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Induction of autotomy in the American bird grasshopper *Schistocerca americana* (Drury) by the ecdysone agonist RH-5849 and investigation of its mode of action

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Abstract. The non-steroidal ecdysone agonist RH-5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) was found to be an effective neurotoxicant on injection into the American bird grasshopper, *Schistocerca americana* (Drury). Treated grasshoppers became immediately hyperactive, followed by loss of coordination, paralysis and eventually death. We also discovered that this compound induced bilateral autotomy of the metathoracic legs during the early stages of intoxication. However, no evidence of ecdysonergic or morphogenetic activities was observed. Synergism studies with neurotoxins of known mode of action suggested that RH-5849 has a mechanism of action similar to that of 4-amino pyridine, which blocks potassium channels.

Key words. Grasshopper; *Schistocerca*; ecdysone agonist; RH-5849; autotomy.

Wing and coworkers reported RH-5849 (1,2-dibenzoyl-1-*tert*-butylhydrazine) as the first non-steroidal ecdysone agonist[1,2]. RH-5849 has been shown to mimic the action of 20-hydroxyecdysone on tissue cultures[3,4] and ecdysone sensitive cell lines[1,5,6], and it was able to induce premature molting in larvae of several lepidopterous species[2,7–13]. These phenomena appear to occur by direct action of RH-5849 on the ecdysone receptors at the target tissues rather than by stimulating an elevation of endogenous ecdysone titers[2]. The ecdysone agonist has also been reported to possess morphogenetic activity, producing nymphal-adult intermediates in the milkweed bug, *Oncopeltus fasciatus*[14], supernumerary larvae and larval-pupal intermediates in several lepidoptorous species[9,13], and premature head capsule apolysis and death during molting due to the inability to shed the old cuticle in the Colorado potato beetle, *Leptinotarsa decemlineata*[13,14]. In addition, ingestion by adults in a wide range of Lepidoptera and some species of Coleoptera and Diptera reduced feeding and oviposition[2,7,12,13,15].

Although premature molting has been reported in larvae of the Japanese beetle, *Popillia japonica*[12], the blowfly *Neobelleria bullata*[14], and the house fly, *Musca domestica*[16], neurotoxicity is often the most important action of RH-5849 in Coleoptera and Diptera[2,7,12,17,18]. In all cases the neurotoxic symptoms involved tremors and movement of appendages, followed by paralysis and death after a few hours. The neurotoxic mode of action of RH-5849 and other diacylhydrazines has been recently related to the block of delayed rectifier voltage-dependent potassium channels. (I_K channels) in neurons as well as in muscle[17,19]. Block of nerve and muscle I_K channels were in all cases significant at concentrations that would be achieved in the insect following injection of acute toxic dose of RH-5849[17,19], indicating that the K^+ channel block is the primary neurotoxic mechanism of action of RH-5849, although it may have other neurotoxic targets as well.

We were curious about whether Orthoptera might show effects similar to that described for other insects. Accordingly, we tested RH-5849 for ecdysonergic, neurotoxic and morphogenetic activities on the American bird grasshopper, *Schistocerca americana* (Drury), and compared them with those of the natural molting hormone 20-hydroxyecdysone, some structurally related benzoyl-phenyl ureas (BPUs) and some neurotoxic insecticides. We were surprised to discover in our first experiments that RH-5849 induced bilateral autotomy of the metathoracic legs. Therefore, we broadened our investigation to include autotomy, and other kinds of ecdysone related activities and/or neurotoxic effects that might be induced by RH-5849 in grasshoppers, and to evaluate its potential as an alternative method for grasshopper and locust control.

