The Mechanism of C-20 Hydroxylation of α -Ecdysone in the Desert Locust, *Schistocerca gregaria*

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1. The C-20 hydroxylation of α -ecdysone to produce β -ecdysone was investigated in the desert locust, *Schistocerca gregaria*. 2. α -Ecdysone C-20 hydroxylase activity was located primarily in the fat-body and Malpighian tubules. The properties of the hydroxylation system from Malpighian tubules were investigated further. 3. The enzyme system was mitochondrial, had a pH optimum of 6.5, an apparent K_m of $12.5 \mu\text{M}$ and required O_2 and NADPH. 4. The activity of the hydroxylation system showed developmental variation within the fifth instar, the maximum activity corresponding to the maximum titre of endogenous moulting hormone. The significance of these results is assessed in relation to the control of the endogenous titre of β -ecdysone. 5. The mechanism of the hydroxylation system was investigated by using known inhibitors of hydroxylation reactions such as CO, metyrapone and cyanide. 6. The CO difference spectrum of the reduced mitochondrial preparation indicated the presence of cytochrome P-450 in the preparation. 7. It is concluded that the α -ecdysone C-20 hydroxylase system is a cytochrome P-450-dependent mono-oxygenase.

The major moulting hormone controlling post-embryonic development in most insects is β -ecdysone (II), which is generally accompanied by smaller amounts of α -ecdysone (I) (Gilbert & King, 1973). However, prothoracic glands, which are the major source of moulting hormone in larval insects, when cultured *in vitro* secrete exclusively α -ecdysone, with no detectable β -ecdysone (Chino *et al.*, 1974; King *et al.*, 1974; Hoffmann *et al.*, 1975). Although α -ecdysone may directly affect certain tissues in some insects, it serves primarily as a precursor of β -ecdysone, the active moulting hormone in most systems (Gilbert & King, 1973). Exogenous labelled α -ecdysone is rapidly transformed into β -ecdysone in all species of insects investigated. In the tobacco horn-

worm, *Manduca sexta*, it has been demonstrated *in vitro* that this transformation can occur in several tissues, including Malpighian tubules, fat-body, body wall and gut, whereas the prothoracic glands, along with muscle, salivary glands and oenocytes, lack this capacity (King, 1972). Therefore, the prothoracic glands secrete α -ecdysone, which is converted by C-20 hydroxylation into the active hormone, β -ecdysone, by some peripheral tissues.

It has been shown in the insects *M. sexta* (Bollenbacher *et al.*, 1975), *Locusta migratoria* (migratory locust) (Koolman *et al.*, 1975) and *Schistocerca gregaria* (desert locust) (Morgan & Poole, 1976) that the ratio β -ecdysone/ α -ecdysone undergoes marked changes during development, attaining a high value at the time of maximum titre of total endogenous moulting hormone. In agreement with this, it has also been demonstrated in several species of insects that the apparent rate of conversion *in vivo* of [^3H] α -ecdysone into β -ecdysone (as determined from the radioactivity recovered in each hormone) is dependent on the developmental stage, the maximum conversion corresponding to the stage of maximum titre of endogenous β -ecdysone (Moriyama *et al.*, 1970; Hoffmann *et al.*, 1974; Feyereissen *et al.*, 1976a; Lagueux *et al.*, 1976). This developmental variation in the apparent rate of conversion of α -ecdysone into β -ecdysone suggests that the changing activity of the C-20-hydroxylating-enzyme system could be one of the factors involved in regulating β -ecdysone concentrations (Lagueux *et al.*, 1976; Bollenbacher

(I) R = H
 (II) R = OH

et al., 1975). However, the further metabolism of both α - and β -ecdysones into several products in studies *in vivo* may complicate the picture. To circumvent such complications, we now report results on the developmental variation in activity of the α -ecdysone C-20-hydroxylase system in mitochondria from Malpighian tubules. The notion that variation in activity of the α -ecdysone C-20-hydroxylase system is involved in regulating β -ecdysone concentrations is disputed by Young (1976), who suggests from kinetic experiments with *Calliphora* (blowfly) that hormone inactivation by the formation of conjugates and an enhanced synthesis of α -ecdysone are more important in regulating β -ecdysone titre.

