Ecdysone 20-mono-oxygenase in the desert locust, *Schistocerca gregaria*

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1. The enzyme catalysing the hydroxylation of ecdysone to 20-hydroxyecdysone, ecdysone 20-mono-oxygenase (EC 1.14.99.22), was investigated in the Malpighian tubules of fifth-instar locusts, *Schistocerca gregaria*. 2. Enzyme activity was optimal at 35°C and pH 6.8–8.0. 3. Under these conditions the mono-oxygenase exhibited an apparent K_m for ecdysone of 7.1×10^{-7} M, a maximal specific activity of 1.1 nmol/h per mg of protein and was competitively inhibited by 20-hydroxyecdysone with an apparent K_i of 6.3×10^{-7} M. 4. Enzyme activity was decreased in the presence of Ca^{2+} , Mg^{2+} , EDTA and non-ionic detergents. 5. The Malpighian tubule ecdysone 20-mono-oxygenase was localized primarily in the subcellular fraction sedimenting at 7500g and, on the basis of marker enzyme profiles, was assigned mainly to the mitochondria. 6. NADPH was required for activity, although addition of NADH together with NADPH had a synergistic effect. 7. NADP⁺-dependent isocitrate dehydrogenase (EC 1.1.1.42) and an energy-dependent NAD(P) transhydrogenase (EC 1.6.1.1.) appeared to be the major sources of reducing equivalents, with the contribution from the 'malic enzyme' (EC 1.1.1.40) being less important. 8. The mono-oxygenase was characterized as a cytochrome *P*-450-containing mixed-function oxidase from the inhibition patterns with metyrapone, CO and cyanide; CO inhibition was reversible with monochromatic light at 450 nm. However, the ecdysone 20-mono-oxygenase shows much lower sensitivity to CO inhibition and to photodissociation of the CO-inhibited complex than do vertebrate cytochrome *P*-450-dependent hydroxylation systems. 9. The concentration of cytochrome *P*-450 in the Malpighian tubule mitochondria was 30 pmol/mg of protein. 10. The properties of the mono-oxygenase are discussed in relation to hydroxylation enzymes from other sources.

Moulting in insects is controlled by steroidal moulting hormones (ecdysteroids). In larvae of most species the prothoracic glands synthesize ecdysone, which is released into the haemolymph and is hydroxylated in certain peripheral tissues yielding the generally predominant 20-hydroxyecdysone. This hydroxylation reaction, catalysed by ecdysone 20-mono-oxygenase [EC 1.14.99.22; ecdysone, hydrogen donor: oxygen oxidoreductase (20-hydroxylating)], forms the hormonally more active 20-hydroxyecdysone, which is considered as the true moulting hormone in most species.

Hydroxylation at C-20 occurs in the Malpighian tubules and fat body in the locusts, *Schistocerca gregaria* (Johnson & Rees, 1977) and *Locusta migratoria* (Feyereisen *et al.*, 1976), and additionally in the body wall and gut in the tobacco hornworm, *Manduca sexta* (King, 1972). The specific activity of ecdysone 20-mono-oxygenase has been shown to undergo developmental variation during the final larval instar of *S. gregaria* (Johnson & Rees, 1977; D. R. Greenwood & H. H. Rees, unpublished work) and *L. migratoria* (Feyereisen & Durst, 1980a,b).

The aim of the present study was to characterize further ecdysone 20-mono-oxygenase from *S. gregaria* Malpighian tubules. A preliminary account of some of this work has appeared (Rees *et al.*, 1981).

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Experimental

Nomenclature

Trivial names are often used. The systematic names are: ecdysone, (22*R*)-2 β ,3 β ,14,22,25-pentahydroxy-5 β -cholest-7-en-6-one; 20-hydroxyecdysone, (22*R*)-2 β ,3 β ,14,20,22,25-hexahydroxy-5 β -cholest-7-en-6-one.

Chemicals

[23,24-³H₄]Ecdysone (2.5 Ci/mmol), a gift from Schering A.-G., Berlin, was purified by silica gel column chromatography and diluted with unlabelled material before use. Samples of ecdysone and 20-hydroxyecdysone were generously given by Dr. G. B. Russell, D.S.I.R., Palmerston North, New Zealand. Glucose-6-phosphate dehydrogenase from yeast, lactate dehydrogenase from beef muscle, alcohol dehydrogenase from yeast, dichlorophenolindophenol, glucose 6-phosphate, cytochrome *c* (type III from horse heart; oxidized), and ATP were purchased from Sigma. Nicotinamide dinucleotide cofactors were from Boehringer Corp. (London). Metyrapone was a gift from Ciba Laboratories, Horsham, Sussex, U.K.

Insects

Locusts were reared as described previously (Dinan & Rees, 1981), the 'day' and 'night' temperatures being 33°C and 27°C, respectively. Johnson & Rees (1977) showed that, in the fifth instar of 8 days duration, the time of maximum ecdysone 20-mono-oxygenase activity occurred at approx. day 5.5. Under the present rearing conditions the duration of the fifth instar was 8.5–9.5 days and maximal mono-oxygenase activity was detected at day 6–6.5 (D. R. Greenwood & H. H. Rees, unpublished work). *S. gregaria* larvae were synchronized ($\pm$ 6h) from the fourth/fifth instar moult and tissues were excised from larvae killed 6–6.5 days later.

