Immunological analysis of developmental changes in ecdysone 20-mono-oxygenase expression in the cotton leafworm, *Spodoptera littoralis*

Jian-Hua CHEN,*‡ Takayuki HARA,† Michael J. FISHER* and Huw H. REES*

*Department of Biochemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, U.K.
and †Department of Food and Nutrition, Nakamura Gakuen College, Fukuoka 814-01, Japan

The developmental changes in ecdysone 20-mono-oxygenase during the sixth larval instar of the cotton leafworm, *Spodoptera littoralis*, were investigated. The specific activity of mitochondrial ecdysone 20-mono-oxygenase in the fat-body exhibited a distinct peak at 72 h, at which time the larvae stop feeding. Immunoblot analyses, using antibodies raised against components of vertebrate mitochondrial steroidogenic enzyme systems [anti-(cytochrome P-450_{scc}), anti-(cytochrome P-$450_{11\beta}$), anti-adrenodoxin and anti-(adrenodoxin reductase) antibodies], revealed the presence of specific immunoreactive polypeptides in fat-body mitochondrial extracts. In addition, these antibodies effectively inhibited fat-body mitochondrial ecdysone 20-mono-oxygenase activity. This suggests that the *S. littoralis* steroid-hydroxylating system(s) may contain polypeptide components analogous to those present in vertebrates. A close correlation between developmental changes in mitochondrial ecdysone 20-mono-oxygenase activity and the abundance of polypeptides (approx. 66 kDa and 50 kDa) recognized by the anti-(cytochrome P-$450_{11\beta}$) antibody and a polypeptide (approx. 52 kDa) recognized by the anti-(adrenodoxin reductase) antibody were observed in both fat-body and midgut. These results suggest that developmental changes in the abundance of components of the ecdysone 20-mono-oxygenase system may play an important role in the developmental regulation of the enzyme expression and, hence, of 20-hydroxyecdysone titre.

INTRODUCTION

Ecdysone is a steroid hormone involved in the regulation of insect development and metamorphosis. In larvae of most insect species, the prothoracic glands synthesize ecdysone, which is released into the haemolymph. Ecdysone undergoes C-20 hydroxylation in peripheral tissues yielding the generally predominant ecdysteroid, 20-hydroxyecdysone (Horn and Bergamasco, 1985; Redfern, 1989). Although ecdysone may have hormonal effects *per se*, it appears to function primarily as the precursor of 20-hydroxyecdysone which is considered to be the true moulting hormone in most species. Accordingly, the conversion of ecdysone into 20-hydroxyecdysone, which is catalysed by ecdysone 20-mono-oxygenase [ecdysone, hydrogen donor: oxygen oxidoreductase (20-hydroxylating), EC 1.14.99.22], has been viewed as an important regulatory step in the production of the moulting hormone [for reviews see Smith (1985) and Weirich (1989)].

Ecdysone 20-mono-oxygenase occurs in several tissues including fat-body, Malpighian tubules and midgut, in either mitochondria or microsomes or both subcellular fractions. The enzyme is an NADPH-requiring cytochrome P-450-dependent steroid hydroxylase analogous to those present in vertebrate steroidogenic tissues, such as the cholesterol side-chain cleavage and steroid 11β-hydroxylase (EC 1.14.15.4) enzymes (Smith, 1985; Weirich, 1989). In both mitochondria and endoplasmic reticulum of mammalian tissues, the cytochrome P-450s are components of distinct multienzyme systems, consisting of NADPH–ferredoxin reductase (e.g. adrenodoxin reductase), ferredoxin (e.g. adrenodoxin) and cytochrome P-450 in mitochondria and NADPH–cytochrome P-450 reductase and cytochrome P-450 in endoplasmic reticulum (Takemori and Kominami, 1984; Jefcoate, 1986). Studies with *Manduca sexta* fat-body mitochondria have provided evidence using e.s.r. spectroscopy for the presence of a ferredoxin-type non-haem iron protein (Smith et al., 1980). Evidence for the involvement of an NADPH–cytochrome P-450 reductase in ecdysone 20-hydroxylation has been furnished in *Diploptera punctata* midgut microsomes (Halliday et al., 1986).

Ecdysone 20-mono-oxygenase activity changes dramatically during insect development, with the occurrence of a tissue-specific peak of activity during the final larval instar (Nigg et al., 1976; Johnson and Rees, 1977; Feyereisen and Durst, 1980; Smith et al., 1983; Halliday et al., 1986). A reversible phosphorylation–dephosphorylation mechanism may be responsible for short-term regulation of the enzyme (Hoggard and Rees, 1988; Hoggard et al., 1989). However, little is known about the mechanism(s) underlying the long-term developmental control of enzyme expression. One of the major difficulties encountered in this type of study has been the absence of antibodies directed against ecdysone 20-mono-oxygenase.