Materials and methods

The ecdysone agonist RH-5849 (analytical standard) was provided by the American Cyanamid Company (Princeton, NJ). The benzoyl-phenyl ureas teflubenzuron (E.C. 5%), chlorofluazuron (E.C. 5%) and XRD-473 (technical 97%) were provided by Dow Chemical (Midland, MI). The neurotoxic insecticides dieldrin (99.8%), aldrin (99%), kepone (86.6%), endosulfan (100%), heptachlor epoxide (99.3%), lindane (99%) and toxaphene (99.3%) were provided by EPA (Research Triangle Park, NC); parathion (98.5%) and malathion

(97.5%) by Pesticides Research Laboratories (Perrine, FL); aldicarb (99.7%) and sevin (99.9%) by Union Carbide Agricultural Products Company (Research Triangle Park, NC); carbofuran (99%) by FMC Chemicals (Middleport, NY); chlordimeform (99.6%) by Norm-Am; EPN (100%) by DU-Pont Nemours & Co. (Wilmington, DE); and p,p'DDT (Tech. 77%) by Nutritional Biochemicals Corporation (Cleveland, OH). Analytical standards of the insecticides o,p'DDT, p,p'DDD, dicofol, EDO (CSIRO), NRDC-157 (Roussel UCLAF), IS-157 (Roussel UCLAF), deltamethrin (Roussel UCLAF), and GH-401 (CSIRO) were a gift from Prof. D. Soderland (Cornell University). The natural molting hormone 20-hydroxyecdysone; the neurotransmitters acetyl choline chloride, L-glutamate, DL-octopamine hydrochloride, and GABA; the neurotoxins aconitine, veratridine, procaine, 4-amino pyridine and tetraethyl ammonium chloride; and dried whole venom from *Apis mellifera* were purchased from Sigma.

A stock population of the grasshopper *S. americana* was reared under crowded conditions with a photoperiodic regimen of 16 hours light and 8 hours dark, at a temperature of 30°C. The grasshoppers were fed fresh wheat seedlings daily, supplemented with wheat germ twice a week, and fresh water weekly. A colony of the grasshopper *Melanoplus differentialis melanistic* Cockerell was reared under identical conditions, except that the eggs were kept at 4°C for 2–3 months to break diapause.

Newly molted last instar nymphs of *S. americana*, within 24 hours of the last ecdysis, were injected in the abdominal sternum with 200, 100, 50, 10 and 5 µg/g of RH-5849 and 200, 50 and 10 µg/g of 20-hydroxyecdysone dissolved in 5 µl of dimethyl sulfoxide (DMSO); or topically treated with 200, 50, and 5 µg/g of RH-5849 dissolved in 5 µl of hexane: mineral oil: acetone (4:1:5, v/v/v). Controls were injected with DMSO only or treated topically with the oil solution. Each treatment was tested in 25–30 nymphs, which were checked daily to evaluate neurotoxic reactions. In addition, the length of the last instar was recorded to determine ecdysonergic activity, and the resulting adults observed for 5 days to establish possible morphogenetic abnormalities. Control and treated nymphs were maintained in goups of ten in glass jars of 3.8 liters capacity, under the environmental conditions already described. A Fisher protected least significance test (Fisher PLSD) was chosen to perform multiple comparisons among the lengths of last instar means.

Neurotoxic responses were further studied by injecting newly molted last instar nymphs of *S. americana* with 400, 360, 280, 240, 200, 160, 120, 80, 40, 20, 10, and 5 µg/g of RH-5849 dissolved in 5 µl DMSO. Each treatment was tested in 30 nymphs, each group comprising half of each sex, maintained individually in transparent plastic cups of 0.25 liter capacity an checked periodically over 48 hours. Newly molted adults and 5–10 day old last instar nymphs of *S. americana* and 5–10 day old last instar nymphs of the grasshopper *M.d. melanistic* were treated in the same way. In this experiment, each treatment was tested on ten grasshoppers, composed of five of each sex. The loss of coordination and equilibrium within the first hour post-treatment (knock-down), the drop of one or both hind legs (autotomy), and paralysis and death at 48 hours (mortality) were recorded as different types of neurotoxic responses. The lethal dose to kill 50% of the specimens (LD50), the effective dose to induce knock-down in 50% of them (KD50), and the effective dose to induce autotomy in 30% of them (AD30) was calculated by Probit analysis[20]. The AD30 was chosen instead of an AD50 because the percentage of autotomy has only a dose-dependent linear response in the range 0–40 to 60%.