Preparation of subcellular fractions

Malpighian tubules were excised from *S. gregaria* larvae, washed in insect Ringer solution (Bodenstein, 1946) and homogenized in 0.02M-potassium phosphate buffer (pH 7.5) containing 0.154M-KCl in a Potter-Elvehjem homogenizer. The homogenate (6 ml) was centrifuged at 600*g* for 5 min, the supernatant was centrifuged at 7500*g* for 10 min and the pellet was resuspended in buffer (3 ml) and recentrifuged. The combined 7500*g* supernatant fractions were centrifuged at 15000*g* for 10 min and the resulting supernatant was centrifuged at 109000*g* for 90 min and the pellet was resuspended in buffer. The foregoing operations were carried out at 0–4°C.

Ecdysone 20-mono-oxygenase activity was de-

termined in individual fractions and was compared with the profiles of succinate dehydrogenase [EC 1.3.99.1; assayed by the method of Singer (1974)] and NADPH-cytochrome *P*-450 (*c*) reductase (EC 1.6.2.4), marker enzymes for mitochondria and the 'microsomal fraction', respectively. Assay of NADPH-cytochrome *P*-450 (*c*) reductase was by the method of Masters *et al.* (1967) with the addition of cyanide to 1 μ M to prevent reoxidation of reduced cytochrome *c* (observed in mitochondrial fractions).

For routine preparation of Malpighian tubule mitochondrial fractions, the post-600*g* supernatant was centrifuged at 8300*g* for 10 min; the resulting pellet was washed in a small volume of buffer and centrifuged at 10000*g* for 10 min. The pellet was resuspended in incubation buffer (1–2 ml) such that Malpighian tubules from 15–50 locusts produced a mitochondrial fraction containing 2–7 mg of protein/ml. In experiments for the determination of sources of reducing equivalents, the mitochondrial pellet was washed twice before final resuspension.

Assay of ecdysone 20-mono-oxygenase

Initially the conditions described by Johnson & Rees (1977) were used to assay ecdysone 20-mono-oxygenase, except that the incubation medium was buffered at pH 6.5 with 0.02M-potassium phosphate. In later work, the standard assay contained the following in an incubation buffer (0.02M-potassium phosphate, pH 7.5, containing 0.154M-KCl), total volume 0.5 ml: [³H]ecdysone (0.08–2 μ M; 0.1–0.3 μ Ci), NADP⁺ (0.05 mg), glucose-6-phosphate (0.5 mg), glucose-6-phosphate dehydrogenase (1 i.u.) and subcellular fraction containing up to 0.5 mg of protein. After incubation at 35°C for 20 min, the reaction was stopped by addition of methanol (4 ml), carrier ecdysone and 20-hydroxyecdysone (20 μ g each) were added, and ecdysteroids were extracted and fractionated by t.l.c. (Johnson & Rees, 1977). The bands corresponding to the two ecdysteroids were either eluted well with chloroform/methanol (1:1, v/v), or scraped directly into scintillation vials, before radioassay. All hydroxylase assays were carried out in duplicate. The percentage conversion of ecdysone into 20-hydroxyecdysone was calculated after subtracting the apparent conversion (0.5–2.0% maximum) observed in boiled-enzyme assays. Since 20-hydroxyecdysone was the only metabolite detected, radioactivity associated with the rest of the chromatogram (typically 5% of that applied) was ignored. Bovine serum albumin was used as standard in protein assays (Lowry *et al.*, 1951).

Radiochemical methods were as described previously (Greenwood & Rees, 1982). The product of the hydroxylation reaction was characterized as

20-hydroxy[^3H]ecdysone by recrystallization, chromatography (t.l.c. and h.p.l.c.), and derivative formation (acetate and acetonide; cf. Galbraith & Horn, 1969).

Assay of NAD(P) $^+$ transhydrogenase (EC 1.6.1.1)

Assay of the reversible transhydrogenase reaction in the mitochondrial fraction was undertaken in both directions using substrate-regenerating systems to maintain the level of one of the components constant (Rydstrom, 1979).

Assay of NAD $^+$ -dependent isocitrate dehydrogenase (EC 1.1.1.41)

A continuous spectrophotometric assay was constructed containing KCN (1 mM), isocitrate (5 mM) and mitochondrial fraction in incubation buffer (total volume 0.9 ml). The sample and reference (no enzyme) cuvettes were incubated at 35°C for 5 min before recording the base line at 340 nm. The reaction was initiated by addition of NAD $^+$ (to 1 mM) and the reduction of this cofactor was monitored. Background reduction due to endogenous substrates was determined in a separate assay.

The addition of 1 mM-NADP $^+$ was substituted for that of 1 mM-NAD $^+$ for the assay of NADP $^+$ -dependent isocitrate dehydrogenase (EC 1.1.1.42).