The hydroxylation of α -ecdysone to β -ecdysone may be considered analogous to the various steroid-hormone hydroxylations occurring in vertebrates. These hydroxylations are catalysed by mixed-function oxygenases containing cytochrome *P*-450 as the terminal oxidase. The presence of cytochrome *P*-450 in insects has been reported (Ray, 1967), and evidence suggests that it participates in insect microsomal hydroxylations of several insecticides (Agosin & Perry, 1974).

Cytochrome *P*-450 involvement in steroid hydroxylations has generally been implicated by inhibition of the enzyme system with CO and the subsequent reversal of this inhibition by irradiation with light at 450 nm (Simpson & Boyd, 1967; Wilson & Harding, 1970; Greengard *et al.*, 1967). In addition, metyrapone (2-methyl-1,2-di-3'-pyridylpropan-1-one) has been shown to inhibit mammalian cytochrome *P*-450-dependent steroid hydroxylations (Chart & Sheppard, 1959), although the oxidative metabolism of many substrates catalysed by cytochrome *P*-450 can either be enhanced or inhibited by the drug (Leibman, 1969). Inhibition of hydroxylation reactions by cyanide is apparently characteristic of systems not involving cytochrome *P*-450 (Gaylor *et al.*, 1973; Gaylor & Mason, 1968) and cytochrome *P*-450-dependent steroid hydroxylations are not generally inhibited by moderately high cyanide concentrations (Schleyer *et al.*, 1973).

The aim of the present study was to elucidate the mechanism of α -ecdysone C-20-hydroxylation and to determine, by using the previously mentioned known inhibitors of hydroxylation reactions, whether the hydroxylation was cytochrome *P*-450-dependent. This required the initial investigation of the tissue and subcellular location of the hydroxylating-enzyme system and the development of a reproducible enzyme assay. Part of this work has been presented in preliminary form (Johnson *et al.*, 1976).

Experimental

Nomenclature

Trivial names are often used. Systematic names are

as follows: α -ecdysone, (22*R*)-2 β ,3 β ,14,22,25-penta-hydroxy-5 β -cholest-7-en-6-one; β -ecdysone, (22*R*)-2 β ,3 β ,14,20,22,25-hexahydroxy-5 β -cholest-7-en-6-one.

Chemicals

Samples of α -ecdysone and β -ecdysone were generously given by Simes S.p.A., Milan, Italy. [23,24-³H]- α -Ecdysone (2.9 Ci/mmol) was a gift from Schering A.G., Berlin, Germany. The purity of the [³H] α -ecdysone was ascertained regularly before use by t.l.c. (see below) and the developed plate was scanned for radioactivity. Metyrapone was a gift from Ciba Laboratories Ltd., Horsham, Sussex, U.K.

T.l.c.

T.l.c. separation of ecdysones (applied in methanol) was performed on silica gel (0.5 mm thick, Kiesel-gel GF₂₅₄; E. Merck A.G., Darmstadt, Germany) developed twice in chloroform/methanol (4:1, v/v). Compounds were detected under u.v. light and eluted thoroughly with chloroform/methanol (1:1, v/v).

High-performance liquid chromatography

A modified model ALC/100 liquid chromatograph was used in conjunction with an M6000 pumping system and a u.v. detector (254 nm), all from Waters Associates Ltd., Northwich, Cheshire, U.K. Samples dissolved in methanol were filtered through a Millipore Fluoropore (polytetrafluoroethylene) membrane filter before injection by using a Waters Associates model U6K septumless high-pressure valve injector. The column used in this work was 1.2 m $\times$ 3.2 mm (4 ft $\times$ $\frac{1}{8}$ in) stainless steel packed with Bondapak C₁₈/Corasil (37–50 μ m) (Waters Associates). Ecdysones were eluted with methanol/water (3:7, v/v) at a flow rate of 1 ml/min. All solvents were de-gassed under vacuum immediately before use.

Radiochemical methods

Radioactivity was measured on an Intertechnique three-channel liquid-scintillation spectrometer, model ABAC SL 30. Samples were dissolved in 10 ml of a dioxan scintillation solution containing 15 g of butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] and 100 g of naphthalene per litre of dioxan. ³H radioactivities are quoted after corrections had been applied for background, counting efficiency and quenching.

Developed t.l.c. plates were scanned for radioactivity, when necessary, by using a Panax RTLS-1A instrument with a P7900A rate-meter.

