Induction of Enzymes Involved in Molting Hormone (Ecdysteroid) Inactivation by Ecdysteroids and an Agonist, 1,2-Dibenzoyl-1-tert-butylhydrazine (RH-5849)*

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Molting in insects is regulated by molting hormones (ecdysteroids). The major active hormone, 20-hydroxyecdysone, is formed by ecdysone 20-monoxygenase-catalyzed hydroxylation of ecdysone. During times of decreasing hormone titers, inactivation occurs by several routes including (i) 26-hydroxylation and further oxidation to the 26-oic acid, (ii) formation of various conjugates (e.g. phosphates), and (iii) in Lepidoptera in particular, ecdysone oxidase-catalyzed formation of 3-dehydroecdysteroid, which is reduced to 3-epiecdysteroid, followed by phosphotransferase-catalyzed formation of phosphate conjugates. Administration of the nonsteroidal ecdysteroid agonist RH-5849 (1,2-dibenzoyl-1-tert-butyldrazine), but not 20-hydroxyecdysone, to tobacco hornworm (Manduca sexta) resulted in induction of midgut cytosolic ecdysone oxidase and ecdysteroid phosphotransferase activities. In addition, both 20-hydroxyecdysone and RH-5849 caused induction of ecdysteroid 26-hydroxylase activity in midgut mitochondria and microsomes, whereas 20-hydroxylase was induced to a lesser extent by 20-hydroxyecdysone in mitochondria and by either RH-5849 or 20-hydroxyecdysone in microsomes. Commensurate with induction of the enzymes by ecdysteroid and RH-5849 is a requirement for RNA and protein synthesis, without precluding indirect mechanisms. These results indicate that molting hormone stimulates at least one universal route of its own inactivation by inducing ecdysteroid 26-hydroxylase activity and are discussed in relation to an analogous phenomenon observed for vitamin D inactivation in vertebrates.

Molting hormones (ecdysteroids) regulate molting in immature stages of insects (1). Conversion of ecdysone into the major active hormone 20-hydroxyecdysone occurs in certain peripheral tissues and is catalyzed by the cytochrome P450-dependent ecdysone 20-monoxygenase (2, 3). The activity of this enzyme undergoes developmental change, exhibiting a distinct peak during the final larval instar in several species (2, 4).

Inactivation of ecdysteroids occurs by various reactions including conversion of ecdysteroid via the 26-hydroxy derivative into the 26-oic acid, formation of various conjugates (e.g. phosphates), and conversion into 3-epi(3α-hydroxy)-ecdysteroids (5, 6). 3-Epiedysteroid formation is prominent in lepidopteran midgut cytosol and involves ecdysone oxidase-catalyzed formation of 3-dehydroecdysteroid followed by NAD(P)H-dependent irreversible reduction to 3-epiecdysteroid, which may also be phosphorylated. The 3-dehydroecdysteroid may also undergo NAD(P)H-dependent reduction back to 3β-hydroxyecdysteroid (for reviews, see Refs. 3 and 7). These enzymatic activities in the midgut cytosol (Fig. 1) also exhibit developmental changes (8, 9).

In the tobacco hornworm (Manduca sexta), the subject of this investigation, midgut ecdysone 20-monoxygenase is localized in both mitochondrial and microsomal fractions (10). During development of the fifth instar, the midgut 20-monoxygenase undergoes a 50-fold increase in activity, temporally coincident with the onset of wandering (11). This occurs within a day of the commitment peak in hemolymph ecdysteroid titer, which causes reprogramming of the larval tissues for pupal development (12). It has been reported that ecdysone, 20-hydroxyecdysone, and the nonsteroidal ecdysteroid agonist RH-5849 (13) induce ecdysone 20-monoxygenase activity in midgut homogenate from M. sexta that has been head-ligated to prevent the normal developmental increase in the 20-monoxygenase activity (14, 15).