Comparative studies of P-450 cytochromes, involving peptide mapping and sequence analysis, together with spectral and immunochemical approaches, have revealed that most P-450 proteins have certain structural similarities (Coon et al., 1992; Nelson et al., 1993). Generally, more structural homology is observed between the corresponding enzyme of different species than among the different enzymes found in a particular species (Black and Coon, 1986). This suggests that specific antibodies raised against components of vertebrate steroidogenic enzymes may be useful as probes of the components of insect ecdysone 20-mono-oxygenase.

In the fat-body of *Spodoptera littoralis* the majority of the ecdysone 20-mono-oxygenase activity occurs in the mitochondria, with only a small amount associated with the microsomal fraction (Hoggard and Rees, 1988). Preliminary experiments have indicated that the specific activity of ecdysone 20-mono-oxygenase in the fat-body undergoes developmental variation

‡ To whom correspondence should be addressed.

during the final larval instar (Zimowska et al., 1989a). In the present paper we have analysed developmental alterations in ecdysone 20-mono-oxygenase activity. In addition, we have investigated the relationship between the insect 20-mono-oxygenase and analogous vertebrate enzyme using antibodies raised against bovine adrenal steroidogenic proteins.

EXPERIMENTAL

Materials

[23,24-$^3H_2$]Ecdysone (82.8 Ci/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Hepes, D-glucose 6-phosphate, BSA, glucose-6-phosphate dehydrogenase, antipain, chymostatin, pepstatin A, 1,10-phenanthroline and prestained SDS/PAGE molecular-mass markers were purchased from Sigma (Poole, Dorset, U.K.). NADPH was from Boehringer-Mannheim (Lewes, East Sussex, U.K.). Chemicals for SDS/PAGE, alkaline phosphatase-conjugated goat anti-rabbit IgG and the alkaline phosphatase-conjugated substrate kit for immunoblot experiments were purchased from Bio-Rad. Nitrocellulose membrane was obtained from Schleicher & Schuell (Dassell, Germany). All other reagents used were of the highest purity grade available from Fluka (Gillingham, Dorset, U.K.) or Fisons (Loughborough, Leics., U.K.).

Animals

Last-instar larvae of the cotton leafworm, *S. littoralis*, were reared on an artificial diet under a photoperiod of 18 h light:6 h dark at 28 °C and 70 % relative humidity. An almost synchronous population of animals was obtained at the end of the fifth instar and resynchronized at the beginning of the sixth instar, which lasts 5 days under our rearing conditions (Hoggard and Rees, 1988). Animals that moulted in the second gate were mainly used (Zimowska et al., 1989b).

Ecdysone 20-mono-oxygenase assay

In order to limit proteolysis during the preparation of mitochondria, protease inhibitors (antipain, chymostatin, pepstatin A and 1,10-phenanthroline) at a final concentration of 1 μg/ml were included in both isotonic and hypotonic Hepes buffers. Fat-body from last-instar larvae was dissected and homogenized using a Potter–Elvehjem homogenizer in ice-cold isotonic Hepes buffer (0.037 M, containing 0.3 M sucrose and 0.1 M KF), pH 7.5. Mitochondria were isolated by differential centrifugation (10000 *g* for 10 min), washed with the same buffer and then resuspended in a hypotonic Hepes buffer (0.037 M, containing 0.05 M sucrose and 0.1 M KF), pH 7.5, as described previously (Hoggard and Rees, 1988). The resulting broken mitochondrial suspension was used for ecdysone 20-mono-oxygenase assay essentially as described previously (Hoggard and Rees, 1988). Briefly, the reaction mixture contained [23,24-$^3H_2$]ecdysone (0.15 μCi, final specific radioactivity 0.5 Ci/mmol), cofactors (0.2 mM NADPH, 2.0 mM glucose 6-phosphate, 0.2 unit of glucose-6-phosphate dehydrogenase) and broken mitochondrial fraction (50 or 100 μl; generally 150–300 μg of protein) in a total volume of 300 μl of hypotonic Hepes buffer, pH 7.5. The reaction, which was carried out at 37 °C, was initiated by addition of cofactors (30 μl) and terminated after 30 min by addition of methanol (300 μl). Under the conditions used, the rate of reaction was linear up to 30 min and using up to 360 μg of protein. The mixture was then centrifuged for 10 min at 8800 *g* (4 °C), and a portion (50 μl) of the assay supernatant was analysed directly by h.p.l.c. The radioactivity of the 20-hydroxyecdysone was monitored using an on-line radioactivity monitor (A-200 FLO-ONE/Beta; Radiomatic Instruments, Canberra–Packard Co.).