In order to determine whether autotomy induction is mediated by the brain, decapitated and normal 5–10 day old last instar nymphs were injected with 400, 200, 100, 50 and 10 µg/g of RH-5849 dissolved in 5 µl DMSO. Each treatment was tested in ten nymphs, which were periodically checked over six hours, before any adverse symptoms were detected in decapitated controls. In a related experiment, newly molted last instar nymphs were injected into the femur of the left or right hindleg with 200 µg/g of RH-5849 dissolved in 5 µl DMSO. Contols were injected into the femur with 5 µl of DMSO or punctured with the microsyringe without injecting anything. Each treatment was tested in 20 nymphs, which were checked after 48 hours to determine which hindleg was induced to drop.

The neurotoxic action of the ecdysone agonist RH-5849 was compared with that of the following neurotoxic insecticides: DDT analogs (p,p'DDT, o,p'DDT, p,p'DDD, dicofol, and EDO), pyrethroids (NRDC-157, IS-157, deltamethrin, and GH-401), organophosphates (malathion, parathion and EPN), carbamates (aldicarb, carbofuran and sevin), amidines (chlordimeform), cyclodienes (aldrin, dieldrin, kepone and endosulfan) and cholorinated hydrocarbons (lindane, toxaphene, and heptachlor epoxide). Some structurally related BPUs (teflubenzuron, chlorofluazuron and XRD-473), neurotransmitters (acetyl choline, L-glutamate, octopamine and GABA), neurotoxins (aconitine, veratridine, procaine, tetraethyl ammonium chloride and 4-amino pyridine), and bee venom were also tested. Each compound was tested in 10–30 newly molted last instar nymphs, composed of half of each sex, by injection into the abdominal sternum with 200, 100 and 50 µg/g dissolved in 5 µl DMSO. If the compound showed neurotoxic activity, the doses were reduced until the activity was lost. If the compound showed no activity, the dose was increased. The pyrethroid NRDC-157 and p,p'DDT were also tested by topical application on the abdomen of ten newly molted last instar nymphs. Each specimen was maintained individually in transparent

plastic cups of 0.25 liter capacity and examined periodically for 48 hours to record autotomy induction at 1 and 24 hours, knock-down at 1 hour, and mortality at 48 hours. The LD50, KD50 and AD30 of the compounds with some of these activities were calculated by Probit analysis[20]. The joint actions of mixtures of RH-5849 with p,p'DDT, 4-amino pyridine (4-AP) or procaine were tested under the same conditions in order to elucidate synergistic and antagonistic effects. The mixtures were formulated according to the relative potencies of their components, each mixture containing equipotent concentrations as calculated by the ratio of their respsective LD50s.

The viability of autotomized grasshoppers was established by comparing newly molted last instar nymphs of *S. americana* with no hindlegs, and with only one hindleg, with normal control adults. Nymphs were physically induced to autotomize by grasping one hindleg at a time until the nymph voluntarily dropped it. Each experimental group consisted of 50 nymphs, composed of half of each sex, that were checked daily until five days after adult ecdysis. The nymphs were maintained in groups of ten in glass jars of 3.8 liters capacity, under conditions identical to the first experiment described. The length of the last instar in each experimental group was compared by a Fisher PLSD test.

