.D. of two preparations for each age.

Incubations of V-4 microsomes with ecdysone produced not only marginal amounts of 20-hydroxyecdysone, but also small amounts of a compound that eluted with the R_t of 26-hydroxyecdysone. Neither microsomes from the two later age groups nor any of the mitochondria yielded detectable amounts of this compound.

DISCUSSION

E20MO activities of microsomes and mitochondria showed a different temporal profile between days four and seven of the fifth larval instar (Fig. 1). Although both E20MOs peaked on day five, the relative increase during the day before and decline during the two days following the peak were quite different. This observation suggests a different mode of control for the activities of the two enzymes.

The K_ms of the microsomal and mitochondrial E20MOs have recently been reported to be 3.67×10^{-7} M and 1.63×10^{-5} M, respectively (Weirich et al., 1996). In the present study E20MO activities were measured at a concentration of 30–32 μM ecdysone, i.e. about twice the K_m for mitochondrial E20MO and almost two orders of magnitude above the K_m for microsomal E20MO. The rates of the hydroxylation were therefore close to V_{max} for the microsomes, and between one half V_{max} and V_{max} for the mitochondria.

Maximal enzymatic reaction rates, by definition requiring infinite substrate concentrations, are never realized under physiological conditions. The hemolymph ecdysone titers between days four and seven of the fifth instar are 36 to 200 ng/ml (7.8×10^{-8} to 4.3×10^{-7} M) (Smith et al., 1983). As we have recently reported, in this concentration range the total E20MO activity of midgut microsomes exceeds that of the mitochondria 8-fold (Weirich et al., 1996).

The activity profile of the microsomes, although not comparable in absolute numbers, showed a close similarity to the E20MO activity profile previously reported for midgut homogenates (Smith et al., 1983). The activities in homogenates were measured at 0.34 μM, a concentration about equal to the peak hemolymph titer during the fifth instar and to the K_m for microsomal E20MO, but far below the K_m of mitochondrial E20MO. The results, therefore, would be expected to reflect mainly microsomal activities (at about one half V_{max}) with only minor contributions by the mitochondria (far below V_{max}), as indeed

they did. However, determinations of V_{max} for midgut homogenates during the same period of development revealed a close temporal and quantitative similarity to the profile obtained at 0.34 μM (Smith et al., 1983). Apparently, even at the higher ecdysone concentrations used for the kinetic analysis there was no significant contribution by the mitochondrial E20MO. The reason(s) for that are not obvious, but osmolarity or other experimental parameters may have been involved.

The fat body of *M. sexta* larvae contains another low K_m, high affinity E20MO, which is located in the mitochondria (Bollenbacher et al., 1977; Smith et al., 1979, 1980). The E20MO activity in the fat body continuously decreases between days four and seven of the fifth instar (Smith et al., 1983), thus representing yet another activity profile, different from those of midgut homogenate, microsomes and mitochondria.

The E20MO activities found in midgut homogenates (peak at 498 pg or 1.07 pmol/min/mg tissue) far exceeded those of fat body homogenates (peak at 63 pg or 0.14 pmol/min/mg tissue; Smith et al., 1983). The total masses of fat body and of midgut tissue are comparable, 0.52 and 0.34 g, respectively, for fifth instar larvae at the end of the feeding period (Williams-Boyce & Jungreis, 1980). Thus, the midgut has a significant potential for ecdysone 20-hydroxylation.

The large increase in E20MO activity of midgut homogenates or microsomes occurs within one day following a small peak of the hemolymph ecdysteroid titer on day four (Smith et al., 1983). This commitment peak is believed to cause reprogramming of the larval tissues for pupal development (Riddiford, 1978) and is probably eliciting the increase of E20MO activity as well. Head or thorax ligation of early fifth instar larvae or injection of cycloheximide or actinomycin D (Keogh et al., 1989) has been shown to prevent the increase in E20MO activity of midgut homogenate, but it can be restored in ligated animals by injection of ecdysone, 20-hydroxyecdysone (Keogh et al., 1989), or the ecdysone agonist RH-5849 (Keogh & Smith, 1991). Injection of sonicated brain-retrocerebral complexes also restored activity in head-ligated, but not in thorax-ligated larvae (Keogh et al., 1989). These observations and the data presented in this paper suggest that the sharp increase in midgut microsomal E20MO activity is directly or indirectly caused by an increase of the ecdysteroid titer in the fifth instar larvae and that the mitochondrial E20MO is not or much less dependent on ecdysone stimulation than the microsomal E20MO.