We report here that RH-5849 causes pronounced induction of ecdysone oxidase and ecdysteroid phosphotransferase activities in M. sexta midgut cytosol. Furthermore, 20-hydroxyecdysone and RH-5849 cause much greater induction of ecdysone 26-hydroxylase than 20-monoxygenase in both mitochondrial and microsomal fractions. 3α-[3H]Ecdysone rather than 20-hydroxyecdysone has been used as substrate in this work, since it allows simultaneous investigation of ecdysone 20-monoxygenase and the ecdysteroid inactivation reactions, which are similar for both ecdysone and 20-hydroxyecdysone (5, 6).

EXPERIMENTAL PROCEDURES

Animals

M. sexta were reared on an artificial diet at 25.5 °C and 60% relative humidity under a 16-h light/8-h dark photoperiod (16). Last instar larvae were used in this study, the instar lasting 11 days under these conditions. Synchronous batches of gate II newly molted fifth instar larvae, which had undergone head capsule slippage during the dark period, were collected. The beginning of that dark period is designated as 0 h and the following day is day 0 (17); under our conditions of rearing, wandering occurred by the end of the dark period (152 h) on day 6.

Ecdysteroids and Agonist

20-Hydroxyecdysone was kindly provided by Dr. G. B. Russell (Department of Scientific and Industrial Research, Palmerston North, New Zealand). 26-Hydroxyecdysone and 20,26-dihydroxyecdysone were from...
**Induction of Ecdysoidal Inactivation**

Dr. M. Feldauer (U.S. Department of Agriculture, Agricultural Research Service, Beltsville, MD). Several ecdysteroid phosphates were previously isolated from locusts in this laboratory (5). 3-Epiecdysone 2-phosphate was synthesized from ecdysone 2-phosphate using ecdysone oxidase and 3-dehydroecdysone 3α-reductase enzymes purified from *Spodoptera littoralis* midgut (18). Epo-20-hydroxyecdysone 3-phosphate isolated from *Pieris brassicae* (19) was kindly provided by Prof. R. Lafont (Ecole Normale Superieure, Department de Biologie, Paris, France). RH-5849 was provided by Dr. G. R. Carlson (Rohn and Haas Co., Spring House, PA).

**Insect Ligation and Injection**

Head ligations were carried out 88 h into the fifth instar (day 3) between the head and prothoracic segments using waxed dental falx. At this stage, larvae weighed approximately 8–10 g. Injections were made via abdominal segments, and the injection sites were sealed with low melting point wax. For routine assay of enzymatic activities on day 5, RH-5849 and 20-hydroxyecdysone were administered to the larvae in methanol as triple injections (4–10 μl/injection) at 88 h (day 3; 5 μg/ml of larva), 112 h (day 4; 5 μg/ml of larva), and 129 h (day 5; 10 μg/ml of larva). Methanol-injected larvae served as controls. In the case of day-6 assays, all injections of ecdysteroid or agonist were given 24 h later for day-5 assays. Day-2 assays (61 h) of ecdysone oxidase were carried out following administration of RH-5849 as a double injection (4–10 μl/injection) at 40 h (day 1; 5 μg/ml of larva) and 57 h (day 2; 10 μg/ml of larva).

For each experimental regime, two or three independent experiments were carried out, with groups of five to eight insects being used for each treatment and enzyme assays carried out in duplicate for the pooled insects.

**Subcellular Fractions**

Four hours after the final injection, midguts of larvae were excised, cleaned, and homogenized using a Potter-Elvehjem homogenizer in 10% (v/v) methanol in water/injection/larva) was injected alone at 108 h and, subsequently, together with RH-5849 (5 μg/ml of larva) in methanol at 112 h and with RH-5849 (10 μg/ml of larva) at 129 h. Cytosol (25 μg in 0.5 μl of 50% (v/v) methanol in water/injection/g of larva) was administered under the same regime for cytosolic D. Cytosol oxidase or actomyosin D were co-injected with RH-5849 when injection times coincided, and the supernatant was used without further processing, whereas for assay of individual enzymes, the supernatant fraction was first dialyzed. Assays were performed essentially as described for the hydroxylation assays, except that different labeled substrates ([23,24-3H2]ecdysone, 0.12 μCi; [23,24-3H2]3-epiecdysone, 0.12 μCi; [23,24-3H2]ecdysone; one, 0.12 μCi; 0.42 Ci/mmol, both prepared from [3H]ecdysone using purified enzyme preparations from *S. littoralis* midgut) or cofactors (2 mM MgCl2, 10 μM NADPH, or 0.2 mM NADPH plus regenerating system as above) were used appropriate.