Results

The ecdysone agonist RH-5849 was tested on newly molted last instar nymphs of *S. americana*, either by injection or topical application (table 1). In a typical sequence of events, the injection of 200 and 100 µg/g of RH-5849 into the abdomen of the nymphs induced convulsive movements of the hind legs and tremors of the body, followed in most cases by loss of coordination and equilibrium (knock-down) within a few minutes. Usually within the first minutes post-injection, nymphs may drop one or both hind legs (autotomy). The process is, in most cases, followed by paralysis after a few hours, and eventually death at 2–3 days. On the other hand, topical application of RH-5849 failed to produce any neurotoxic activities at the concentrations tested nor did injection of 20-hydroxyecdysone (table 1). In all cases, mortality prior to and during adult ecdysis and the number of morphogenetic abnormalities in the resulting adults were very low, occurring at levels similar to the controls. Neither ecdysone or RH-5849 induced premature molting, and the duration of the last nymphal instar was similar in treated and control nymphs, except for the survivors following the injection of 200 and 100 µg/g of RH-5849. These treated nymhs needed about one more day on average to molt to adults. This difference was statistically significant for nymphs injected with 100 µg, but not for the only survivor after injection with 200 µg of RH-5849 (table 1).

Table 1. Neurotoxic reactions, morphological abnormalities and ecdysonergic activity induced by the ecdysone agonist RH-5849 and the molting hormone 20-hydroxyecdysone in newly molted last instar nymphs of *S. americana*.

Treatment	Doses (µg/g)	N	Autotomy	Paralysis & death	Dead prior & during ecdysis	Molt to adults	Normal adults	Adults with curled wings	Length of last instar (days) (mean ± SE)
RH-5849 (injection)	200	30	21	29	0	1	1	0	11^{AB}
	100	30	14	13	1	16	15	1	10.4 ± 0.2^{B}
	50	29	3	0	0	29	27	2	9.7 ± 0.2^{A}
	10	29	0	2	0	27	27	0	9.7 ± 0.2^{A}
	5	29	0	0	0	29	28	1	9.7 ± 0.2^{A}
	control	29	0	0	1	28	27	1	9.8 ± 0.2^{A}
RH-5849 (topical)	200	26	0	0	2	24	23	1	9.8 ± 0.1^{A}
	50	29	0	0	1	28	28	0	9.6 ± 0.2^{A}
	5	28	0	0	2	26	25	1	9.6 ± 0.1^{A}
	control	30	0	0	1	29	29	0	9.6 ± 0.1^{A}
20-hydroxyecdysone (injection)	200	29	0	0	0	29	27	2	9.7 ± 0.2^{A}
	50	30	0	0	0	30	30	0	9.8 ± 0.2^{A}
	10	30	0	0	1	29	28	1	9.8 ± 0.2^{A}
	control	28	0	0	0	28	28	0	9.7 ± 0.2^{A}

Length of last instar means with the same capital letter ($^{A, B}$) within each treatment group are not significantly different from each other ($\alpha = 0.05$, Fisher PLSD test).

Table 2a. Reactions induced in last instar nymphs of *S. americana* by injection of several neurotoxins.