In experiments reported by Chen et al. (1994b) injection of ecdysone, 20-hydroxyecdysone, or the ecdysteroid agonist RH-5849 into *Spodoptera littoralis* larvae (intact or ligated) caused induction of ecdysone 26-monooxygenase rather than E20MO. These results suggested that injected ecdysteroids and ecdysteroid agonists open a molting hormone inactivation pathway. Our experiments, however, have shown that midguts of untreated *M. sexta* larvae at the time of the peak hemolymph ecdysteroid titer (day 7; WL+2) do not contain any ecdysone 26-monooxygenase activity.

It is interesting to note that not only the total ecdysteroid titer, but also the ratio of 20-hydroxyecdysone to ecdysone in *M. sexta* hemolymph starts to increase substantially on day five (Smith et al., 1983), the day of peak E20MO activities. Although the titers are undoubtedly influenced by other processes, such as catabolism and excretion, the increase in the proportion of 20-hydroxyecdysone is probably facilitated by the high midgut E20MO activity, especially that of the microsomes.

The increases of the E20MO activities in *M. sexta* midgut occur at a time when the total amounts of mitochondrial and microsomal protein decrease 46 and 39%, respectively (Table 1). Thus, while the total supply of mitochondria and microsomes is reduced (perhaps a reflection of the diminishing demands for digestion), these subcellular components are still being modified for specific metabolic requirements. The increases in the E20MO activities could be caused by activation of existing enzymes or the de novo synthesis of new, perhaps more active cytochromes P-450 and/or other components of the E20MO systems and their incorporation into mitochondrial or microsomal membranes. The involvement of gene transcription and protein synthesis has been demonstrated by experiments showing that the increase of E20MO activity in midgut homogenates is inhibited by actinomycin D and cycloheximide (Keogh et al., 1989). On the other hand, the increased activity in homogenates apparently did not result from the production of a higher affinity cytochrome P-450 because the K_m did not change significantly (Smith et al., 1983).

The decreases in E20MO activities between days five and seven again reflect specific modifications of the enzymatic characteristics of mitochondrial and microsomal membranes. At the same time the total amounts of mitochondrial and microsomal proteins continue to decrease, and the midgut weight also decreases (Weirich et al., 1993).

Feeding of an ecdysone-supplemented diet was shown to increase the E20MO activity in mitochondria, but not in microsomes, of *Musca domestica* larvae (Srivatsan et al., 1987). Although mitochondria and microsomes of ecdysone-treated larvae each contained six forms of cytochrome P-450 with E20MO activity, only one mitochondrial form, based on its high activity, was presumed to be responsible for ecdysone 20-hydroxylation under physiological conditions (Srivatsan et al., 1990; Agosin & Srivatsan, 1991).

The apparent responsiveness of the *M. sexta* midgut E20MO to ecdysteroid stimulation and the high affinity of the microsomal enzyme for ecdysone support the conclusion that the microsomes of the fifth instar *M. sexta* midgut contain an ecdysone-specific cytochrome P-450, i.e. a true E20MO, and that it is this enzyme, rather than the mitochondrial E20MO, that is primarily responsible for changes in the physiological rate of ecdysone 20-hydroxylation in the midgut and thereby for the control of the 20-hydroxyecdysone titer.

ACKNOWLEDGEMENTS. I thank Virginia P. Williams and Lynda J. Liska for their dedicated technical assistance, and Govindan Bhaskaran, David J. Chitwood, and Paul D. Shirk for reviewing the manuscript.

REFERENCES

AGOSIN M. & SRIVATSAN J. 1991: Role of microsomal cytochrome P-450 in the formation of ecdysterone in larval house fly. *Comp. Biochem. Physiol. (B)* **99**: 271–274.

BECKAGE N.E. & TEMPLETON T.J. 1986: Physiological effects of parasitism by Apanteles congregatus in terminal-stage tobacco hornworm larvae. *J. Insect Physiol.* **32**: 299–314.

BOLLENBACHER W.E., SMITH S.L., WIELGUS J.J. & GILBERT L.I. 1977: Evidence for an α-ecdysone cytochrome P-450 mixed function oxidase in insect fat body mitochondria. *Nature* **268**: 660–663.

CHEN J.-H., HARA T., FISHER M.J. & REES H.H. 1994a: Immunological analysis of developmental changes in ecdysone 20-monooxygenase expression in the cotton leafworm, Spodoptera littoralis. *Biochem. J.* **299**: 711–717.

CHEN J.-H., KABBOUH M., FISHER M.J. & REES H.H. 1994b: Induction of an inactivation pathway for ecdysteroids in larvae of the cotton leafworm, Spodoptera littoralis. *Biochem. J.* **301**: 89–95.

DARVAS B., REES H.H. & HOGGARD N. 1993: Ecdysone 20-monooxygenase systems in flesh-flies (Diptera: Sarcophagidae), Neobellieria bullata and Parasarcophaga argyrostoma. *Comp. Biochem. Physiol. (B)* **105**: 765–773.

