RH 5849, a Nonsteroidal Ecdysone Agonist: Effects on Larval Lepidoptera

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The ecdysone agonist RH 5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) causes the premature initiation of molting at all stages of larval development of the tobacco hornworm, *Manduca sexta*. This phenomenon occurs without an increase in the endogenous ecdysone (20-hydroxyecdysone) titers. RH 5849 likewise provokes the initiation of molting in larval abdomens in the absence of a source of endogenous hormone. Although substantially less active than 20-hydroxyecdysone in vitro, RH 5849 was 30 to >670 times as active as the authentic molting hormone in bioassays with isolated larval abdomens or intact hornworms. This reversal in potency can be attributed to the superior transport properties and metabolic stability of RH 5849 relative to 20-hydroxyecdysone. Thus RH 5849 and its analogs are relatively persistent ecdysone agonists that halt feeding in larval lepidoptera by forcing an ultimately lethal, developmentally premature molt.

IN THE ACCOMPANYING REPORT THE ecdysonergic actions of RH 5849 in *Drosophila* K_c cells and ecdysone receptor extracts are described (*1*). In these in vitro bioassays where differential metabolic and transport effects were inconsequential, RH 5849 was consistently less active than 20-hydroxyecdysone. However, RH 5849 is extraordinarily potent at promoting molting in larvae of the tobacco hornworm (*Manduca sexta*), and this is due to a direct action of the compound on target tissues and not because of elevation of endogenous ecdysone titers. In addition, this is the insecticidally significant mode of action of RH 5849 and many of its analogs in lepidopteran larvae.

Lepidoptera display the onset of a larval molt by slippage of the head capsule down over the mandibles during formation of an underlying pharate head capsule; this morphological marker accompanies epidermal apolysis, which ordinarily occurs at or near peak molting hormone titers (*2*). RH 5849 caused a rapid, premature apolysis in newly ecdysed fifth instar day 0 (L5D0) *M. sexta* larvae (Fig. 1); the well-formed pharate head capsule is clearly visible. Although this photograph was taken 3 days after initiating treatment, clear signs of head capsule apolysis were evident 24 hours after the initiation of feeding.

The effects of RH 5849 on immunoreactive ecdysone titers and weights of developing L4 *M. sexta* larvae are shown in Fig. 2. The treated larvae underwent capsule slippage after only 24 hours, without any elevation of ecdysone titers; simultaneously, further feeding and weight gain ceased. RH 5849–treated larvae were unable to ecdyse (shed their old cuticle) successfully; hemorrhage of hemolymph and molting fluid took place, and the pharate larvae eventually died without completing the molt.

The relative potency of 20-hydroxyecdysone and RH 5849 at initiating development in isolated abdomen or intact hornworm assays is shown in Table 1. The prothoracic glands are the only known source of 20-hydroxyecdysone precursors in hornworm larvae (*3*), and after ligation and removal of the thorax the abdomens are convenient preparations of target tissues. When injected into L4D1 abdomens 20-hydroxyecdysone elicited apolysis of spiracular cuticle and the formation of new fifth instar larval crochets (sclerotized hooks at the bottom of the proleg). However, RH 5849 is 27 times as potent in this assay as the molting hormone (*4*). When injected into L5D3 abdomens, RH 5849 was again 28 times as potent as 20-hydroxyecdysone at initiating events related to pupal formation. These results imply that RH 5849 can stimulate the epidermal cells so that they undergo apolysis and synthesize the appropriate proteins necessary for either larval or pupal cuticle formation under the modulating influence of juvenile hormone, in a manner precisely analogous to stimulation by 20-hydroxyecdysone (*5*).

The dosage difference between the two compounds was even more dramatic in bioassays on intact (unligated) L5D0 hornworms; if the compounds are injected, RH 5849 was 53 times as effective as 20-hydroxyecdysone, whereas if administered orally it was more than 670 times as potent (Table 1).