For determination of the effects of antibodies raised against bovine adrenal steroidogenic proteins on *S. littoralis* fat-body ecdysone 20-mono-oxygenase activity, broken mitochondrial suspension (about 30 μg of protein) was mixed with each antibody (about 450 μg of protein) in the presence of [23,24-$^3H_2$]ecdysone substrate and pre-incubated at 37 °C for 30 min. Enzyme activity was then assayed by addition of cofactors in a final volume of 50 μl of hypotonic Hepes buffer, pH 7.5 (final concentrations of the substrate and cofactors were the same as those stated above) and terminated by the addition of 50 μl of methanol after 30 min incubation at 37 °C, as described above. Enzyme activity was also measured in the presence of a preimmune rabbit IgG fraction following the same protocol as that for the antibodies used.

Preparation of antibodies and immunoblot analyses

IgG fractions directed against components of bovine adrenal mitochondrial steroidogenic enzymes, which are designated as anti-*P*-450_{scc} [anti-(cytochrome *P*-450 cholesterol side-chain cleavage)], anti-*P*-$450_{11\beta}$ [anti-(cytochrome *P*-450 steroid 11β-hydroxylase)], anti-adrenodoxin and anti-(adrenodoxin reductase), were prepared as described previously (Hara and Kimura, 1989a,b; Watabe et al., 1993). Broken mitochondrial suspension, prepared as described above, was treated with an equal volume of double-concentration sample buffer [0.125 M Tris, 4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol, 0.004 % (w/v) Bromophenol Blue, pH 6.8] followed by heating in a boiling water bath for 3 min (Hames, 1990). Proteins were separated by SDS/PAGE using 10 % polyacrylamide gels essentially as described by Laemmli (1970) and electrophoretically transferred to nitrocellulose (Towbin et al., 1979). Immunoblotting was performed with the antibodies at a final dilution of 1:750. Immunoblots were developed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit IgG at a final dilution of 1:3000. This was followed by incubation in 15 ml of alkaline phosphatase colour-development buffer (100 mM $NaHCO_3$, pH 9.8) to which was added 150 μl of Bio-Rad AP colour reagent A (Nitroblue Tetrazolium in aqueous dimethylformamide containing $MgCl_2$) and 150 μl of Bio-Rad AP colour reagent B (5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide) (McGaedy, 1970). Immunoreactive protein was quantified using a Molecular Dynamics Densitometer. A linear relationship between absorbance and protein load was established using a series of mitochondrial extracts in serial dilutions. The molecular mass of each immunoreactive polypeptide was determined by comparison of its electrophoretic mobility with that of a range of prestained SDS/PAGE molecular-mass markers.

Protein assay

Protein was determined by the method of Lowry et al. (1951) using BSA as standard.

RESULTS

Developmental change in the specific activity of ecdysone 20-mono-oxygenase in fat-body mitochondria during the last larval instar

Determination of ecdysone 20-mono-oxygenase in fat-body mitochondria revealed that the enzyme specific activity changes

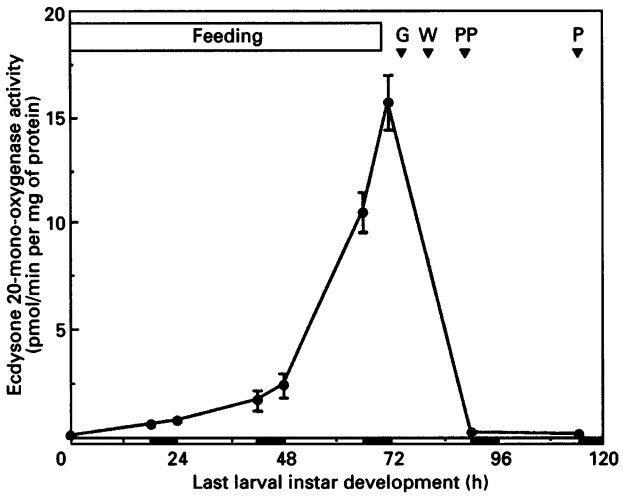

Figure 1 Profile of fat-body mitochondrial ecdysone 20-mono-oxygenase activity during the last larval instar of *S. littoralis*