FEYEREISEN R. & DURST F. 1980a: Development of microsomal cytochrome P-450 monooxygenases during the last larval instar of the locust, Locusta migratoria: Correlation with the hemolymph 20-hydroxyecdysone titer. *Mol. Cell. Endocrinol.* **20**: 157–169.

FEYEREISEN R. & DURST F. 1980b: Control of cytochrome P-450 monooxygenases in an insect by the steroid moulting hormone. In Coon M.J., Conney A.H., Estabrook R.W., Gelboin H.V., Gillette J.R. & O'Brien J.P. (eds.): *Microsomes, Drug Oxidations, and Chemical Carcinogenesis, Vol. 1.* Academic Press, New York, pp. 595–598.

HALLIDAY W.R., FARNSWORTH D.E. & FEYEREISEN R. 1986: Hemolymph ecdysteroid titer and midgut ecdysone 20-monooxygenase activity during the last larval stage of Diploptera punctata. *Insect Biochem.* **16**: 627–634.

HOGGARD N. & REES H.H. 1988: Reversible activation-inactivation of mitochondrial ecdysone 20-monooxygenase: A possible role for phosphorylation-dephosphorylation. *J. Insect Physiol.* **34**: 647–653.

HOGGARD N., FISHER M.J. & REES H.H. 1989: Possible role for covalent modification in the reversible activation of ecdysone 20-monooxygenase activity. *Arch. Insect Biochem. Physiol.* **10**: 241–253.

JOHNSON P. & REES H.H. 1977: The mechanism of C-20 hydroxylation of α-ecdysone in the desert locust, Schistocerca gregaria. *Biochem. J.* **168**: 513–520.

KAPLANIS J.N., WEIRICH G.F., SVOBODA J.A., THOMPSON M.J. & ROBBINS W.E. 1980: Moulting hormones of the tobacco hornworm and the milkweed bug: Their chemistry and biochemistry. In Hoffmann J.A. (ed.): *Progress in Ecdysone Research.* Elsevier/North Holland Biomedical Press, Amsterdam, pp. 163–186.

KEOGH D.P. & SMITH S.L. 1991: Regulation of cytochrome P-450 dependent steroid hydroxylase activity in Manduca sexta: Effects of the ecdysone agonist RH-5849 on ecdysone 20-monooxygenase activity. *Biochem. Biophys. Res. Commun.* **176**: 522–527.

KEOGH D.P., JOHNSON R.F. & SMITH S.L. 1989: Regulation of cytochrome P-450 dependent steroid hydroxylase activity in Manduca sexta: Evidence for the involvement of a neuroendocrine-endocrine axis during larval-pupal development. *Biochem. Biophys. Res. Commun.* **165**: 442–448.

LIEBRICH W., DÜRNBERGER B.B. & HOFFMANN K.H. 1991: Ecdysone 20-monooxygenase in a cricket (Gryllus bimaculatus Ensifera, Gryllidae) – activity throughout adult life cycle. *Comp. Biochem. Physiol. (A)* **99**: 597–602.

MAYER R.T., SVOBODA J.A. & WEIRICH G.F. 1978: Ecdysone 20-hydroxylase in midgut mitochondria of Manduca sexta (L.). *Hoppe-Seyler's Z. Physiol. Chem.* **359**: 1247–1257.

MITCHELL M.J. & SMITH S.L. 1988: Ecdysone 20-monooxygenase activity throughout the life cycle of Drosophila melanogaster. *Gen. Comp. Endocrinol.* **72**: 467–470.

NIGG H.N., SVOBODA J.A., THOMPSON M.J., DUTKY S.R., KAPLANIS J.N. & ROBBINS W.E. 1976: Ecdysone 20-hydroxylase from the midgut of the tobacco hornworm (Manduca sexta L.). *Experientia* **32**: 438–439.

PETERSON G.L. 1977: A simplification of the protein assay of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**: 346–356.

RIDDIFORD L.M. 1978: Ecdysone-induced change in cellular commitment of the epidermis of the tobacco hornworm, Manduca sexta, at the initiation of metamorphosis. *Gen. Comp. Endocrinol.* **34**: 438–446.

SMITH S.L. 1985: Regulation of ecdysteroid titer: Synthesis. In Kerkut G.A. & Gilbert L.I. (eds): *Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 7.* Pergamon Press, Oxford, pp 295–341.

SMITH S.L. & MITCHELL M.J. 1986: Ecdysone 20-monooxygenase systems in a larval and an adult dipteran: An overview of their biochemistry, physiology and pharmacology. *Insect Biochem.* **16**: 49–55.