Hemolymph concentrations of [^{14}C]RH 5849 were measured by feeding L5D0 hornworm larvae an artificial diet containing the labeled compound. At specified intervals thereafter measured volumes of hemolymph were collected, extracted, and analyzed by thin-layer chromatography and high-performance liquid chromatography. Administration of 10 μg of compound per gram of live weight (about a 95% effective dose for premature molting at this stage) resulted in peak hemolymph concentrations of 16 μ*M* at 6 hours after the initiation of feeding (Fig. 3). Blood levels then declined rapidly, but remained at a residual level of about 3

Table 1. Relative potency of 20-hydroxyecdysone and RH 5849 at inducing the onset of molting in ligated *M. sexta* abdomens and L5D0 whole larvae. L4 animals 36 hours after ecdysis were ligated, decapitated, and next day injected with varying doses of compound in 1 to 2 μl of DMSO. They were scored after 2 days as follows: 0, normal L4 abdomens with no L5 crochets or spiracle apolysis; 1, exposed dorsal vessel (response to moderate levels of molting hormone in the presence of low juvenile hormone levels); 2, L5 crochets formed but no spiracle apolysis; 3, L5 crochets formed and mild spiracle apolysis; 4, strong molting response, both L5 crochets and spiracle cuticle synthesized. These methods are an adaptation of (*16*). Injection and scoring of L5D3 abdomens (weighing 7.3 to 8.3 g) are also adaptations of previous methods (*17*). Mature feeding stage fifth instar animals weighing 8.5 to 9.5 g were ligated and decapitated, and immediately injected with RH 5849 or 20-hydroxyecdysone in 2 to 5 μl of DMSO. Animals were held over moistened filter paper and were scored after 3 days. Scoring was as follows: 0, normal undeveloped blue-green L5D3 abdomen; 1, light dorsal vessel exposure with mild cuticular blanching, abdominal contraction; 2, dorsal vessel well exposed with abdominal contraction; 3, complete prepupal cuticle formation with strong abdominal contraction and cuticular blanching. For both L4 and L5 abdomens, the dose for 50% of maximal response (ED_{50}) was determined by plotting the mean response for all individuals as a function of dose, then determining the dose that gives a score of 2.0 and 1.5, respectively. L5D0 animals were either allowed to feed on treated commercial black cutworm diet or injected with drug in a proleg and scored after 48 hours. The ED_{50} was calculated by plotting percentage of the population bearing a prematurely apolysed head capsule as a function of dose on log-logit paper. For all experiments in this table at least five different doses with ten animals per dose were used. Data are means ± SD for three separate determinations.

Bioassay	ED_{50}	
	20-Hydroxyecdysone	RH 5849
L4D1 abdomens (μg injected per abdomen)	35.2 ± 6.2	1.3 ± 0.3
L5D3 abdomens (μg injected per abdomen)	30.7 ± 5.0	1.1 ± 0.09
L5D0 larvae, injected (μg/g body weight)	181.0 ± 19.0	3.4 ± 0.4
L5D0 larvae, oral (ppm in diet)	>2000.0	3.0 ± 0.4

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μM for at least 36 hours thereafter. By contrast, we were unable to elicit head capsule slippage in L5D0 hornworm larvae by feeding up to 2000 ppm 20-hydroxyecdysone, indicating that hemolymph ecdysone titers were well below those usually observed during a molt (about 6 μM).

Although the apparent binding affinity of RH 5849 to K_c cell extracts was 1/30 that of 20-hydroxyecdysone, RH 5849 is clearly much more potent at eliciting whole-animal effects, apparently due to its longevity in the hemocoele after ingestion. The peak concentrations of RH 5849 in the hemolymph at an oral dose of 10 μg per gram of live weight exceeded by three- to fivefold those required for a 50% response in process elaboration in whole K_c cells and in displacement of [^{3}H]ponasterone A from its receptor in K_c cell extracts (*1*). By contrast orally administered 20-hydroxyecdysone is probably metabolized and cleared rapidly (*6*).

The relative potencies of 28 RH 5849 analogs were tested in vitro on *Drosophila* K_c cell receptor binding and compared with their ability to promote premature head capsule apolysis in L3D0 hornworm larvae (Fig. 4). Apparent receptor binding affinity is strongly related to insecticidal activity in vivo. These data indicate that, at least for this population of compounds, the toxicologically significant mode of action is ecdysonergic and that the 1,2-diacyl-1-alkylhydrazine binding domains of both the dipteran K_c cell receptor and the lepidopteran epidermal receptors are similar.