Growth of insects

The locusts (*Schistocerca gregaria*) were reared on a diet of fresh grass and rolled oats and kept under a 12 h 'day' period during which the temperature was maintained at 35°C and a corresponding 12 h 'night' period maintained at 25°C. Under these conditions, the fifth-instar stage lasted for 8 days. Larvae that

were 5–6 days into the fifth instar should be at a stage of maximum endogenous moulting-hormone titre and were used for experiments (unless otherwise specified in the text).

Incubation of tissues

Fat-bodies, guts, Malpighian tubules and body walls were excised from five dissected locusts, freed from surrounding tissues, and washed with insect Ringer solution (Bodenstein, 1946). They were then sliced on a glass slide with a scalpel blade, weighed, and placed in an incubation mixture of the following composition (Burststein *et al.*, 1974): 500 μ l of 0.02 M-potassium phosphate buffer (pH 7.3), 0.05 mg of NADP⁺, 0.5 mg of glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase and 0.05 μ Ci of [³H] α -ecdysone.

The tissues were incubated for 3 h at 25°C with moderate agitation. The total incubation mixture was then homogenized in a Potter–Elvehjem homogenizer and extracted several times with butan-1-ol. The butanol extract was evaporated to dryness under N₂ and separated by t.l.c. on silica gel (Kieselgel GF₂₅₄) developed twice in chloroform/methanol (4:1, v/v). The developed plates were scanned for radioactivity, and percentages of the recovered radioactivity associated with the various compounds present after incubation were determined.

Subcellular fractionation

Malpighian tubules were excised from ten locusts and homogenized by hand in a Potter–Elvehjem homogenizer (60 strokes) in 10 ml of 0.154 M-KCl/1 mM-EDTA at pH 7.0 (Van den Bergh, 1967). A sample was taken, and the rest of the homogenate was centrifuged for 5 min at 500g and the supernatant re-centrifuged for 20 min at 10000g. This supernatant was centrifuged for 45 min at 214000g (Morello *et al.*, 1971), after which the pellet and supernatant were both retained. Samples of each fraction were added to an incubation medium of the following composition: 200 μ l of 0.154 M-KCl plus 1 mM-EDTA (pH 7.0), 0.05 mg of NADP⁺, 0.5 mg of glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase and 0.05 μ Ci of [³H] α -ecdysone.

Each incubation was carried out with moderate agitation at 25°C for 1 h, after which the mixture was extracted three times with butan-1-ol, the combined butanol extracts evaporated to dryness, separated by t.l.c. and the developed plates were scanned for radioactivity. The percentages of recovered radioactivity associated with α -ecdysone and its metabolites were again determined. Each subcellular fraction was also assayed for protein by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The subcellular composition of the various preparations was ascertained by the assay of each fraction for the marker enzymes succinate dehydrogenase

(EC 1.3.99.1) and cytochrome *c* reductase (EC 1.6.99.3).

Assay of succinate dehydrogenase

This enzyme assay was performed as a test for the presence of mitochondria. A slight modification of the method of Zeijlemaker *et al.* (1969) was developed for use with insect preparations. As there was a possibility that the insect mitochondria were not permeable to succinate (Sacktor, 1974), pyruvate and proline were substituted for succinate in the modified assay mixture, which contained in a total volume of 3 ml: 120 μ mol of potassium phosphate buffer (pH 7.5), 0.5 μ mol of KCN, 10 μ mol of potassium ferricyanide, 60 μ mol of pyruvate, 60 μ mol of proline and the subcellular fraction (max. vol. 0.1 ml). The reaction was initiated by the addition of the subcellular fraction, which had been preincubated for 5 min at 30°C. The reduction of ferricyanide was followed at 420 nm at room temperature (21°C) against a reagent blank. The activity of the enzyme was calculated by using a $\Delta\epsilon_{\text{mM}}$ (oxidized minus reduced) of 1.03 (Zeijlemaker *et al.*, 1969).

*Assay of cytochrome *c*-NADPH reductase*

This enzyme assay was used to detect the presence of a microsomal fraction in the subcellular preparations. The assay mixture contained, in a final volume of 3 ml: 75 μ mol of potassium phosphate buffer (pH 7.5), 0.2 μ mol of cytochrome *c*, 0.75 μ mol of NADPH and the subcellular fraction (max. vol. 0.1 ml). The reaction was initiated by the addition of the subcellular fraction and the reduction of cytochrome *c* was followed at 550 nm (Haas, 1955). The activity of the enzyme was calculated by using $\Delta\epsilon_{\text{mM}}$ (reduced minus oxidized) of 27.7 (Haas, 1955).