Mitochondrial fraction was prepared from fat-body tissues by differential centrifugation at various developmental times, and the enzyme activity was determined under standard assay conditions (see the text). Each point represents the mean ± S.E.M. for three different tissue preparations; where not shown, error bars are within the symbol. Characteristic changes during development are indicated: gut purge (G), wandering (W), pharate pupal formation (PP) and pupation (P). Light and dark periods are indicated by open (□) and black (■) boxes respectively on the time scale.

dramatically during the last larval instar of *S. littoralis* (Figure 1). Activity was barely detectable at the beginning of the sixth instar. Thereafter it increased gradually during the feeding stage and exhibited a distinct peak at 72 h when the larvae stop feeding. By the onset of pharate pupal development (90 h), fat-body ecdysone 20-mono-oxygenase had dropped sharply to basal levels and remained low for the rest of the instar.

Immunoblot analysis of the fat-body mitochondrial proteins during the last larval instar

In initial experiments, immunoblot analyses, using antibodies raised against bovine adrenal cytochrome *P*-450$_{scc}$, cytochrome *P*-450$_{11\beta}$, adrenodoxin and adrenodoxin reductase revealed unique immunoreactive polypeptides in fat-body mitochondrial extracts (Figure 2). It is relevant that there was no distinct immunoreactivity, against these antibodies, in a mitochondrial fraction from *Schistocerca gregaria* leg muscle tissue which lacks ecdysone 20-mono-oxygenase activity (results not shown). In *S. littoralis* fat-body, the abundance of polypeptides detected with anti-*P*-450$_{11\beta}$ and anti-(adrenodoxin reductase) underwent increases to a peak at 72 h with a sharp decrease thereafter (Figures 2 and 3). These immunoreactivity profiles are essentially similar to that for the specific activity of ecdysone 20-mono-oxygenase during the final larval instar (see Figure 1). In contrast, the abundance of specific polypeptides detected with anti-*P*-450$_{scc}$ and anti-adrenodoxin antibodies remained fairly constant during the feeding stage with a slight decline thereafter (Figures 2 and 3).

The approximate molecular masses of the polypeptides detected with the anti-*P*-450$_{11\beta}$ (66 kDa and 50 kDa) and anti-(adrenodoxin reductase) (52 kDa) are reasonably close to those