In parallel tests the two benzoylphenylurea insecticides Dimilin and CGA-112913 failed to elicit premature molting in intact hornworms or isolated abdomens. These results corroborate those described for *Drosophila* K_c cells and their ecdysone receptor extracts (*1*) and provide further evidence that RH 5849 and the benzoylphenylureas differ markedly in their mode of action. Although both classes of compound cause ecdysis failure, the benzoylphenylureas disrupt normal cuticle lamellar deposition (*7*), while it appears that RH 5849 causes a forced, untimely synthesis of cuticle before the animal is developmentally competent to molt. In addition, high residual levels of an ecdysone agonist may inhibit the release of and sensitivity to eclosion hormone, which normally requires a peak of 20-hydroxyecdysone followed by its precipitous decline (*8*).

RH 5849 at $1 \times 10^{-4} M$ is not significantly bound by our antiecdysone antiserum (thus, the binding affinity for 20-hydroxyecdysone is >3125 times as great as that for RH 5849). Consequently, RH 5849 can be used as an agonist in physiological systems, while ecdysone-specific immunoassays are used to measure steroid levels. For example, ecdysone titers in RH 5849–treated L4D0 animals are significantly depressed relative to control levels within 4 hours after the initiation of feeding (*9*). These findings imply that in hornworm larvae ecdysone ago-

Fig. 1. Close-up photographs of 3-day-old fifth instar *Manduca sexta* larvae treated since ecdysis with either (**bottom**) 10 ppm RH 5849 in artificial diet (Black Cutworm diet, Bioserv) or (**top**) control diet. Note the pharate head capsule (arrow) in the treated animal.

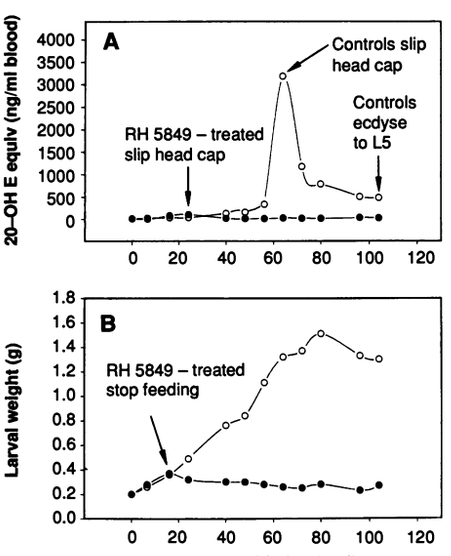

Fig. 2. Effects of 10 ppm RH 5849 administered in an artificial diet to newly ecdysed fourth instar (L4) *M. sexta*: (**A**) immunoreactive hemolymph ecdysteroid titers and (**B**) weight gain. Ecdysteroid titers were measured by a radioimmunoassay adapted from Warren *et al.* (*18*) with protein A–*Staphylococcus aureus* suspension (Sigma) as the reagent to separate bound from free ligand. The antiserum against ecdysone was formed by reaction of 20-hydroxyecdysone (20-OH E) with carboxymethoxyamine, which resulted in the corresponding ecdysteroid 6-oxime acid. The antiserum recognizes α-ecdysone with an affinity 2.7 times as great as that for 20-hydroxyecdysone, with commercially available ^{3}H-labeled α-ecdysone as the radioligand (New England Nuclear). Data are expressed in 20-hydroxyecdysone equivalents, for which the log-logit standard curve commonly had a correlation coefficient $r > 0.99$. All samples were diluted appropriately in borate buffer (*p*H 8.0) and assayed in duplicate. For both ecdysteroid titer and weight determinations ten individuals were used, and all data points are means. All larvae described in this report were held in a long-day photoperiod (16L:8D, 25°C) and were purchased as eggs (Carolina Biological Supply Co.). ○, Solvent controls; ●, larvae received 10 ppm RH 5849.