**Enzyme Assays**

**Ecdysone Oxidase Assay—**Dialyzed cytosol was incubated with [3H]ecdysone in the absence of cofactors to prevent reduction of 3-dehydroecdysone product.

**3-dehydroecdysone 3α-Reductase and 3β-Reductase Assays—**Dialyzed cytosol was incubated with [14C]3-dehydroecdysone in the presence of NADPH (incorporating a regenerating system) or NADH as appropriate. Assays were executed anaerobically under N2 to prevent any conversion of ecdysone product back to 3-dehydroecdysone by cytosole oxidase activity.

**Ecdysteroid Phosphotransferase Assay—**Dialyzed cytosol was incubated with [3H]3-epiecdysone in the presence of Mg2+-ATP. Enzymatic products were analyzed by HPLC.

**Protein Assay—**Protein was determined by the method of Bradford (21) using bovine serum albumin as standard.

**Enzymatic Hydrolysis of Ecdysteroid Conjugates**

Putative polar ecdysteroid conjugates were purified by reversed-phase HPLC and dissolved in 1 ml of 0.1 M MES buffer, pH 5.5. A crude hydrolyase preparation (250 units) from *Helix pomatia* (so-called "arylsulphatase, Sigma) was added, the mixture was incubated at 37 °C for 24 h, and the reaction was terminated by addition of methanol (4 ml). The protein precipitate was sedimented by centrifugation, and the ecdysteroids were extracted three times with methanol (4 ml); subsequently the extracts were combined and evaporated to dryness. The hydrolyzed ecdysteroids were applied to a Sep-Pak C18 cartridge (Waters Associates, Watford, Hertfordshire, United Kingdom) in 10% (v/v) methanol/water (2 ml); any unhydrolyzed polar ecdysteroids eluted with 25% (v/v) methanol (4 ml), and the free ecdysteroids released from conjugates eluted with 60% (v/v) methanol/water (6 ml).

**High-Performance Liquid Chromatographic Analyses**

HPLC analyses of free ecdysteroids were carried out on a Nova-Pak C18 cartridge (10 cm × 5 mm; particle size, 4 μm, Waters Associates) employing a linear gradient over 1 h of 35–60% (v/v) methanol/water at 1 ml/min (system 1) or isocratic elution with 22% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid in water at 1 ml/min (system 2). Radioactivity was detected with an on-line radioactivity monitor (Radiomatic). Adsorption HPLC was carried out using an octylpropyl silicone-Hypersil column (25 cm × 4.6 mm; particle size, 5 μm; Shandon Southern Products, Runcorn, Cheshire, United Kingdom) eluted isocratically with 8% (v/v) methanol in 1.2-dichloroethane at 2 ml/min (system 3a) or with 6% (v/v) methanol in 1.2-dichloroethane at 2 ml/min (system 3b). In this case, fractions collected at 1-min intervals were evaporated to dryness and assayed by liquid scintillation counting.
Enzymatic activities were determined in either day-5 or day-6 last instar larvae. Values are means ± range for two independent experiments. In each experiment, pools of five to eight insects were used for each treatment, and all assays were carried out in duplicate. Experiments i, ii, and iii represent routine doses of inducing compounds. The detailed regimes for injection of inducing compounds are given under “Experimental Procedures.” For experiment iv, inducing compounds were administered as double injections (112 and 129 h) and the amounts per g weight per injection were: 20.3 μg of ecdysone plus 1.2 μg of 20-hydroxyecdysone, 100 ng of ecdysone, or 50 ng of RH-5849.