Assay of α -ecdysone C-20 hydroxylation

The standard assay mixture contained in a total volume of 0.5 ml: 77 μ mol of KCl, 0.5 μ mol of EDTA, 0.1 μ mol of NADPH, 0.025 μ mol (0.1–0.5 μ Ci) of [³H] α -ecdysone and the Malpighian-tubule mitochondrial preparation (approx. 0.1 mg of protein). The assay mixture was adjusted to pH 6.5 before the addition of the mitochondrial preparation. The mitochondrial preparation, KCl, EDTA and NADPH (total vol. 0.45 ml) were preincubated at 35°C for 5 min and the reaction was started by the addition, from a graduated syringe, of the substrate (0.05 ml) dissolved in 0.154 M-KCl/1 mM-EDTA (pH 6.5). Incubation was performed aerobically at 35°C for 20 min. The reaction rate was linear with time over a period of 35 min.

The assay was terminated by the addition of 2 ml of methanol with immediate mixing. After a period of 15 min, precipitated protein was removed by centrifugation at 2500g for 5 min and re-extracted with 1 ml of methanol. Carrier β -ecdysone (10 μ g) was added

to the combined methanol extract, which was then evaporated to dryness under a stream of N_2 . The extract was fractionated by t.l.c., and the β -ecdysone band, as well as the remaining area of the plate, were carefully removed and eluted. Radioactivity associated with β -ecdysone band was expressed as a percentage of the total radioactivity recovered from the whole plate. By this procedure the total recovery of radioactivity was approx. 80%.

Apparent conversions of approx. 0.5% were consistently obtained with both boiled-enzyme and no-enzyme control incubations. A no-enzyme control incubation was performed in each experiment, and the apparent percentage conversion obtained was subtracted from percentage conversions (usually in the range 4–5%) found in the experiment. By using these corrected percentage conversions in conjunction with duplicate protein assays, the enzyme specific activity was determined and expressed as μmol of α -ecdysone converted into β -ecdysone/h per mg of protein.

In initial assays for α -ecdysone C-20-hydroxylase activity in mitochondria, the assay product (t.l.c. band containing β -ecdysone) was subjected to high-performance liquid chromatography on a reverse-phase column of Bondapak C_{18} /Corasil with collection of fractions every 2 min for radioassay. Significant radioactivity was only associated with β -ecdysone.

When it was necessary to carry out anaerobic incubations, the water used to prepare the assay mixture was de-gassed under vacuum and the assay mixture was sealed under an atmosphere of N_2 by using Suba-Seal caps.

Effect of inhibitors on α -ecdysone C-20-hydroxylation

The effect of CO on the conversion of α -ecdysone into β -ecdysone was investigated by carrying out enzyme incubations in CO/O_2 (9:1). Parallel control incubations were performed by substituting N_2 for CO in the gaseous mixture. The gaseous mixture was prepared by passing CO (or N_2) and O_2 from gas cylinders, through flow-meters connected in parallel into a glass chamber for mixing. The mixed gas was then bubbled through the assay mixture for 30s and the incubation mixture was sealed. After a preincubation period of 5 min the reaction was started by the introduction of the radioactive substrate.

The inhibition of α -ecdysone C-20-hydroxylation by CO was reversed by irradiating assay mixtures with monochromatic light at 450 nm by using a DGO Metrospec monochromator (100 W). Assay mixtures were placed in specially designed optical cells of path length less than 1 mm after the CO/O_2 had been applied as described above. The optical cell was placed in the beam of light (450 nm) produced by the monochromator. The effect of irradiating assay mixtures to which N_2/O_2 had been applied was also observed as a control.

The effects of metyrapone and KCN on the hydroxylation reaction were investigated by adding the inhibitors in various concentrations (0–10 mM) to standard enzyme assay mixtures, which were then preincubated for 5 min before the addition of the radioactive substrate.