Effect of CO on ecdysone C-20 hydroxylation

Varying ratios of CO in O $_2$ were prepared in gas burettes, maintaining the O $_2$ concentration at 4% by making up the balance of CO with N $_2$. The gas mixtures (water-saturated) were then bubbled into the incubation mixtures and substrate ([^3H]ecdysone) was added after a 5 min preincubation period.

Effect of light on CO-inhibited ecdysone 20-mono-oxygenase

An apparatus similar to that of Estabrook *et al.* (1963) was used to study the effect of monochromatic light at 450 nm on the activity of the mono-oxygenase inhibited by CO. In this case, the reaction was irradiated with monochromatic light (36 $\mu\text{einstein} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$) derived from a tungsten filament light source (750 W) via an interference filter (max. transmittance, 55% at 451 nm; 10 nm band width).

Measurement of cytochrome P-450

The cytochrome P-450 content of Malpighian tubule mitochondria was determined according to the method of Omura & Sato (1964).

Results and discussion

Basic properties of Malpighian tubule ecdysone 20-mono-oxygenase

The basic properties of the ecdysone 20-mono-oxygenase from *S. gregaria* Malpighian tubules were investigated. 20-Hydroxyecdysone was the exclusive metabolite detected following incubation (<3 h) of [^3H]ecdysone with Malpighian tubule tissue or homogenate. A non-polar metabolite previously observed (Johnson & Rees, 1977) in similar incubations was not detected.

Whereas either potassium phosphate or Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffers were equally effective in the incubation medium, Tris/HCl was inhibitory (see Fig. 2; D. R. Greenwood & H. H. Rees, unpublished work).

Subcellular location

In view of apparently conflicting reports that the Malpighian tubule ecdysone 20-mono-oxygenase from the locusts *S. gregaria* (Johnson & Rees, 1977) and *Locusta migratoria* (Feyereisen & Durst, 1978) are located primarily in the mitochondrial and 'microsomal' fractions, respectively, the subcellular location of the enzyme system in the former tissue was investigated further by differential centrifugation of a homogenate. The distribution of the mono-oxygenase and representative mitochondrial and 'microsomal' marker enzymes is shown in Fig. 1. A considerable proportion of the total activities of all three enzymes assayed was present in the cell debris/nuclear fraction (N), presumably owing to incomplete homogenization of the tissue. The majority of the total ecdysone 20-mono-oxygenase activity in the post-cell debris/nuclear fraction was present in the mitochondrial fraction, in agreement with the succinate dehydrogenase fractionation profile and quite distinct from the fractionation of 'microsomal' membranes as assayed by NADPH-cytochrome P-450 (c) reductase.

The relatively high recoveries for succinate dehydrogenase and NADPH-cytochrome P-450 (c) reductase suggest that some form of latency is occurring, whereby separation of subcellular fractions increases the activity of the enzymes relative to that present in the homogenate. The presence of NADPH-cytochrome P-450 (c) reductase in the cell sap fraction (S) is probably indicative of insufficient centrifugal force to sediment the entire 'microsomal' membrane population (Morello *et al.*, 1971).

From electron-microscopic examination (D. R. Greenwood & H. H. Rees, unpublished work), the intermediate fraction (7500–15000 g; L, Fig. 1) consisted almost entirely of double-membraned

Fig. 1. Subcellular fractionation of *S. gregaria* Malpighian tubule homogenate by differential centrifugation: comparison of ecdysone 20-mono-oxygenase activity with the profiles of mitochondrial (succinate dehydrogenase) and microsomal [NADPH-cytochrome P-450 (c) reductase] marker enzymes

The ordinate scale (relative specific activity) is obtained by dividing the enzyme activity in the fraction (expressed as a percentage of the total activity in all the fractions) by the percentage of protein in that fraction. The values in parentheses indicate the percentage recoveries of the summed enzyme activities in the fractions relative to that present in the initial homogenate. Fractions: N, cell debris/nuclear (600g pellet); M, mitochondrial (7500g pellet); L, intermediate (15000g pellet); P, microsomal (109000g pellet); S, cell sap (109000g supernatant).

vesicular structures which were tentatively identified as re-annealed mitochondrial fragments minus the electron-dense matrix. Some remnants of mitochondrial cristae were also apparent. The specific activity of the mono-oxygenase was comparatively high in this intermediate fraction, as would be expected for membrane-bound mitochondrial enzymes. However, succinate dehydrogenase, a recognized ubiquitous mitochondrial inner membrane enzyme, was not correspondingly enriched. In this respect it is relevant that populations of mitochondria differing in their succinate dehydrogenase, cytochrome *b* and α -glycerophosphate dehydrogenase activities, as well as in size, have been separated from rat liver by differential centrifugation and density gradient ultracentrifugation (see Wilson & Cascarano, 1972). Similar cases of mitochondrial heterogeneity have been reported in insect tissues (Tsuyama & Miura, 1979; Novak *et al.*, 1979). Although insect flight muscle mitochondria are impermeable to succinate (Sacktor, 1974), the relative lack of organelle integrity in our preparations, as revealed by electron microscopy, probably precludes this factor from significantly affecting the succinate dehydrogenase assay; an intact membrane is not essential for enzymic activity (Singer, 1974).