### TABLE I

<table>
<thead>
<tr>
<th>Stage and inducing compound</th>
<th>Mitochondria</th>
<th>Microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ecdysteroid 26-hydroxylase</td>
<td>Ecdysone 20-monooxygenase</td>
</tr>
<tr>
<td>(i) Day 5 (ligated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (methanol)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>20-Hydroxyecdysone</td>
<td>31.1 ± 3.1</td>
<td>14.2 ± 3.0</td>
</tr>
<tr>
<td>RH-5849</td>
<td>37.0 ± 1.4</td>
<td>nd</td>
</tr>
<tr>
<td>(ii) Day 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (methanol)</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>20-Hydroxyecdysone</td>
<td>28.8 ± 0.4</td>
<td>7.7 ± 1.9</td>
</tr>
<tr>
<td>RH-5849</td>
<td>36.7 ± 1.8</td>
<td>nd</td>
</tr>
<tr>
<td>(iii) Day 5 (ligated)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecdysone plus 20-hydroxyecdysone</td>
<td>15.1 ± 0.4</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>(iv) Day 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecdysone</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>RH-5849</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

* nd, not detected (<0.5 pmol/min/mg protein).

Polar ecdysteroid conjugates were identified by reversed-phase HPLC on the same Nova-Pak C18 cartridge using two linear 30-min gradient elution systems at 1 ml/min: (i) 20–70% (v/v) methanol in 20 mM Tris-perchlorate buffer, pH 7.5 (system 5); and (ii) 8–40% (v/v) acetonitrile in 20 mM Tris-perchlorate buffer, pH 7.5 (system 5).

**RESULTS**

*Induction of Ecdysteroid 26-Hydroxylase—Under our conditions of rearing *M. sexta*, the natural activity (mean ± range for two independent experiments) of ecdysone 20-monooxygenase was low on day 5 (<0.5 pmol/min/mg protein) and high on day 6 (14.5 ± 1.8 pmol/min/mg protein, mitochondrial; 23.5 ± 1.4 pmol/min/mg protein, microsomal) of the fifth larval instar. Injection of RH-5849 or 20-hydroxyecdysone into final larval instar *M. sexta* preceding hydroxylase assay on day 5 (133 h) or day 6 (157 h) led to induction of both midgut mitochondrial and microsomal ecdysteroid 26-hydroxylase activity in normal and head-ligated larvae (Table I, i–iii). Ecdysteroid 26-hydroxylase activity was undetectable in methanol-injected controls (Table I). Lower induction of ecdysone 20-monooxygenase activity was observed in mitochondria and microsomes from day-5 larvae observed in mitochondria and microsomes from day-5 larvae treated with RH-5849 and day-6 larvae treated with RH-5849 and 20-hydroxyecdysone-treated larvae decreased compared with control activity, whereas ecdysteroid 26-hydroxylase activity remained at the levels seen in day-5 induced larvae (Table I, iii). This decrease in 20-monooxygenase activity may be due to physiological perturbation of the larvae (e.g. premature cessation of feeding) caused by the inducing agents. In the foregoing work, the reaction products were characterized by co-chromatography on HPLC with authentic samples on both reversed-phase (system 1; retention times (Rt): 20,26-dihydroxyecdysone, 11.0 min; 20-hydroxyecdysone, 17.7 min; 26-hydroxyecdysone, 19.7 min) and adsorption (system 3a; Rt: 20,26-dihydroxyecdysone, 5 min; 26-hydroxyecdysone, 10 min; 20,26-dihydroxyecdysone, 15 min) columns.

Both actinomycin D and cycloheximide, inhibitors of transcription and protein synthesis, respectively, gave nearly complete inhibition of the appearance of ecdysteroid 26-hydroxylase activity (Table II). The dose of cycloheximide was 8–10-fold that used for actinomycin D, since the same dose was ineffective. Preinjection of inhibitors 4 h before the first RH-5849 injection as well as subsequent co-injection with the agonist was vital for effective inhibition of enzymatic induction. Similarly, RH-5849 induction of ecdysone 20-monooxygenase activity in microsomes was completely abolished by both inhibitors (Table II).