CO difference spectrum of the mitochondrial preparation

The mitochondrial (10000g) pellet from differential centrifugation was prepared from ten locusts as described above and resuspended in 2 ml of 0.1 M-potassium phosphate buffer (pH 7.0) by using a loose-fitting homogenizer. A sample was removed for protein assay and the remaining mitochondrial preparation was reduced with dithionite. The mixture was divided into two portions; CO was gently bubbled through one sample for 30s and the CO difference spectrum was recorded between 400 nm and 500 nm on a Unicam SP.1800 spectrophotometer, with the other sample as reference. The cytochrome *P*-450 concentration in the mitochondrial preparation was calculated from the CO difference spectrum by using a value of $91 \text{ cm}^{-1} \cdot \text{mm}^{-1}$ for the increment of molar absorption between 450 nm and 490 nm (Omura & Sato, 1964).

Results and Discussion

The basic properties of the α -ecdysone C-20-hydroxylation system were investigated.

Tissue distribution

Of the tissues investigated, under the conditions used, conversion of α -ecdysone into β -ecdysone was only apparent in the fat-body and the Malpighian tubules (Table 1). Significant hydroxylation of α -ecdysone to β -ecdysone was not observed in incubations of gut and body-wall tissue slices. These results are essentially in agreement with a report on the tissue distribution of α -ecdysone hydroxylase activity in the migratory locust, *Locusta migratoria* (Feyereissen *et al.*, 1976b).

All the tissues investigated in the present work converted α -ecdysone into an unidentified less-polar compound of R_F 0.57 (α -ecdysone, R_F 0.43) on t.l.c. In contrast with the situation in locusts, conversion of α -ecdysone into β -ecdysone has been demonstrated in Malpighian tubule, fat-body, gut and body-wall tissues from the tobacco hornworm, *Manduca sexta* (King, 1972). These apparent differences are probably based on the wide taxonomic distinction between *M. sexta* and locusts. In the present work with *S. gregaria*, Malpighian tubules were used in all subsequent studies, as the activity of the α -ecdysone C-20-hydroxylase per unit weight of tissue was much higher in that organ than in fat-body, and also because the

Table 1. *Metabolism of [3H] α -ecdysone in various tissues and in subcellular fractions of Malpighian tubules from *S. gregaria**
For details see the text. —, Activity not detectable.

Tissue	Weight (mg)	Distribution of radioactivity among incubation products (%)			α -Ecdysone C-20-hydroxylase activity (pmol/h)
		α -Ecdysone	β -Ecdysone	Compound at R_f 0.57	
Gut	332	27	—	73	—
Body wall	352	46	—	54	—
Malpighian tubules	93	25	33	42	1.87
Fat-body	286	24	22	54	1.26

Subcellular fraction of Malpighian tubules*	Weight (mg)	α -Ecdysone	β -Ecdysone	Compound at R_f 0.57	α -Ecdysone C-20-hydroxylase activity (pmol/h per mg of protein)
Homogenate	0.108	36	13	51	20.7
500g pellet	0.210	—	69	31	>56.5
10000g pellet	0.051	—	100	—	≥337
214000g pellet	0.020	84	16	—	138
214000g supernatant	0.017	100	—	—	—

* Different amounts of each fraction were used for each assay.

Table 2. *Marker-enzyme activities of subcellular fractions of *S. gregaria* Malpighian tubules*
For details see the text. —, Activity not detectable.

Fraction	Protein	Enzyme activity (nmol/min per mg of protein)	
		Succinate dehydrogenase	Cytochrome <i>c</i> -NADPH reductase
Homogenate	0.108	50.0	1.57
500g pellet	0.210	20.5	1.00
10000g pellet	0.050	42.0	2.20
214000g pellet	0.020	—	4.50
214000g supernatant	0.017	—	0.59

former tissue was the easier to dissect and obtain uncontaminated with other material.