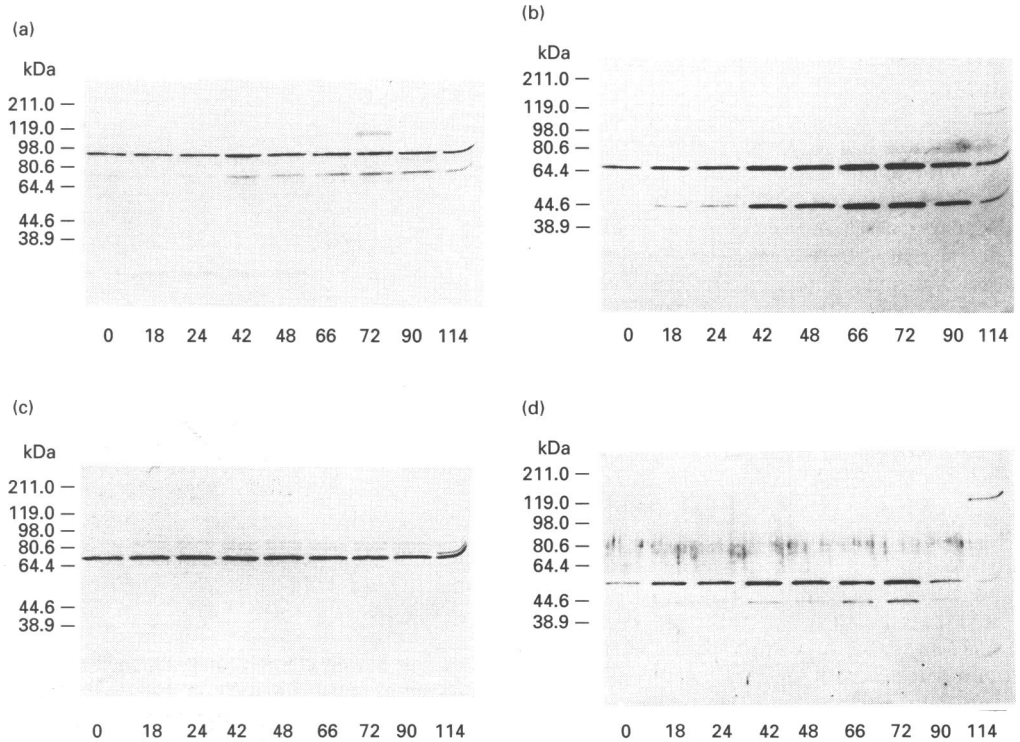

Figure 2 Immunoblot analysis of *S. littoralis* mitochondrial proteins

Fat-body mitochondrial extracts (5 μg of protein/lane) prepared at various times (hours as indicated) into the last larval instar of *S. littoralis* were separated by SDS/PAGE using 10% gels, electrophoretically transferred to nitrocellulose membrane and probed with the anti-*P*-450$_{scc}$ (**a**), anti-*P*-450$_{11\beta}$ (**b**), anti-adrenodoxin (**c**) and anti-(adrenodoxin reductase) (**d**) antibodies at a final dilution of 1:750. The positions and relative molecular masses of a series of prestained protein standards are indicated on the left.

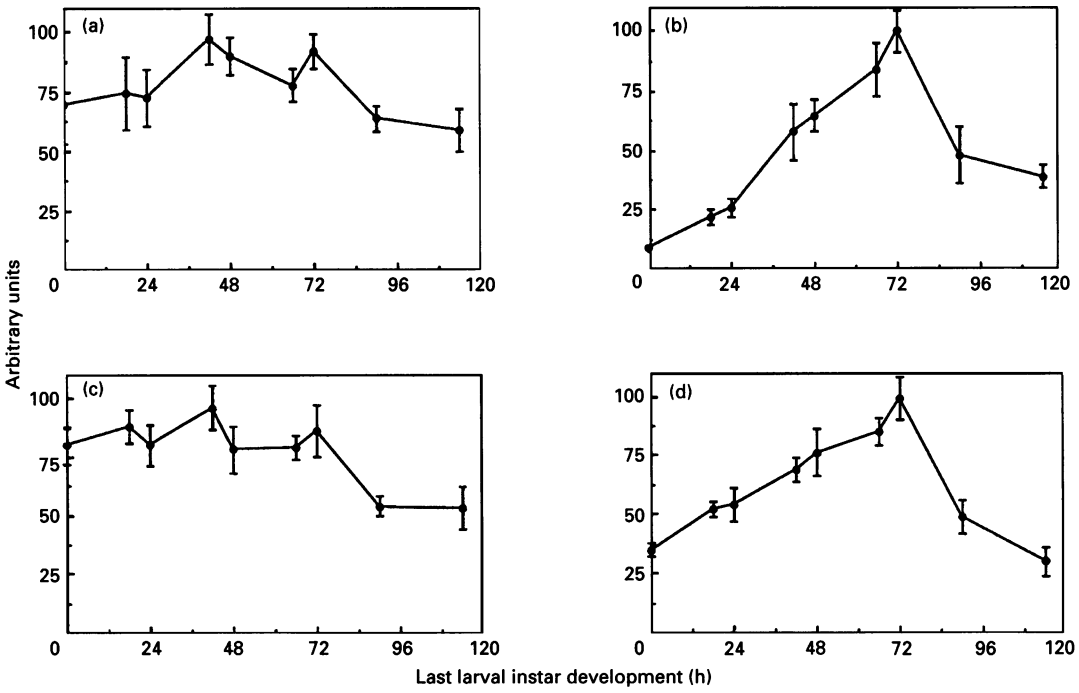

Figure 3 Relative abundance of immunoreactive polypeptides in *S. littoralis* fat-body mitochondria during last-instar development

Relative abundance of immunoreactive polypeptides in *S. littoralis* fat-body mitochondria detected with the anti-*P*-450$_{scc}$ (**a**), anti-*P*-450$_{11\beta}$ (**b**), anti-adrenodoxin (**c**) and anti-(adrenodoxin reductase) (**d**) were quantified by scanning the blots with a computing densitometer. Each point represents the mean values (with range) from two independent experiments, except in the case of (**b**), where values are the mean of three experiments.

reported for the mammalian antigenic proteins [cytochrome *P*-450s generally 45–57 kDa (Black and Coon, 1986); adrenal *P*-450$_{11\beta}$, 46 kDa (Katagiri et al., 1978), 48 kDa (Waterman et al., 1986); adrenodoxin reductase, 51 kDa (Waterman et al., 1986), 54 kDa (Hara and Kimura, 1989a)]. In our immunoblotting of bovine adrenal cortex mitochondrial proteins, the anti-*P*-450$_{11\beta}$ and anti-(adrenodoxin reductase) revealed unique polypeptides with apparent molecular masses of 54 kDa and 53 kDa respectively [results not shown; for reference see Watabe et al. (1993)]. In *S. littoralis* fat-body mitochondria, the molecular masses of polypeptides detected with the anti-*P*-450$_{scc}$ (82 kDa) and the anti-adrenodoxin (73 kDa) were significantly higher than the mammalian counterparts [*P*-450$_{scc}$, 51.7 kDa (Black and Coon, 1986), 49 kDa (Waterman et al., 1986); adrenodoxin, 12 kDa (Waterman et al., 1986)]. In comparison, immunoblotting of bovine adrenal cortex mitochondrial extract with the anti-*P*-450$_{scc}$ and anti-adrenodoxin revealed polypeptides of apparent molecular mass 56 kDa and < 20 kDa respectively [not shown; for reference see Watabe et al. (1993)]. The exact reason for the slight discrepancy between the molecular masses of the adrenal steroidogenic components observed in the present work as compared with some literature values is uncertain.