The results taken together indicate that the majority (85%) of the ecdysone 20-mono-oxygenase

activity in the post-cell debris fraction was sedimented along with the mitochondria at 15000g, whereas 75% of the microsomal marker enzyme activity remained in suspension. However, the occurrence of some mono-oxygenase activity in the 'microsomal' fraction cannot be precluded. This is in agreement with the study by Johnson & Rees (1977). Similar results have been reported for *M. sexta* midgut (Mayer *et al.*, 1978) and fat body (Bollenbacher *et al.*, 1977), indicating primarily a mitochondrial location for ecdysone 20-mono-oxygenase, although in fat body some activity was also apparently associated with the 'microsomal' fraction and in midgut appreciable activity was detected in this fraction (Kaplanis *et al.*, 1980). The mono-oxygenase from *L. migratoria* Malpighian tubules and fat body predominated in the 100000g fraction, although an appreciable amount was sedimented at 10000g (Feyereisen & Durst, 1978). In the current investigation there was little apparent microsomal contamination of the mitochondrial fraction as assayed by NADPH-cytochrome P-450 (c) reductase and by electron-microscopic examination.

All subsequent experiments with *S. gregaria* Malpighian tubule ecdysone 20-mono-oxygenase were performed on the subcellular fraction sedimenting at 8300g, hereafter referred to as the 'mitochondrial fraction'.

Table 1. *Effect of divalent metal ions and EDTA on ecdysone 20-mono-oxygenase activity from S. gregaria Malpighian tubules*
For details see the text. Values are means and range of two determinations.

Additions	Mono-oxygenase activity (nmol/h per mg of protein)	Decrease (%)
Control	0.498 ± 0.005	—
Mg ²⁺ (7 mM)	0.413 ± 0.009	17
Ca ²⁺ (7 mM)	0.373 ± 0.003	25
Mg ²⁺ + Ca ²⁺ (both 7 mM)	0.300 ± 0.005	39
EDTA (1 mM)	0.198	60

General properties

Table 1 shows the effect of Ca²⁺, Mg²⁺ and EDTA on ecdysone 20-mono-oxygenase activity. In this instance 0.02 M-Hepes was substituted for potassium phosphate buffer to alleviate salt precipitation. EDTA at 1 mM markedly inhibited the mono-oxygenase (60% inhibition) and was not included in the assay medium subsequent to this. In contrast, the activity of the microsomal mono-oxygenase from *L. migratoria* is greatly enhanced by EDTA (Feyereisen & Durst, 1978). Both divalent metal ions showed an apparent inhibitory effect at 7 mM. An analogous inhibition by Mg²⁺ of mitochondrial ecdysone 20-mono-oxygenase activity has been reported for both *M. sexta* fat body (Smith *et al.*, 1979) and midgut (Mayer *et al.*, 1978). This is in contrast to the majority of mammalian mitochondrial steroid hydroxylases which are activated in the presence of these cations (Hayano & Dorfman, 1953; Björkhem & Gustafsson, 1974). An increase in NADPH-dependent mitochondrial steroid hydroxylation in the presence of Ca²⁺ has been explained by mitochondrial swelling (Peron & McCarthy, 1968), which presumably facilitates transfer of NADPH into the mitochondria. With the low degree of mitochondrial integrity in our preparations, the site of hydroxylation should be accessible to NADPH (see Harding *et al.*, 1968).

The ecdysone 20-mono-oxygenase activity was optimal at 35°C and showed an apparent broad pH optimum (Fig. 2) in the region pH 6.8–8.0, somewhat higher than observed previously (Johnson & Rees, 1977). Although the possibility always exists that the actual intramitochondrial pH could be different from that in the medium, in view of the comparatively low degree of mitochondrial integrity of the preparation, this may not be a major complication. Under these conditions mono-oxygenase activity was linear for up to 25 min and at a protein concentration of up to 0.9 mg/ml.

The possibility of enhancing ecdysone 20-mono-oxygenase activity by treatment of mitochondrial preparations with detergent (cf. Takikawa *et al.*, 1978) or by sonication was explored. However, the

Fig. 2. *Effect of pH on ecdysone 20-mono-oxygenase activity in mitochondria from S. gregaria Malpighian tubules*

Constant ionic strength ($I = 0.2$) potassium phosphate and Tris/HCl buffers were used over the pH range 6.0–8.5. Values are means and range of two or three estimations.

non-ionic detergents, Tween 20, Tween 80 and Triton X-100 all strongly inhibited enzyme activity over the concentration range tested (0.4–2%, v/v). Similarly, sonication resulted in loss of activity (presumably by disruption of the structural integrity of the enzyme system), except for very short time intervals (<5s).