Subcellular location

Subcellular fractionation of a homogenate of Malpighian tubules from *S. gregaria* by differential centrifugation indicated that the hydroxylation of α -ecdysone to β -ecdysone occurred maximally in the 10000g pellet (Table 1). Marker-enzyme studies on the subcellular fractions indicated that this fraction consisted predominantly of mitochondria (Table 2). The α -ecdysone hydroxylase activity observed in the 500g pellet fraction probably reflects the presence of unbroken cells and/or mitochondria, as reflected by the activity of the mitochondrial enzyme, succinate dehydrogenase. Some hydroxylase activity was also observed in the 214000g pellet, although this is much lower than that in the mitochondrial fraction, especially as the latter activity is probably grossly under-

estimated owing to lack of substrate. The small hydroxylase activity in the 214000g pellet probably reflects contamination with mitochondria, but, surprisingly, no detectable succinate dehydrogenase (mitochondrial) activity was observed in this fraction. Although complete absence of α -ecdysone C-20-hydroxylase activity from the microsomal fraction cannot be ascertained, it is clear that the specific activity of the hydroxylase is much greater in the mitochondrial 10000g pellet than in the microsomal 214000g sediment. It is noteworthy that the unknown product (R_f 0.57) was only produced in the crude homogenate and in the 500g pellet; β -ecdysone was the only detectable metabolite formed from α -ecdysone in the mitochondrial fraction. A mitochondrial location of the α -ecdysone C-20-hydroxylase system has also been observed in mid-gut (Nigg *et al.*, 1976) and fat-body (L. I. Gilbert, personal communication) tissues from *M. sexta*. A conflicting report has implicated the microsomal fraction as the

site for C-20 hydroxylation of α -ecdysone (Lagueux *et al.*, 1976), although, in another study, α -ecdysone was not converted into β -ecdysone in insect microsomal fraction (Terriere & Yu, 1976).

Properties

The mitochondrial α -ecdysone C-20-hydroxylase system from Malpighian tubules of *S. gregaria* had optimal activity at pH 6.5 and 35°C. This optimal pH value is in agreement with the generally slightly acidic nature of insect haemolymph. The activity of the mitochondrial α -ecdysone-hydroxylation system was directly proportional to the enzyme concentration, demonstrating that no endogenous inhibitors or activators were present. The hydroxylation system exhibited Michaelis-Menten kinetics and had an apparent K_m with respect to α -ecdysone of 1.25 μ M and V_{max} of 0.15 μ mol/h per mg of protein. The maximum concentration of α -ecdysone in *S. gregaria* haemolymph, as estimated from the titre in whole fifth-instar locusts (Morgan & Poole, 1976), is approx. 25 nM. This appears to be low compared with the apparent K_m quoted above. However, it could reasonably be expected that the local concentration of substrate in the tissues performing this conversion would be much higher than this.

When NADPH was omitted from the standard assay mixture or incubations were carried out anaerobically, the α -ecdysone C-20-hydroxylase activity was considerably decreased (Table 3). This O_2 - and NADPH-dependency demonstrates that the enzyme system is a mixed-function oxygenase. The residual

Table 3. *Properties of α -ecdysone C-20-hydroxylase system in mitochondria from Malpighian tubules of S. gregaria*

Conditions	α -Ecdysone C-20-hydroxylase activity (nmol/h per mg of protein)
(a) O_2 and NADPH requirement	
Control (for details see the text)	22
No NADPH	5
No O_2	14
(b) Effect of CO	
Air (Control)	24
N_2/O_2 (9:1)	23
CO/O_2 (9:1)	12
(c) Reversibility by light of CO inhibition	
N_2/O_2 (9:1)	
Dark	9.8
Irradiated at 450 nm	9.9
CO/O_2 (9:1)	
Dark	5.6
Irradiated at 450 nm	9.5

Fig. 1. *Activity of the Malpighian-tubule α -ecdysone C-20-hydroxylase system during the development of fifth-instar S. gregaria*

Mitochondria from Malpighian tubules of *S. gregaria* taken at different stages of development during the fifth instar were incubated with [3H] α -ecdysone under standard conditions for assay of C-20-hydroxylase activity. For details, see the text. Each experimental point represents a single determination of hydroxylase activity in mitochondria from Malpighian tubules pooled from several insects.

hydroxylation activity in the anaerobic assay probably reflects inefficiency in obtaining completely anaerobic conditions.