The lower-molecular-mass species (approximately 50 kDa) seen in the immunoblot detected with the anti-*P*-450$_{11\beta}$ may be a proteolytic product of the 66 kDa species (see Figure 2b). We observed that the 66 kDa species was predominant when samples for immunoblot analysis were freshly prepared from fat-body extracts. In contrast, in samples prepared from the extracts after freeze–thawing, the 66 kDa species was barely detectable and the 50 kDa species was abundant (results not shown). Protease inhibitors, such as antipain, chymostatin, pepstatin A and 1,10-phenanthroline, appeared unable to prevent this proteolysis completely (not shown).

Table 1 Effect of antibodies raised against bovine steroidogenic proteins on *S. littoralis* fat-body ecdysone 20-mono-oxygenase activity

Enzyme activity was determined under the assay conditions in the presence of the antibodies as described in the text. Enzyme activity measured in the presence of a preimmune IgG fraction is used as control. Values represent means (with range) for two independent experiments. The percentage of control activity is shown in parentheses.

Antibody treatment	Ecdysone 20-mono-oxygenase activity (pmol/min per mg of protein)
Preimmune IgG fraction	6.4 ± 0.5
Anti-*P*-450$_{scc}$	3.1 ± 0.4 (48%)
Anti-*P*-450$_{11\beta}$	2.0 ± 0.2 (31%)
Anti-adrenodoxin	1.2 ± 0.2 (19%)
Anti-(adrenodoxin reductase)	2.7 ± 0.3 (42%)

Effects of bovine steroidogenic antibodies on ecdysone 20-mono-oxygenase activity in the *in vitro* assay

To obtain evidence for the possible involvement of the mitochondrial immunoreactive peptides in the ecdysone 20-mono-oxygenase-catalysed reaction, the effects of the bovine steroidogenic antibodies on the ecdysone 20-mono-oxygenase activity were determined. In contrast with the negligible effect of preimmune IgG on the enzyme activity, the antibodies were effective

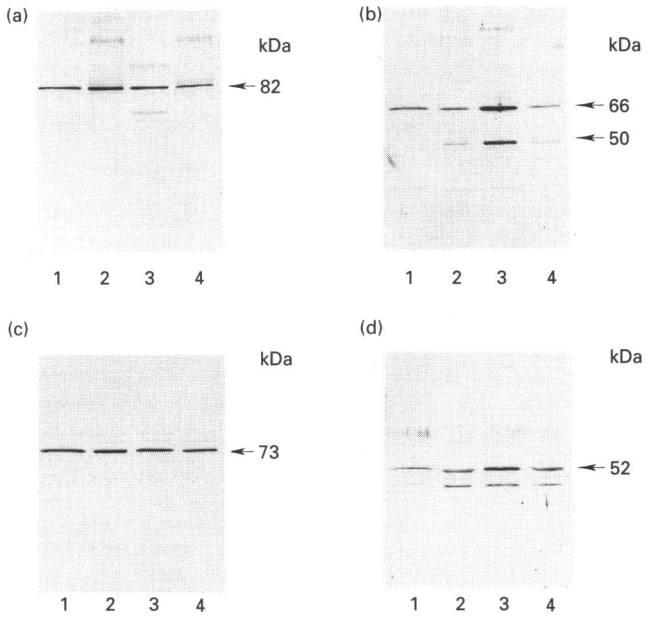

Figure 4 Immunoblot analysis of *S. littoralis* mitochondrial proteins in fat-body and midgut

Mitochondrial proteins (5 μg) were subjected to immunoblot analysis as indicated in Figure 2 with the anti-*P*-450$_{scc}$ (**a**), anti-*P*-450$_{11\beta}$ (**b**), anti-adrenodoxin (**c**) and anti-(adrenodoxin reductase) (**d**). Lanes 1 and 3, 18 h and 72 h fat-body respectively; lanes 2 and 4, 18 h and 72 h midgut respectively. The molecular masses of the immunoreactive polypeptides, determined by analysis of relative mobilities of a series of prestained molecular-mass markers, are indicated on the right of each blot.