Ecdysteroids were also administered at concentrations similar to those occurring in the hemolymph near the maximum titer of the hormone in the late fifth larval instars and during the commitment peak prior to wandering (11, 12) to further evaluate the physiological significance of induction of the 20- and 26-hydroxylases. The ecdysone/20-hydroxyecdysone hemolymph titers are approximately 0.5 μM (0.23 μg/g of larva) ecdysone and 2.5 μM (1.2 μg/g of larva) 20-hydroxyecdysone in the region of the peak titer (11, 12). Double administration of such concentrations of an ecdysone/20-hydroxyecdysone mixture led to marked induction of mitochondrial and microsomal 26-hydroxylase activity but less induction of the 20-monooxygenase (Table I, iv). During the small commitment peak of ecdysteroid occurring prior to wandering, there is a slight preponderance of 20-hydroxyecdysone compared with ecdysone, with a total ecdysteroid concentration in the region of 0.16 μM (approximately 75 ng/g of larva) (11, 12). Double administration of similar concentrations of ecdysone/20-hydroxyecdysone mixture led to marked induction of mitochondrial and microsomal 26-hydroxylase activity but less induction of the 20-monooxygenase (Table I, iv). The specificity of the mitochondrial and microsomal 26-hydroxylase for ecdysteroid substrate was demonstrated by co-incubation of the subcellular fractions with ecdysone and cholesterol or vitamin D3. For this, mitochondria or microsomes from nonligated, day-5, RH-5849-induced larvae that contained ecdysteroid 26-hydroxylase activity were incubated under the standard assay conditions with varying amounts of the competing sterol. At least 100 μM cholesterol or vitamin D3 was required to elicit any inhibitory effect, and at 1 μM, activities of mitochondrial ecdysteroid 26-hydroxylase and microsomal ecdysteroid 26-hydroxylase were 55–60 and 80% of control values, respectively (data not shown).
Induction of Ecdysteroid Inactivation

Indicators and RH-5849 were administered as described under “Experimental Procedures” and the enzymatic activities determined in mitochondrial, microsomal, or dialyzed cytosolic supernatant fractions from midgut of day-5 larvae. Values are means ± range for two independent experiments. In each experiment, pools of five to eight insects were used for each treatment, and all assays were carried out in duplicate.

Inhibitors and RH-5849 were administered as described under “Experimental Procedures” and the enzymatic activities determined in mitochondrial, microsomal, or dialyzed cytosolic supernatant fractions from midgut of day-5 larvae. Values are means ± range for two independent experiments. In each experiment, pools of five to eight insects were used for each treatment, and all assays were carried out in duplicate.

**TABLE II**

Effect of actinomycin D and cycloheximide on induction of enzymatic activities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzymatic activities</th>
<th>Mitochondria</th>
<th>Microsomes</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ecdysteroid 26-</td>
<td>Control</td>
<td>RH-5849</td>
<td>RH-5849 + actinomycin D</td>
</tr>
<tr>
<td></td>
<td>hydroxylase</td>
<td>Edcysone 26-hydroxylase</td>
<td>Edcysone 20-monooxygenase</td>
<td>3-Epiedcysone phosphotransferase</td>
</tr>
<tr>
<td>Control (methanol)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>RH-5849</td>
<td>34.5 ± 1.9</td>
<td>37.0 ± 2.1</td>
<td>9.8 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>RH-5849 + actinomycin D</td>
<td>1.3 ± 1.1</td>
<td>2.3 ± 0.3</td>
<td>3.3 ± 0.5</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>RH-5849 + cycloheximide</td>
<td>1.1 ± 1.1</td>
<td>2.9 ± 0.2</td>
<td>6.3 ± 0.5</td>
<td>6.0 ± 0.2</td>
</tr>
</tbody>
</table>

* nd, not detected (<0.5 pmol/min/mg protein).

RH-5849 has been shown to inhibit ecdysone 20-monoxygenase activity in vitro in *M. sexta* midgut homogenates in a dose-dependent manner (15). To investigate whether the concentration of RH-5849 in midgut cells was sufficiently high to induce ecdysone 20-monoxygenase in subcellular fractions after its intracellular induction, midgut mitochondria and microsomes from day-5, non-ligated, 20-hydroxyecdysone-induced larvae (which contained ecdysone 20-monoxygenase and ecdysoid 26-hydroxylase in both subcellular fractions) were incubated under standard assay conditions with increasing amounts of RH-5849. Inhibition of ecdysone 20-monoxygenase and ecdysteroid 26-hydroxylase began to occur at concentrations of 100–250 μM RH-5849 (Fig. 2).