Developmental variation

Determination of the α -ecdysone C-20-hydroxylase activity of mitochondrial preparations of Malpighian tubules prepared from *S. gregaria* of different developmental stages taken at daily intervals during the fifth larval instar gave the results shown in Fig. 1. Hydroxylase activity showed marked developmental changes, being low in the early stages of the instar but rising to a broad maximum on the fifth day, with a subsequent slow decrease in activity towards the end of the instar. This peak of activity slightly precedes the maximum titre of moulting hormone during the fifth instar of *S. gregaria* (Morgan & Poole, 1976). The variation in the rate of conversion *in vitro* of α -ecdysone into β -ecdysone by mitochondria from insects of differing age provides direct evidence that the α -ecdysone C-20-hydroxylase system is at least one of the factors involved in regulating the titre of the active form of the moulting hormone, β -ecdysone, and hence the development of the insect. It has also been demonstrated that the mitochondrial α -ecdysone hydroxylase system from mid-gut of *M. sexta* undergoes marked developmental changes in activity (Nigg *et al.*, 1976). The factor(s) controlling the changes in α -ecdysone C-20-hydroxylase activity are obscure at present.

The developmental changes in α -ecdysone C-20-hydroxylation probably explains to some extent the

Fig. 2. CO difference spectrum of a dithionite-reduced mitochondrial preparation from *S. gregaria*. For details, see the Experimental section. ----, Baseline; —, difference spectrum.

variation in activity of the mitochondrial preparations between experiments in the present study. It is difficult to obtain precisely synchronized experimental animals even when care is taken to achieve this aim.

Involvement of cytochrome P-450

The possible involvement of cytochrome P-450 in the conversion of α -ecdysone into β -ecdysone was investigated. Table 3 shows that when incubations were carried out in CO/O₂ (9:1) the conversion of α -ecdysone into β -ecdysone was decreased to 52% of the activity in the control incubation. This inhibition by CO was reversed by irradiating the enzyme assay mixture with monochromatic light at 450 nm (Table 3). The enzyme activities recorded in Table 3(c) are lower than those recorded in other parts of this study, as the use of the DGO Metrospec monochromator necessitated the performance of the enzyme assay at room temperature (21°C), which is considerably lower than that used in all other experiments (35°C).

Metrapone inhibited α -ecdysone C-20-hydroxylation when added to the assay mixture; 0.001 mM gave 20% inhibition, and at high concentrations (10 mM) the inhibition increased to 80%.

The inhibition of α -ecdysone C-20-hydroxylation by CO, the subsequent reversal of this inhibition by irradiation with light at 450 nm and the inhibition of the hydroxylation by metrapone have been clearly demonstrated. From these results it can be concluded that cytochrome P-450 is involved in the conversion of α -ecdysone into β -ecdysone. However, low concentrations of KCN (1 mM) were found to produce a 25% inhibition of α -ecdysone C-20-hydroxylation. As cyanide inhibition is thought to be indicative of hydroxylation not dependent on cytochrome P-450, the significance of this result is unclear.

The CO difference spectrum of the dithionite-reduced mitochondrial preparation from *S. gregaria* was recorded (Fig. 2). The peak at 450 nm demon-

strated the presence of cytochrome P-450 in the preparation. The minor peak at 430 nm is probably due to other haemoproteins and has also been observed in similar difference spectra from *Musca domestica* (housefly) (Morello *et al.*, 1971). From this difference spectrum, the concentration of cytochrome P-450 was estimated as 0.91 nmol/mg of protein, which is of the same order of magnitude as concentrations reported in houseflies (Morello *et al.*, 1971).

In conclusion, it is clear that, in common with many hydroxylations of steroid regulatory hormones in mammals, the hydroxylation of α -ecdysone to β -ecdysone in Malpighian tubules of *S. gregaria* is catalysed by a mitochondrial cytochrome P-450-dependent mono-oxygenase. The involvement of cytochrome P-450 in the C-20-hydroxylation of α -ecdysone in mitochondria from fat-body of *M. sexta* has also been demonstrated (L. I. Gilbert, personal communication).