The mono-oxygenase exhibited Michaelis–Menten kinetics and had an apparent K_m with respect to ecdysone of 7.1×10^{-7} M and a maximal specific activity of 1.1 nmol/h per mg of protein. The apparent K_m value was appreciably lower than that obtained by Johnson & Rees (1977) under different conditions (1.25×10^{-5} M), but is of the same order of magnitude as those reported for the mono-oxygenases from *M. sexta* fat body mitochondria (1.6×10^{-7} M; Smith *et al.*, 1979) and *L. migratoria* microsomes (2.7×10^{-7} M; Feyereisen & Durst,

1978). In contrast, Mayer *et al.* (1978) reported a K_m of 1.83×10^{-5} M for the *M. sexta* midgut mono-oxygenase. It is always possible that differences in apparent K_m may reflect not only affinity of the enzyme for substrate, but also to some extent, differences in assay conditions. A maximal specific activity of 15 nmol/h per mg of protein recorded by Johnson & Rees (1977) is appreciably higher than that obtained in the present study; the reason for this is obscure. The apparent K_m values are similar to the maximum titre of ecdysone in haemolymph of fifth-instar *S. gregaria* (2.1×10^{-7} M; Gande *et al.*, 1979).

S. gregaria ecdysone 20-mono-oxygenase was competitively inhibited by the product of the reaction, 20-hydroxyecdysone (apparent K_i 6.3×10^{-7} M), in agreement with the mono-oxygenase from *M. sexta* fat body (2.7×10^{-5} M; Smith *et al.*, 1979) and *L. migratoria* (7.5×10^{-7} M; Feyereisen & Durst, 1978). Since this apparent K_i is of the same order of magnitude as the 20-hydroxyecdysone concentration detected in haemolymph during peak titre in fifth instar *S. gregaria* (9.0×10^{-7} M; Gande *et al.*, 1979), product feedback inhibition may be physiologically important in regulating ecdysone 20-mono-oxygenase activity and hence modulating the ratio of ecdysone to 20-hydroxyecdysone in this species. The possibility always exists that the apparent kinetic properties (K_m and K_i) of the mono-oxygenase, a particulate enzyme, may have been influenced by limitations in the transport of the added ecdysteroid.

Potential sources of reducing equivalents

NADPH can serve as a source of reducing equivalents for the ecdysone 20-mono-oxygenase

from *S. gregaria* Malpighian tubules (Johnson & Rees, 1977). The nicotinamide nucleotide cofactor requirement for this reaction has now been investigated further with a washed mitochondrial preparation (Table 2). In the absence of cofactors or on the addition of NADP⁺, no hydroxylase activity was observed, indicating that no endogenous substrates were present for NADPH generation. NADPH restored mono-oxygenase activity to the level of the control (NADPH-generating system). Substitution of NADPH with NADH was only minimally effective in supporting mono-oxygenase activity, but the presence of NADPH and NADH together resulted in activity even above (143%) the value for NADPH addition (111%); an analogous situation has been reported for the *Locusta* microsomal enzyme (Feyereisen & Durst, 1978). This is probably accounted for either by regeneration of NADPH via a proposed NADH/NADP⁺-linked transhydrogenase and/or by NADH preventing reoxidation of NADPH by competitive binding to scavenging oxidases, which were detected in the mitochondrial preparations (D. R. Greenwood & H. H. Rees, unpublished work). The presence of 13% relative mono-oxygenase activity after the addition of NADH is probably due to a transhydrogenase (see below) passing reducing equivalents from NADH to an endogenous pool of NADP⁺.

Mitochondrial steroid hydroxylases are localized on the mitochondrial inner membrane (Mitani *et al.*, 1982) and several possible routes for the production of intramitochondrial NADPH for steroid hydroxylation exist (Rydstrom *et al.*, 1976). Oxidation of isocitrate by NADP⁺-specific isocitrate dehydrogenase [*threo*-D₅-isocitrate:NADP⁺

Table 2. Possible sources of reducing equivalents for ecdysone 20-mono-oxygenase from Malpighian tubules of *S. gregaria*. For details see the text. NADP⁺, NADH, NADPH and CN⁻ were added at 1 mM; glucose 6-phosphate, 1 mg/ml; glucose-6-phosphate dehydrogenase, 1 i.u.; ATP, 2 mM; tricarboxylic acid cycle intermediates, 5 mM. Values are means and range of two determinations.