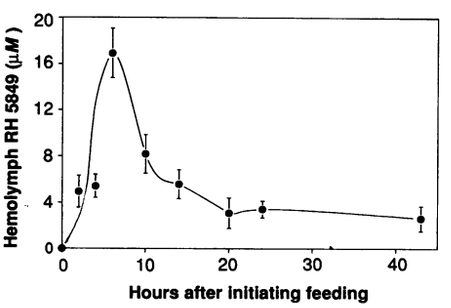

Fig. 3. Hemolymph titers of [^{14}C]RH 5849 in L5 hornworms after feeding on the radiolabeled compound (10 μg/g of live weight). Newly ecdysed L5 *M. sexta* larvae were starved for 12 hours and then offered 350-mg chunks of artificial diet containing 13 μg of the radiolabeled RH 5849 (1-[*carboxy*-^{14}C]benzoyl-2-benzoyl-1-*tert*-butylhydrazine, >99% radiochemically pure, 3.09 mCi/mmol, synthesized at Rohm and Haas Research Labs). At specified times thereafter larvae were bled into calibrated tubes, and the hemolymph volume measured. To recover RH 5849 from the hemolymph, we added excess NaCl in 500 μl of 1% aqueous HCl and extracted three times with 1 ml of ethyl acetate : acetonitrile (2 : 1), with vigorous vortexing and centrifugation between washes. The pooled organic layers were evaporated to dryness and spotted on 500-μm normal-phase silica gel thin-layer chromatography plates (TLC) (Analtech) and developed in dichloromethane : acetonitrile : acetic acid (67:33:1). Radioactivity was analyzed by either autoradiography, scraping the spot corresponding to parent compound, and liquid scintillation spectrometry; or by using a Berthold LB 2842 Linear Analyzer and comparison to a [^{14}C]RH 5849 calibration curve. When the radioactivity corresponding to the parent on TLC was scraped, extracted, and run on reversed-phase high-performance liquid chromatography (35% acetonitrile in water, 35°C, Alltech Applied Sciences, 3-μm particle size C18 Adsorbosphere column), the radioactivity almost quantitatively eluted with authentic RH 5849 standard. Data are means of five to eight individuals ± SEM.

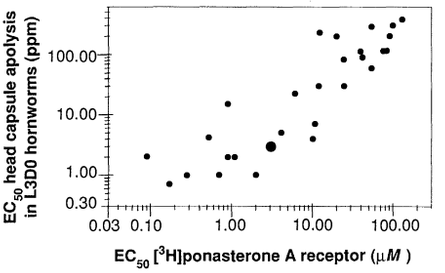

Fig. 4. Relation of apparent K_c cell ecdysteroid receptor affinity and induction of premature molting in L3D0 *M. sexta* larvae for 28 RH 5849 analogs. [^{3}H]Ponasterone A receptor displacement assays were as described (*1*). Premature molt induction of L3D0 *M. sexta* was measured by admixing different concentrations of analogs in 0.5% DMSO:acetone (1:1) into artificial diet, then allowing the larvae to feed for 48 hours (ten individuals per treatment). The concentration to elicit a 50% of maximal response (ED_{50}) was determined by plotting percentage of the treatment population bearing a prematurely slipped head capsule as a function of dose in the diet. The linear relation is highly significant ($P < 0.001$). The enlarged data point represents RH 5849.

nists may exert a negative feedback inhibition on hormone biosynthesis, as has been suggested for *Pieris brassicae* pupae (*10*).

We have observed the induction of premature head capsule apolysis by RH 5849 in all larval stages of *M. sexta*. This phenomenon has also been observed in larval Lepidoptera of the families Noctuidae, Pyralidae, and Pieridae. RH 5849 controls dipteran larvae (both houseflies and mosquitoes) and certain coleopteran larvae and has been shown to inhibit ovariole development in all three orders (*11*). This latter activity is consistent with its putative ecdysonergic mechanism of action (*12*).

The 1,2-diacyl-1-alkylhydrazines are thus a novel class of "ecdysonoids" and are representative members of the third generation insecticides having their genesis in insect hormones (*13*). C. M. Williams coined the term "hyperecdysonism," an experimentally induced state first described in *Samia cynthia* pupae (*14*); clearly, many of the phenomena reported here for RH 5849 are aptly described by this term. Other modes of action may be encountered in these compounds; for example, we have noted significant neurotoxic symptoms in certain Coleoptera in vivo and housefly larval muscle in vitro (*15*). However, it is clear that RH 5849 and its analogs are behaving as nonsteroidal ecdysone agonists in *Drosophila* K_c cells and in hornworm larvae, and it is anticipated that these findings may be extended to other invertebrate systems utilizing ecdysones.

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