SMITH S.L., BOLLENBACHER W.E., COOPER D.Y., SCHLEYER H., WIELGUS J.J. & GILBERT L.I. 1979: Ecdysone 20-monooxygenase: Characterization of an insect cytochrome P-450 dependent steroid hydroxylase. *Mol. Cell. Endocrinol.* **15**: 111–133.

SMITH S.L., BOLLENBACHER W.E. & GILBERT L.I. 1980: Studies on the biosynthesis of ecdysone and 20-hydroxy-ecdysone in the tobacco hornworm, Manduca sexta. In Hoffmann J.A. (ed): *Progress in Ecdysone Research.* Elsevier/North Holland Biomedical Press, Amsterdam, pp. 139–162.

SMITH S.L., BOLLENBACHER W.E. & GILBERT L.I. 1983: Ecdysone 20-monooxygenase activity during larval-pupal development of Manduca sexta. *Mol. Cell. Endocrinol.* **31**: 227–251.

SRIVATSAN J., KUWAHARA T. & AGOSIN M. 1987: The effect of α-ecdysone and phenobarbital on the α-ecdysone 20-monooxygenase of house fly larva. *Biochem. Biophys. Res. Commun.* **148**: 1075–1080.

SRIVATSAN J., WEIRICH M. & AGOSIN M. 1990: Cytochrome P-450-catalyzed formation of 20-hydroxy-ecdysone in larval house fly mitochondria. *Biochem. Biophys. Res. Commun.* **166**: 1372–1377.

SVOBODA J.A., WEIRICH G.F. & FELDLAUFER M.F. 1991: Recent advances in insect steroid biochemistry. In Patterson G.W. & Nes W.D. (eds.): *Physiology and Biochemistry of Sterols.* American Oil Chemists' Society, Champaign, pp. 294–323.

THOMPSON M.J., WEIRICH G.F. & SVOBODA J.A. 1990: Metabolism of insect molting hormones: Bioconversion and titer regulation. In Gupta A.P. (ed): *Morphogenetic Hormones of Arthropods, Vol. 1.* Rutgers University Press, New Brunswick, pp. 325–360.

WEIRICH G.F. 1985: Ecdysone 20-monooxygenase. In Law J.H. & Rilling H.C. (eds): *Methods in Enzymology, Vol. 111.* Academic Press, Orlando, pp. 454–458.

WEIRICH G.F. 1989: Enzymes involved in ecdysone metabolism. In Koolman J. (ed): *Ecdysone.* Georg Thieme Verlag, Stuttgart, pp. 174–180.

WEIRICH G.F. & ADAMS J.R. 1984: Microsomal marker enzymes of Manduca sexta (L.) midgut. *Arch. Insect Biochem. Physiol.* **1**: 311–321.

WEIRICH G.F. & SVOBODA J.A. 1992: 3-Oxoecdysteroid reductases in Manduca sexta midgut. *Arch. Insect Biochem. Physiol.* **21**: 91–102.

WEIRICH G.F., SVOBODA J.A. & THOMPSON M.J. 1984: Ecdysone 20-monooxygenases. In Hoffmann J. & Porchet M. (eds): *Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones.* Springer-Verlag, Berlin, pp. 227–233.

WEIRICH G.F., SVOBODA J.A. & THOMPSON M.J. 1985: Ecdysone 20-monooxygenase in mitochondria and microsomes of Manduca sexta (L.) midgut: Is the dual localization real? *Arch. Insect Biochem. Physiol.* **2**: 385–396.

WEIRICH G.F., FELDLAUFER M.F. & SVOBODA J.A. 1993: Ecdysone oxidase and 3-oxoecdysteroid reductases in Manduca sexta: Developmental changes and tissue distribution. *Arch. Insect Biochem. Physiol.* **23**: 199–211.

WEIRICH G.F., WILLIAMS V.P. & FELDLAUFER M.F. 1996: Ecdysone 20-hydroxylation in Manduca sexta midgut: Kinetic parameters of mitochondrial and microsomal ecdysone 20-monooxygenases. *Arch. Insect Biochem. Physiol.* **31**: 305–312.

WILLIAMS-BOYCE P.K. & JUNGREIS A.M. 1980: The larval-pupal transformation of Manduca sexta: changes in body and tissue weights and amount of hemolymph contaminating the tissues. *Ann. Entomol. Soc. Am.* **73**: 602–608.

ZHU X.X., GFELLER H. & LANZREIN B. 1983: Ecdysteroids during oogenesis in the ovoviviparous cockroach Nauphoeta cinerea. *J. Insect Physiol.* **29**: 225–235.

Received November 15, 1995; accepted March 18, 1996