	200 µg/g			100 µg/g			50 µg/g			10 µg/g			5 µg/g			1 µg/g			0.5 µg/g			0.1 µg/g			AD30 (µg/g)	KD50	LD50
	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M			
Pyrethroids																											
NRDC-157**	47/10	100	100	70/17	100	93	80/10	100	90	40/20	83	57	50/17	87	40	47/30	67	13	50/3	77	13	3/3	33	0	0.27	0.20	6.2
IS-157*	50/10	70	30	10/0	50	20	10/20	0	0	0	0	0													92	122	204
DTM**	7/3	100	100	17/3	100	93	23/7	100	90	20/7	97	60	10/3	90	30	13/10	73	3	10/0	77	7	7/7	80	0	-	<0.1	8.8
GH-401**	53/10	100	93	37/13	97	93	33/30	100	80	50/10	80	47	37/3	67	20	23/13	43	7	3/0	23	0	0	3	0	2.3	1.9	13
Cyclodienes																											
Aldrin*	0/50	0	100	0/20	0	100	0/20	0	100	0	0	100	0	0	70	0	0	60	0	0	10				106	-	1.5
Deildrin*	0/30	0	100	0/20	0	100	0/20	0	100	0/10	0	100	0	0	90	0	0	40	0	0	10				-	-	1.5
Kepone*	0/20	0	90	0/10	0	100	0	0	90	0	0	0													-	-	32
Endosulfan*	0/50	20	100	0	0	40	0	0	20																171	-	87
Other chlorinated hydrocarbons																											
Lindane*	0/60	100	100	0/20	100	100	0/10	40	100	0/20	0	90	0	0	30	0	0	10							95	42	4.4
Toxaphene*	0	0	80	0/10	0	90	0	10	60	0	0	0													-	-	56
Heptachlor epoxide*	0/30	10	100	0/20	0	100	0/20	0	100	0/20	0	100	0/10	0	70	0	0	30							-	-	1.9
Organophosphates																											
Malathion*	0/30	100	100	0	90	100	0/10	50	60	0	20	0	0	0	0										-	31	38
Parathion*	0/10	100	100	0	100	100	0/20	80	100	0/20	60	100	0/30	50	100	0	0	20	0	0	10				-	10	1.4
EPN*	0/10	100	100	0/10	80	100	0/30	80	100	0/60	30	90	0/60	0	60	0	0	10	0	0	0				-	20	3.4
Carbamates																											
Aldicarb*	0	100	100	0	100	100	0	100	100	0	90	100	0	20	80	0	0	30	0	0	10				-	6.9	1.9
Carbofuran*	0	100	100	0	100	100	0	100	100	0/10	100	100	0/20	100	100	0/50	100	100	0/70	100	100	10/10	30	10	-	0.14	0.19
Sevin*	0/20	100	100	0/40	100	100	0/50	100	100	10/50	100	90	0/20	50	30	0	0	0							-	4.0	6.6
Other neurotoxins																											
Aconitine**	30/20	100	60	23/7	93	27	47/17	80	10	17/7	20	0	7/3	10	0										14	21	164
Veratridine**	0/3	100	100	3/7	97	100	3/10	100	93	0/20	73	57	0/27	87	53	0	53	7							-	<1	6.5

A: percentage autotomy induction at 1 h/24 h; K: percentage knockdown at 1 h; M: percentage paralysis and mortality at 48 h. *N = 10; **N = 30.

Table 2b. Reactions induced in last instar nymphs of *S. americana* by injection of several neurotoxins.

	4 mg/g			2 mg/g			800 µg/g			400 µg/g			200 µg/g			100 µg/g			50 µg/g			10 µg/g			AD30 (µg/g)	KD50	LD50
	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M	A	K	M			
Ecdysone agonist																											
RH-5849**										43/10	100	97	57/7	77	90	13/7	7	13	10/7	0	3	3/0	0	0	97	192	143
Neurotransmitters																											
Acethyl choline*										0	0	0	0	0	0	0	0	0	0	0	0				-	-	-
L-glutamate*										0	0	0	0	0	0	0	0	0	0	0	0				-	-	-
Octopamine*										0	0	0	0	0	0	0	0	0	0	0	0				-	-	-
GABA*										0	0	0	0	0	0	0	0	0	0	0	0				-	-	-
DDT analogs																											
p,p'DDT**							36/3	97	97	43/13	33	60	30/10	13	23	17/10	10	17	3/3	0	0				146	333	261
o,p'DDT*										0/20	10	50	0	0	0	0	0	0	0	0	0				-	-	-
p,p'DDD*										20/30	20	70	20/20	30	30	0	0	0	0/10	0	0				237	-	283
Dicofol*										0	0	0	0	0	0	0	0	0	0	0	0				-	-	-
EDO*										60/10	50	80	50/20	40	70	30/30	20	30	0	0	0				100	364	150
Amidines																											
Chlordimeform*													0	0	10	0	0	0	0	0	0				-	-	-
Benzoyl phenyl ureas																											
Teflubenzuron*													0	0	0	0	0	0	0	0	0				-	-	-
Chlorofluazuron*													0	0	0	0	0	0	0	0	0				-	-	-
XRD-473*													0	0	0	0	0	0	0	0	0				-	-	-
*Bee venom**													0	60	100	0	10	100	0	0	70	0	0	0	-	179	36
Other neurotoxins																											
Procaine**	0	53	97	0	43	43	0	47	10	0	33	3	0	0	0										-	2169	1664
4-amino pyridine**							33/7	73	80	17/3	50	57	3/0	13	7	0	0	3							594	459	428
TEA*							0	10	30	0	0	0	0	0	0	0	0	0							-	-	-
Equipotent mixtures of neurotoxins																											
4-AP (75%)/																											
RH-5849 (25%)**							33/0	93	97	43/3	63	53	3/0	30	27										336	296	312
p,p'DDT (65%)/																											
RH-5849 (35%)**										43/10	87	90	60/7	80	77	27/3	23	33							102	150	133
Procaine (92%)/	(3040 µg/g)			(2280 µg/g)			(1520 µg/g)																				
RH-5849 (8%)**	0	67	93	0	50	60	0/7	23	20																-	2326	1998