Additions		Relative mono-oxygenase activity
Control (NADP ⁺ , glucose 6-phosphate, glucose-6-phosphate dehydrogenase)		100 ± 8
(a)	No cofactors	0
	NADP ⁺	0
	NADPH	111 ± 18
	NADH	13 ± 7
	NADPH + NADH	143 ± 8
(b)	NADP ⁺ + NADH	38 ± 0.4
	NADP ⁺ + NADH + ATP	69 ± 3
	NADP ⁺ + NADH + CN ⁻	44 ± 0.5
(c)	NADP ⁺ + isocitrate	14 ± 1.7
	NADP ⁺ + succinate	0
	NADP ⁺ + malate	1.6 ± 0.3

oxidoreductase (decarboxylating); EC 1.1.1.42] would yield NADPH directly, as would the oxidation of malate to pyruvate via the mitochondrial 'malic' enzyme [L-malate:NADP⁺ oxidoreductase (oxaloacetate decarboxylating); EC 1.1.1.40]. On the other hand, NADH could be produced from tricarboxylic acid cycle intermediates, including 2-oxoglutarate, malate and isocitrate, as well as succinate and substrates of other flavin-linked dehydrogenases via energy-linked reversed electron flow through the respiratory chain. Transfer of reducing equivalents from NADH to NADP⁺ could then occur via an energy-linked NAD(P)⁺ transhydrogenase (NADPH:NAD⁺ oxidoreductase; EC 1.6.1.1). The relative contribution to the mitochondrial NADPH pool of each of these reactions was investigated by using the mono-oxygenase assay *in vitro* as a monitor for NADPH production.

The existence of an NAD(P)⁺ transhydrogenase within the mitochondrial fraction was suggested on incubation with NADP⁺ and NADH (Table 2b); inhibition of reoxidation of NADH by addition of cyanide had minimal effect. Addition of ATP caused a doubling of mono-oxygenase activity suggesting the operation of an energy-linked process.

Of the three tricarboxylic acid cycle intermediates, isocitrate, succinate and malate, only isocitrate sustained mono-oxygenase activity to any significant extent in the presence of NADP⁺

(Table 2c). Due to the comparatively low degree of integrity of the mitochondrial preparations, membrane permeability of these acids should not be a major problem. However, the low rates of NADPH production could reflect loss of mitochondrial matrix enzymes and endogenous cofactors.

Nicotinamide nucleotide transhydrogenases are integral proteins of the mitochondrial inner membrane (Rydstrom *et al.*, 1976) and have been reported in insect gut mitochondria (Horie & Chefurka, 1966; Mayer *et al.*, 1978). The results shown in Table 3a confirm the presence of an active NAD(P)⁺ transhydrogenase in Malpighian tubules. The reduction of NAD⁺ was favoured over the reduction of NADP⁺ by some 4–6-fold. In a separate experiment, ATP increased the detectable transhydrogenase activity producing NADPH by over 4-fold (Table 3b), suggesting that the energetic status of the mitochondrion may control the flux of reducing equivalents between the two nicotinamide nucleotide cofactors.

The effects of isocitrate and malate were examined in greater detail in an attempt to distinguish between mitochondrial NADP⁺- and NAD⁺-linked activities (Table 4). In this case, the 100% control figure (Expt. 1) gives the basal level of mono-oxygenase activity with NADPH being generated by transhydrogenase activity and NADH oxidation by the respiratory chain being minimized by the addition of cyanide. Isocitrate

Table 3. Assay for NAD(P)⁺ transhydrogenase in mitochondrial fractions of *S. gregaria* Malpighian tubules
Components (i) were prepared in 0.02M-potassium phosphate buffer, pH 7.5, containing 0.154M-KCl (total volume 3ml) and incubated at 30°C prior to addition of component (ii) to start the reaction. The A₃₄₀ was monitored. (a) Comparison of the reaction rates in both directions; (b) effect of ATP on the generation of NADPH by the transhydrogenase.

Reaction ... NADH + NADP ⁺ → NAD ⁺ + NADPH		NADPH + NAD ⁺ → NADP ⁺ + NADH	
(NADH regenerated by alcohol dehydrogenase)		(NADPH regenerated by glucose-6-phosphatase dehydrogenase)	(NAD ⁺ regenerated by lactate dehydrogenase)
Composition of incubations			
(i) KCN (6 μmol)		KCN (6 μmol)	KCN (6 μmol)
Ethanol (390 μmol)		Glucose 6-phosphate (15 μmol)	Pyruvate (10 μmol)
Hydrazine (18 μmol)		Glucose-6-phosphate dehydrogenase (1 i.u.)	Lactate dehydrogenase (0.2 i.u.)
Alcohol dehydrogenase (1.2 i.u.)		NADPH (0.6 μmol)	NADPH (0.6 μmol)
NADP ⁺ (1.5 μmol)			
(ii) NADH (0.15 μmol)		Mitochondrial preparation (0.2ml; 0.63mg of protein)	
Transhydrogenase activity (nmol/min per mg of protein)		NAD ⁺ (1.5 μmol)	NAD ⁺ (1.5 μmol)
(a)	2.0	11.1	7.8
(b) - ATP	0.7		
+ ATP (15 μmol)	3.0		

Table 4. *Involvement of isocitrate and malate in the supply of NADPH for ecdysone 20-mono-oxygenase activity*
For details see the text. NADP⁺, NAD⁺, NADH, pyruvate and CN⁻ were at 1 mM (except where stated); isocitrate and malate were at 5 mM.