**Induction of Cytosolic Enzymes Involved in Ecdysteroid 3-Epimerization and Phosphorylation**—In preliminary experiments, possible induction of the midgut cytosolic enzymes catalyzing conversion of ecdysone into 3-epiedcysone (ecdysone oxidase and 3-dehydroecdysone 3α-reductase) and into 3-epiedcysone phosphate (ecdysoid phosphotransferase) either by RH-5849 or 20-hydroxyecdysone was examined in undialyzed cytosol supplemented with either NADP or Mg2+-ATP. In these experiments, significant induction of 3-epiedcysone and 3-epiedcysone phosphate formation was only observed using RH-5849, with a negligible effect using 20-hydroxyecdysone even at a molar dose approximately 12-fold that of the agonist. Consequently, induction of the individual enzymes as well as of 3-dehydroecdysone 3α-reductase was examined using dialyzed midgut cytosol fractions from “control” and RH-5849-induced larvae. The products of the ecdysone oxidase and 3-dehydroecdysone 3α- and 3β-reductase assays were analyzed using HPLC system 2 (R<sub>t</sub> ecdysone, 10.2 min; 3-dehydroecdysone, 5 min; 3-epiedcysone, 8 min). Analysis of the polar ecdysteroid phosphate products of the 3-epiedcysone phosphotransferase assays by two ion-suppression HPLC systems revealed the presence of two components, both of which yielded only 3-epiedcysone on *Helix* hydrolase treatment. The later eluting component (15% of the total radioactivity) co-chromatographed with authentic 3-epiedcysone 2-phosphate on both HPLC systems 4 (R<sub>t</sub> 23.8 min) and 5 (R<sub>t</sub> 19.0 min), corroborating its identity. The earlier eluting compound (30% of total radioactivity; R<sub>t</sub> 22.7 min and 17.9 min on systems 4 and 5, respectively) was suspected to be 3-epiedcysone 3-phosphate. Since no authentic marker was available, the 20-hydroxylated equivalent was made by incubating dialyzed cytosol from RH-5849-induced larvae with 20-hydroxyecdysone, NADPH, ATP, and Mg2+; a parallel incubation was conducted with ecdysone substrate. The relevant 20-hydroxylated product co-chromatographed by HPLC with authentic 3-epiedcysone 3-phosphate (19) on both systems 4 (R<sub>t</sub> 21.5 min) and 5 (R<sub>t</sub> 16.2 min). This indicates that the corresponding 3-epiedcysone phosphotransferase product is 3-epiedcysone 3-phosphate. The identification of 3-epiedcysone 2-phosphate and 3-epiedcysone 3-phosphate as products of the 3-epiedcysone phosphotransferase in *M. sexta* midgut is consistent with the reported detection of two 3-epiedcysone phosphoconjugates in this system (22).

The results (Table III) clearly show that ecdysone oxidase and 3-epiedcysone phosphotransferase(s) are induced by RH-5849, whereas the reductases are not. Thus, 3-epiedcysone formation in cytosol obtained from RH-5849-treated insects is due to the formation of 3-dehydroecdysone by the ecdysone oxidase. Overall, the activities of both 3-dehydroecdysone 3α- and 3β-reductases were higher using NADPH; this may be due to NADH not being replenished, as in the case of NADPH incubations, which contained an NADPH regenerating system.

Since assays of cytosolic enzymes from induced insects were undertaken on day 5, with the natural ecdysone oxidase activity beginning to show an increase during development on day 6, the possibility that RH-5849 advanced the animals to a developmental stage at which ecdysone oxidase activity was higher was examined. For this, larvae were injected with RH-5849 and the ecdysone oxidase activity assayed in cytosol from day-2 insects. The results showed that induction of ecdysone oxidase is not accounted for by accelerated insect development (induced, 29.4 ± 1.0 pmol/min/mg protein; control, no activity, mean ± range for two independent experiments).

Actinomycin D and cycloheximide caused appreciable inhibition of induction of ecdysone oxidase and 3-epiedcysone phosphotransferase by RH-5849 (Table II), demonstrating the involvement of gene transcription and protein synthesis in this process.