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References

- Agosin, M. & Perry, A. S. (1974) in *The Physiology of Insecta*, 2nd edn. (Rockstein, M., ed.), vol. 5, pp. 537–596, Academic Press, New York.
- Bodenstein, D. (1946) *Biol. Bull.* **90**, 148–157.
- Bollenbacher, W. E., Vedeckis, W. V., Gilbert, L. I. & O'Connor, J. D. (1975) *Dev. Biol.*, **44**, 46–53.
- Burstein, S., Middleditch, B. S. & Gut, M. (1974) *Biochem. Biophys. Res. Commun.* **61**, 692–697.
- Chart, J. J. & Sheppard, H. J. (1959) *J. Med. Pharm. Chem.* **1**, 407–413.
- Chino, H., Sakurai, S., Ohtaki, T., Ikekawa, N., Miyazaki, H., Ishibashi, M. & Abuki, H. (1974) *Science* **183**, 529–530.
- Feyereissen, R., Joly, L. & Hoffmann, J. A. (1976a) *C. R. Hebd. Séances Acad. Sci. Ser. D* **282**, 901–904.
- Feyereissen, R., Lagueux, M. & Hoffmann, J. A. (1976b) *Gen. Comp. Endocrinol.* **29**, 319–327.
- Gaylor, J. L. & Mason, H. S. (1968) *J. Biol. Chem.* **243**, 4966–4972.
- Gaylor, J. L., Hsu, S.-T., Delwiche, C. V., Comai, K. & Seifried, H. S. (1973) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S. & Morrison, M., ed.), vol. 2, pp. 431–450, University Park Press, Baltimore.
- Gilbert, L. I. & King, D. S. (1973) in *The Physiology of Insecta*, 2nd edn. (Rockstein, M., ed.), vol. 1, pp. 249–370, Academic Press, New York.
- Greengard, P., Psychoyos, S., Tallan, H. H., Cooper, D. Y., Rosenthal, O. & Estabrook, R. W. (1967) *Arch. Biochem. Biophys.* **121**, 298–303.
- Haas, E. (1955) *Methods Enzymol.* **2**, 699–703.

- Hoffmann, J. A., Koolman, J., Karlson, P. & Joly, P. (1974) *Gen. Comp. Endocrinol.* **22**, 90-97
- Hoffmann, J. A., Koolman, J. & Beyler, C. (1975) *C. R. Hebd. Séances Acad. Sci. Ser. D* **280**, 733-736
- Johnson, P., Rees, H. H. & Goodwin, T. W. (1976) *Abstr. Int. Congr. Biochem.* 10th, 646
- King, D. S. (1972) *Gen. Comp. Endocrinol. Suppl.* **3**, 221-227
- King, D. S., Bollenbacher, W. E., Borst, D. W., Vedeckis, W. V., O'Connor, J. D., Ittycheriah, P. I. & Gilbert, L. I. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 793-796
- Koolman, J., Hoffmann, J. A. & Dreyer, M. (1975) *Experientia* **31**, 247-249
- Lagueux, M., Perron, J. M. & Hoffmann, J. A. (1976) *J. Insect Physiol.* **22**, 57-62
- Leibman, K. C. (1969) *Mol. Pharmacol.* **5**, 1-9
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Morello, A., Bleecker, W. & Agosin, M. (1971) *Biochem. J.* **124**, 199-205
- Morgan, E. D. & Poole, C. F. (1976) *J. Insect Physiol.* **22**, 885-889
- Moriyama, H., Nakanishi, K., King, D. S., Okauchi, T., Siddall, J. B. & Hafferl, W. (1970) *Gen. Comp. Endocrinol.* **15**, 80-87
- Nigg, H. N., Svoboda, J. A., Thompson, M. J., Dutky, S. R., Kaplanis, J. N. & Robbins, W. E. (1976) *Experientia* **32**, 438-439
- Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2379-2385
- Ray, R. W. (1967) *Biochem. Pharmacol.* **16**, 99-108
- Sacktor, B. (1974) in *The Physiology of Insecta*, 2nd edn. (Rockstein, M., ed.), vol. 4, pp. 271-353, Academic Press, New York
- Schleyer, H., Cooper, D. Y. & Rosenthal, O. (1973) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S. & Morrison, M., eds.), vol. 2, pp. 469-491, University Park Press, Baltimore
- Simpson, E. R. & Boyd, G. S. (1967) *Eur. J. Biochem.* **2**, 275-285
- Terriere, L. C. & Yu, S. J. (1976) *Insect Biochem.* **6**, 109-114
- Van den Bergh, S. (1967) *Methods Enzymol.* **10**, 117-122
- Wilson, L. D. & Harding, B. W. (1970) *Biochemistry* **9**, 1615-1621
- Young, N. L. (1976) *Insect Biochem.* **6**, 1-12
- Zeijlemaker, W. P., Dervartanian, D. V., Veeger, C. & Slater, E. C. (1969) *Biochim. Biophys. Acta* **178**, 213-224