Expt.	Additions	Relative mono-oxygenase activity
1	Control: NADP ⁺ + NADH + CN ⁻	100
2	Isocitrate + CN ⁻	4.7
3	Isocitrate + NADP ⁺ + CN ⁻	45
4	Isocitrate + NADP ⁺ (0.1 mM) + CN ⁻	77
5	Isocitrate + NAD ⁺ + CN ⁻	12
6	Isocitrate + NADP ⁺ + NAD ⁺ + CN ⁻	47
7	Malate + NADP ⁺	3.7
8	Malate + NADP ⁺ + NAD ⁺ + CN ⁻	12
9	Malate + NADP ⁺ + NAD ⁺ + pyruvate + CN ⁻	11

supported a low level of mono-oxygenase activity (Expt. 2), indicating the presence of some endogenous cofactors. This activity was markedly increased when NADP⁺ was also added (Expt. 3), indicating the existence of an active mitochondrial NADP⁺-dependent isocitrate dehydrogenase (EC 1.1.1.42). When the NADP⁺ concentration was decreased 10-fold (Expt. 4), a further increase in mono-oxygenase activity occurred, suggesting some form of inhibition by NADP⁺ within the system. The basal level of mono-oxygenase activity with isocitrate was marginally increased on addition of NAD⁺ (Expt. 5), suggesting the presence of some NAD⁺-linked isocitrate dehydrogenase (EC 1.1.1.41) activity in the preparation. Addition of NAD⁺ to NADP⁺ plus isocitrate (Expt. 6) did not enhance mono-oxygenase activity significantly, suggesting that the NAD⁺-linked isocitrate dehydrogenase is not as effective as the NADP⁺-linked enzyme for providing reducing equivalents for the hydroxylation. Addition of malate and NADP⁺ (Expt. 7) resulted in very low mono-oxygenase activity, suggesting that the mitochondrial matrix 'malic' enzyme [NADP⁺: malate dehydrogenase (oxaloacetate decarboxylating), EC 1.1.1.40] was not present to any significant extent in the mitochondrial preparations. Addition of NAD⁺ and cyanide (Expt. 8) increased the level of mono-oxygenase activity 3-fold. Since the effect of cyanide cannot alone account for this increase (see Table 2), the presence of the tricarboxylic acid cycle enzyme, malate dehydrogenase (L-malate: NAD⁺ oxidoreductase, EC 1.1.1.37) is indicated. The activity of this enzyme was not increased on addition of pyruvate (Expt. 9). However, both these enzyme activities were low in comparison with NADP⁺-dependent isocitrate dehydrogenase.

To ascertain the relative activities of the two isocitrate dehydrogenases in Malpighian tubule mitochondria, each enzyme was assayed directly

by measuring reduction of the respective cofactor. However, the presence of transhydrogenase plus endogenous cofactors could cause complications. Apparent NADP⁺-dependent activity (3.0 nmol/min per mg of protein) was 6-fold greater than apparent NAD⁺-dependent activity (0.49 nmol/min per mg of protein), similar to the relative proportion of these two enzyme activities in liver mitochondria (Smith & Plaut, 1979). Some loss of these isocitrate dehydrogenases, being mitochondrial matrix enzymes (Goebell & Klingenberg, 1963), may have occurred during isolation of the mitochondrial preparation.

The comparatively low degree of integrity of the mitochondrial preparations, although permitting direct access of nicotinamide nucleotide cofactors and tricarboxylic acid cycle intermediates, precluded the accurate comparison of individual reaction rates, since loss of mitochondrial matrix contents was apparent from electron-microscopic examination. In addition, the NADPH generating systems were assayed under conditions optimal for ecdysone 20-mono-oxygenase activity *in vitro*. Owing to these considerations, and the probable operation of stringent regulatory mechanisms, the relative quantitative importance of the various sources of NADPH *in vivo* must remain an open question.

Involvement of cytochrome P-450

It has been shown that ecdysone 20-mono-oxygenase from *S. gregaria* Malpighian tubules is cytochrome P-450-dependent (Johnson & Rees, 1977); further investigation of the involvement of cytochrome P-450 is now reported.

The steroid hydroxylase inhibitor, metyrapone, was completely inhibitory above 10⁻⁵ M (Fig. 3), the *I*₅₀ (concentration of inhibitor to decrease enzyme activity by 50%) being 10⁻⁶ M. This result is in accord with that for the mono-oxygenase from *M. sexta* fat body, where metyrapone exhibited

Fig. 3. Effect of metyrapone on ecdysone 20-mono-oxygenase activity

For details see the text. Values are means and range of two estimations.

Fig. 4. Effect of various ratios of CO/O₂ on ecdysone 20-mono-oxygenase activity

For details see the text. Values are means and range of two estimations.

apparent competitive inhibition (I_{50} 5.5×10^{-6} M; Bollenbacher *et al.*, 1977; Smith *et al.*, 1979) and that for the enzyme from *L. migratoria*, where an I_{50} of 4×10^{-6} M was recorded (Feyereisen & Durst, 1978). No inhibition of the *S. gregaria* mono-oxygenase by cyanide over the concentration range 10^{-7} – 10^{-2} M was detected, in agreement with observations made on the *L. migratoria* microsomal hydroxylase by Feyereisen & Durst (1978).