**DISCUSSION**

The results demonstrate that enzymes responsible for inactivation of ecdysteroids and ecdysone 20-monoxygenase can be induced in *M. sexta* by 20-hydroxyecdysone and the ecdysteroid agonist RH-5849. Changes in cytochrome P450-mediated enzyme activities are generally accompanied by concomitant changes in cytochrome P450 levels, and there is evidence that this is the case for fat body mitochondrial ecdysone 20-monoxygenase in *S. littoralis* (4). Inhibition of the *M. sexta* midgut mitochondrial ecdysone 26-hydroxylase activity by carbon monoxide and ketocazol (23) indicates that this enzyme is cytochrome P450-dependent. Thus, the RNA- and protein synthesis-dependent induction of ecdysteroid 20- and 26-hydroxylases in *M. sexta* might be expected to result at least in

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part from alterations in transcription and synthesis of cytochrome P450 species. The observation that RH-5849 also induced ecdysone 20-monoxygenase in microsomes but not in mitochondria is unlikely to be due to the presence of sufficiently high levels of lipophilic RH-5849 in the mitochondria to inhibit the 20-monoxygenase assay, since inhibition in vitro was only apparent at RH-5849 concentrations above 100 μM. A somewhat analogous situation exists in larvae of the housefly (Musca domestica), in which ecdysone induces the 20-monoxygenase activity in mitochondria but not in microsomes (24).

RH-5849 also induced edysteroid 26-hydroxylase activity in both mitochondria and microsomes from M. sexta fat body. Induction of 26-hydroxylase activity in M. sexta by RH-5849 and 20-hydroxyecdysone is consistent with observations in S. littoralis, although in the latter case there was no significant induction of ecdysone 20-monoxygenase activity in fat body or midgut (20). Furthermore, it has been reported that edysteroids and RH-5849 induce ecdysone 20-monoxygenase activity in midgut mitochondria of ligated M. sexta, but induction of the 26-hydroxylase activity was not discerned in that case (14, 15).

Although substantially less active than 20-hydroxyecdysone in various in vitro systems, RH-5849 is far more active and persistent in various in vivo assays, presumably owing to the analog’s superior transport properties and metabolic stability (13, 25). In fact, RH-5849 has been observed to have greater potency to induce edcsyone hydroxylating activities than 20-hydroxyecdysone and ecdysone (15, 20). That this was less marked in the current work could possibly be explained if the dose of 20-hydroxyecdysone used were sufficiently high not to be unduly affected by such factors.

A mixture of ecdysone and 20-hydroxyecdysone at concentrations occurring near peak edcsyoid titer in fifth instar M. sexta led to considerable induction of 26-hydroxylase activity and a smaller induction of 20-monoxygenase activity (Table I, iv). This indicates that induction of 26-hydroxylation, followed by oxidation to the 26-oic acid, may at least partly contribute to the sharp decline in hormone titer at the end of the instar. Interestingly, it has not been possible in various species to demonstrate 26-hydroxylation in homogenates or subcellular fractions of tissues that otherwise effect the reaction (26). In final instar larvae of S. littoralis, appreciable edcsyoid 26-oic acids are formed in vivo at the time of declining edcsyoid titer.

It is noteworthy that concentrations of ecdysone or RH-5849 intended to simulate the commitment peak of edcsyoid prior to wandering led only to low induction of ecdysone 20-monoxygenase in the microsomal fraction (Table I, iv). Although this would not account for the large in vivo 50-fold increase in 20-monoxygenase purported to be triggered by the commitment peak of edcsyoid, it is likely that exogenous edcsyoid is less effective than endogenous hormone, possibly owing to more rapid inactivation/clearance (14).

RH-5849 also induces a second edcsyoid inactivation route, viz, the cytosolic conversion of ecdysone via 3-dehydroecdysone into 3-epiecdysone and the phosphorylation of the latter compound. The observation that “control” phosphotransferase activity was enhanced when 3-epiecdysone was used as substrate compared with ecdysone indicates that the former is the preferred substrate. Within this pathway, RH-5849 induced ecdysone oxidase and edcsyoid phosphotransferase activities (Fig. 1, Table III), since RNA and protein synthesis are required for increased activity (Table II). The formation of 3-dehydroecdysone in the RH-5849-induced system resulted in some 3-epiecdysone being formed by constitutive 3-dehydroecdysone 3α-reductase.