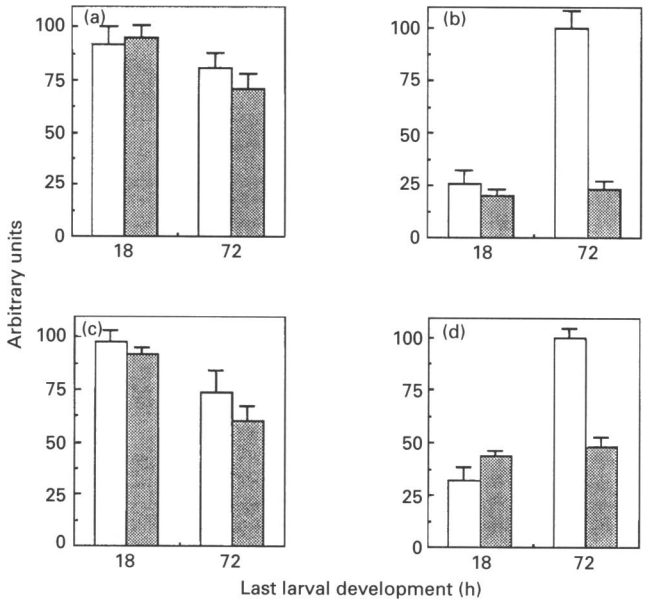

Figure 5 Relative abundance of immunoreactive polypeptides in *S. littoralis* mitochondrial extracts from fat-body and midgut

Relative abundance of immunoreactive polypeptides in *S. littoralis* mitochondrial extracts from fat-body and midgut detected with the anti-*P*-450$_{scc}$ (**a**), anti-*P*-450$_{11\beta}$ (**b**), anti-adrenodoxin (**c**) and anti-(adrenodoxin reductase) (**d**) were quantified by scanning the blots with a computing densitometer. □, Relative absorbances of immunoreactive polypeptides detected in fat-body mitochondria; ▨, relative absorbances of immunoreactive polypeptides detected in midgut mitochondria. Mean values ± S.E.M. ($n \geqslant 3$) from independent experiments are presented.

Table 2 Ecdysone 20-mono-oxygenase activities in fat-body and midgut of *S. littoralis*

Mitochondrial fractions were prepared and the ecdysone 20-mono-oxygenase activities were determined under standard assay conditions as described in the text. Values represent means ± S.E.M. ($n \geqslant 3$) from independent experiments.

	Ecdysone 20-mono-oxygenase activity (pmol/min per mg of protein)	
Last larval development (h)...	18	72
Fat-body	1.2 ± 0.2	14.6 ± 2.9
Midgut	1.7 ± 0.3	1.9 ± 0.4

in inhibiting the mitochondrial ecdysone 20-mono-oxygenase from fat-body (Table 1).

Comparison of the specific activity of ecdysone 20-mono-oxygenase and abundance of immunoreactivity in fat-body and midgut

In contrast with the substantial enzyme activity associated with fat-body mitochondria, only a low ecdysone 20-mono-oxygenase activity was detected in midgut. This midgut activity showed no apparent developmentally associated alterations during the last larval instar (results not shown). In midgut mitochondria, all four antibodies detected only specific polypeptides having the same molecular masses as those observed in mitochondria from fat-body (Figure 4). Ecdysone 20-mono-oxygenase activity and the relative abundance of immunoreactive polypeptides in mitochondrial extracts from fat-body and midgut were determined after 18 h and 72 h of development (Table 2 and Figure 4). The results indicated that the abundance of the specific polypeptides, detected with anti-*P*-450$_{11\beta}$ and anti-(adrenodoxin reductase) antibodies, correlate closely with the relative specific activity of ecdysone 20-mono-oxygenase in mitochondrial fractions of these tissues at these developmental time points (Table 2 and Figure 5). No immunoreactive polypeptides were found in postmitochondrial fractions from these two tissues (results not shown).

DISCUSSION

The mitochondrial ecdysone 20-mono-oxygenase activity in the fat-body of *S. littoralis* exhibits a major peak during the last larval instar. In contrast with the locust, *Locusta migratoria* (Hirn et al., 1979) and *Drosophila melanogaster* (Mitchell and Smith, 1988), the fluctuation in *S. littoralis* fat-body 20-mono-oxygenase activity is not temporally coincident with the major haemolymph ecdysteroid titre peak which occurs in the 5th light period when enzyme activity has dropped to a basal level (Cole, 1993). This finding, together with similar observations in *M. sexta* (Smith et al., 1983), suggest that the biological function of this enzyme system is not straightforward. Although the physiological significance of fluctuations in ecdysone 20-mono-oxygenase activity in *S. littoralis* are not fully understood, this fat-body enzyme does provide a useful model for investigating the regulation of insect cytochrome *P*-450 steroid hydroxylase systems.