Inhibition by 50% (I_{50}) of the *S. gregaria* ecdysone 20-mono-oxygenase by CO was achieved at a CO/O₂ ratio of 8:1 (Fig. 4), identical with that for the *M. sexta* fat body mitochondrial enzyme (Smith *et al.*, 1979), but is considerably lower than that for the *L. migratoria* microsomal enzyme (I_{50} 1:1 CO/O₂; Feyereisen & Durst, 1978) and for the majority of vertebrate steroid hydroxylase systems (Rosenthal & Cooper, 1967).

Warburg's partition constant $K\{K = [\text{CO}]/[\text{O}_2] \times n/(1-n)\}$, where n is the ratio of the reaction rate in the presence of CO to that in the absence of CO (Rosenthal & Cooper, 1967) gave an average value of 11 ± 4 (S.D.) for the ecdysone 20-mono-oxygenase at different gas ratios. K values for mixed function oxidases are generally considered to be in the range of 0.5–2.0 (Cooper *et al.*, 1965), well separated from the value obtained in the current study. The reason for this is obscure, but a similar value was obtained for the fat body mitochondrial ecdysone 20-mono-oxygenase from *M. sexta* ($K = 14.4$; Smith *et al.*, 1979). In contrast, the

microsomal hydroxylase from *L. migratoria* had a partition constant of 1.5 (Feyereisen & Durst, 1978). Reports have appeared quoting higher than expected partition constants for hydroxylase systems (see Wilson & Harding, 1970; Cooper *et al.*, 1977), and it appears that several factors may be responsible. The higher K values for the *M. sexta* fat body and *S. gregaria* enzymes suggest that these hydroxylases are less sensitive to CO.

Inhibition by CO of ecdysone 20-mono-oxygenase activity could be relieved up to 40% by irradiation with light at 450 nm (Table 5). Partition constants for the 'dark' reaction ($K_d = 14.5$) and 'light' reaction ($K_l = 30.6$) were calculated and agree with the corresponding values for the *M. sexta* fat body enzyme ($K_d = 14.4$, $K_l = 31.3$; Smith *et al.*, 1979). From these values of K in the current study, the light sensitivity factor, L , can be calculated [$L = 1/i \times K/K_d = (K_l - K_d)/K_d \times 1/i$, where i is the light intensity; $i = 36 \mu\text{einstein} \cdot \text{s}^{-1} \cdot \text{cm}^{-2}$]. For the *S. gregaria* enzyme, L has a value of $5.1 \times 10^2 \text{ cm}^2 \cdot \text{min/mol}$ of quanta at 35°C, indicating a low response (light sensitivity) of the mono-oxygenase to photodissociation of the CO-inhibited complex. In contrast, values of L for vertebrate steroid hydroxylases are considerably higher [$(2-4) \times 10^6 \text{ cm}^2 \cdot \text{min/mol}$ of quanta; Estabrook *et al.*, 1963; Wilson & Harding, 1970].

The concentration of cytochrome P-450 in *S. gregaria* Malpighian tubule mitochondria was 30

Table 5. *Reversal of CO inhibition of ecdysone 20-mono-oxygenase by irradiation with monochromatic light at 450 nm*
For details see the text. Values of activity are means and range of two determinations.

Incubation atmosphere	Incubation	Mono-oxygenase activity (nmol/h per mg of protein)	Inhibition (%)	n	K
N ₂ /O ₂ (9:1)	Dark	0.14	—		
N ₂ /O ₂ (9:1)	Light	0.16 ± 0.02	—		
CO/O ₂ (9:1)	Dark	0.09 ± 0.01	40	0.62	14.5
CO/O ₂ (9:1)	Light	0.12 ± 0.01	25	0.77	30.6

(±1s.d.) pmol/mg of protein, similar to that reported for *M. sexta* midgut mitochondria (10–15 pmol/mg of protein; Mayer *et al.*, 1978) and *M. sexta* fat body mitochondria (10 pmol/mg of protein; Smith *et al.*, 1979). In all three cases, the largest peak in the CO difference spectrum was at 420–430 nm, which may be attributable to cytochrome P-420 and/or cytochrome oxidase. Johnson & Rees (1977), reported a value of 0.91 nmol of cytochrome P-450/mg of protein for mitochondria from abdomens of *S. gregaria*. This higher value approached the haem P-450 content of vertebrate adrenal cortex mitochondria (1.5 nmol/mg of protein; Cammer & Estabrook, 1967).

In conclusion, ecdysone 20-mono-oxygenase from *S. gregaria* Malpighian tubule mitochondria exhibits similar basic characteristics to vertebrate P-450-dependent hydroxylation systems, namely inhibition by CO, light-mediated reversal of inhibition by CO, inhibition by metyrapone and the absence of inhibition by cyanide. Nevertheless, low sensitivity to inhibition by CO and to photo-dissociation of the CO-inhibited complex are specific differences which have also been observed for the ecdysone 20-mono-oxygenase from *M. sexta* fat body (Smith *et al.*, 1979).

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