Induction of edcsyoid-metabolizing enzymes in M. sexta

<table>
<thead>
<tr>
<th>Inducing compound</th>
<th>Ecdysone oxidase</th>
<th>3-Epiecdysone phosphotransferase</th>
<th>3-Dehydroecdysone 3α-reductase</th>
<th>3-Dehydroecdysone 3β-reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pmol/min/mg protein)</td>
<td>(pmol/min/mg protein)</td>
<td>(pmol/min/mg protein)</td>
<td>(pmol/min/mg protein)</td>
</tr>
<tr>
<td>Control (methanol)</td>
<td>0.4 ± 0.1</td>
<td>3.7 ± 0.4</td>
<td>19.5 ± 0.7</td>
<td>11.3 ± 0.2</td>
</tr>
<tr>
<td>RH-5849</td>
<td>26.6 ± 1.4</td>
<td>15.5 ± 0.5</td>
<td>21.9 ± 0.3</td>
<td>11.1 ± 0.1</td>
</tr>
</tbody>
</table>

I. Davies and H. H. Rees, unpublished results.
by 20-hydroxyecdysone and RH-5849 is not merely a nonspecific effect, since phenobarbital, a known inducer of several forms of cytochrome P450 (24, 27), although induction by phenobarbital and various allelochemicals has been observed for the midgut microsomal enzyme from Spodoptera frugiperda (28).

Induction of these ecdysteroid-metabolizing enzymes by RH-5849 is not likely to be a nonspecific response to an exogenous metabolite, since in the case of ecdysteroid 26-hydroxylase induction, the enzyme can also be induced to the same extent by 20-hydroxyecdysone. Furthermore, the ecdysteroid 26-hydroxylase seems not to be a general sterol/steroid 26-hydroxylase, since a 100-fold excess of cholesterol or vitamin D3 over the ecdysone substrate is required to exhibit any inhibition. In contrast, in the case of porcine mitochondria, 26-hydroxylation of 25-hydroxyvitamin D3 and cholesterol is apparently catalyzed by the same species of cytochrome P450 (29). It is possible that RH-5849 mimics high ecdysteroid levels and that the enzyme is required for the midgut microsomal enzyme from Spodoptera frugiperda (28).

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Induction of ecdysteroid inactivation pathways by 20-hydroxyecdysone and RH-5849 is reminiscent of the regulation of vitamin D inactivation in vertebrates, in which 1α,25-dihydroxyvitamin D3 stimulates its own degradation by inducing vitamin D mitochondrial 24-hydroxylase activity via a vitamin D response element, since in the case of ecdysteroid 26-hydroxylase, the ecdysone substrate is required to exhibit any inhibition. In contrast, in the case of porcine mitochondria, 26-hydroxylation of 25-hydroxyvitamin D3 and cholesterol is apparently catalyzed by the same species of cytochrome P450 (29). It is possible that RH-5849 mimics high ecdysteroid levels and that the insect responds by induction of several ecdysteroid-metabolizing enzymes.

Induction of ecdysteroid inactivation pathways by 20-hydroxyecdysone and RH-5849 is reminiscent of the regulation of vitamin D inactivation in vertebrates, in which 1α,25-dihydroxyvitamin D3 stimulates its own degradation by inducing vitamin D mitochondrial 24-hydroxylase activity via a vitamin D response element to initiate degradation to excretory metabolites (30–32). We cannot discount the possibility that at least some of the observed induction events we now report may be indirect, for example, affecting regulatory proteins involved in transcription of enzyme-encoding genes (33) or post-translational processes (34). It is also always conceivable that tissues may not be fully primed for premature developmental induction or that the induction process may also require other unknown development-specific factors.

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Induction of Enzymes Involved in Molting Hormone (Ecdysteroid) Inactivation by Ecdysteroids and an Agonist, 1,2-Dibenzoyl-1-\textit{tert}-butylhydrazine (RH-5849)  
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