Cytochrome *P*-450-dependent hydroxylation systems in insects may be structurally similar to the mammalian ones. Character-

ization of cytochrome *P*-450s in insects such as *Musca domestica* (Feyereisen et al., 1989, 1990), *Blaberus discoidalis* (Bradfield et al., 1991) and *D. melanogaster* (Gandhi et al., 1992; Waters et al., 1992) has revealed that insect *P*-450s are homologous to vertebrate cytochrome *P*-450 proteins (Nelson et al., 1993). In the present study, we have shown that specific antibodies against components of bovine adrenal steroidogenic enzyme systems detect unique immunoreactive polypeptides in mitochondrial extracts from both fat-body and midgut of *S. littoralis*. Our results suggest that the *S. littoralis* ecdysone 20-mono-oxygenase contains components analogous to those found in vertebrate adrenal steroid hydroxylation systems. The molecular basis of the significantly higher molecular masses observed for the polypeptides detected with the bovine anti-P-450_{scc} and anti-adrenodoxin antibodies in *S. littoralis* fat-body and midgut mitochondria than for those in mammalian tissue remains to be elucidated. The anti-P-450_{scc} antibody also shows weak cross-reactivity with a 66 kDa polypeptide possibly that recognized by the anti-P-$450_{11\beta}$ (Figure 2). This would not be surprising, as certain vertebrate adrenal steroidogenic *P*-450s show some catalytic activity in more than one hydroxylation reaction (Miller, 1988), which further attests to the similarity in the structures of many *P*-450 species.

Although it is always possible that all four major immunoreactive polypeptides detected by the respective antibodies employed in this work may not necessarily be components of the ecdysone 20-mono-oxygenase system, our evidence, taken together, suggests that this is not the case. For example, each antibody effectively inhibited the fat-body ecdysone 20-mono-oxygenase activity (Table 1). Although quantitative conclusions cannot be drawn from this type of experiment it does suggest a functional significance for these polypeptides in the 20-mono-oxygenase-catalysed reaction.

The close correlation of the developmental profile of the immunoreactive polypeptides detected by the anti-P-$450_{11\beta}$ and anti-(adrenodoxin reductase) antibodies with that for the specific activity of ecdysone 20-mono-oxygenase in fat-body mitochondria is consistent with the involvement of these polypeptides in the reaction. This is further supported by the demonstration that the amounts of polypeptides detected by these two antibodies in midgut mitochondria reflected the ecdysone 20-mono-oxygenase activity. These observations suggest the possibility that the variation of the ecdysone 20-mono-oxygenase activity in *S. littoralis* may be regulated by changes in abundance of cytochrome *P*-450 and/or adrenodoxin reductase. In this respect, it is relevant that in the case of microsomal ecdysone 20-mono-oxygenase from locust Malpighian tubules, the quantitative profile, determined by spectroscopy, of total cytochrome *P*-450 and the activity of NADPH–cytochrome *c* (cytochrome *P*-450) reductase also showed correlation with the mono-oxygenase activity during development of the last larval instar (Feyereisen and Durst, 1980). As might be expected, in both the *Spodoptera* and locust systems, the increase in components of the hydroxylase reactions slightly preceded that of the ecdysone 20-mono-oxygenase activity.

The activities of cytochrome *P*-450-dependent systems undergo marked changes under various conditions. In many vertebrate systems, changes in such enzyme activities reflect changes in cytochrome *P*-450 levels (John et al., 1985; Waterman et al., 1986; Imaoka et al., 1991; Addya et al., 1991; Aflalo and Meidan, 1993). Similarly, in insects, variations in the activities of several microsomal xenobiotic-metabolizing *P*-450 systems (e.g. demethylases, epoxidases, hydroxylases) have been noted to occur at specific stages in the insect life cycle and in different insecticide-resistant strains [for review see Hodgson (1985)]. Elevated levels of certain cytochrome *P*-450 species involved in mechanisms of insecticide resistance are commonly encountered [for references see Waters et al. (1992) and Scott and Lee (1993)]. Furthermore, levels of certain cytochrome *P*-450 species in insects may be regulated by other factors, such as starvation and hormonal factors (Bradfield et al., 1991).

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