A: percentage autotomy induction at 1 h/24 h; K: percentage knockdown at 1 h; M: percentage paralysis and mortality at 48 h. *N = 10; **N = 30.

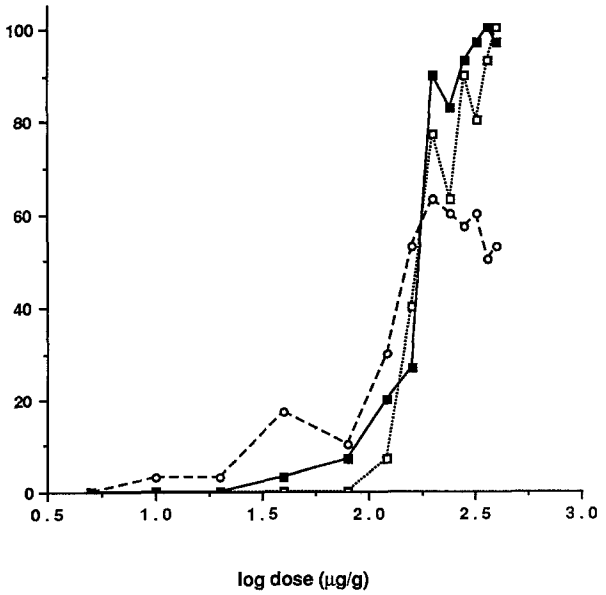

Figure 1. Neurotoxic responses induced by the injection of RH-5849 into newly molted last instar nymphs of *S. americana*. ○ = % autotomy; □ = % knock-down; ■ = % mortality; AD30 = 97 µg/g; KD50 = 192 µg/g; LD50 = 143 µg/g.

The neurotoxic responses induced by the injection of RH-5849 into newly molted last instar nymphs of *S. americana* were dose-depenent, but the percentage of autotomy was never higher than 60% (fig. 1). The effective doses to induce autotomy (AD30), knock-down (KD50) and mortality (LD50) were 97, 192 and 143 µg/g respectively. A very similar pattern of neurotoxic responses was induced by the injection of RH-5849 into older last instar nymphs (AD30 = 83 µg/g; KD50 = 195 µg/g; LD50 = 140 µg/g) and adults (AD30 = 146 µg/g; KD50 = 183 µg/g; LD50 = 167 µg/g) of *S. americana*, and last instar nymphs of a different species of grasshoppers, *M.d. melanistic* (AD30 = 201 µg/g; KD50 = 209 µg/g; LD50 = 216 µg/g). Autotomy and the other neurotoxic responses were also induced by the injection of RH-5849 into third instar nymphs of *S. americana* and *M.d. melanistic*, and in nymphs and adults of several unidentified species of grasshoppers. The injection of RH-5849 into nymphs and adults of the house cricket, *Acheta domestica*, and into adults of the milkweed bug and the American cockroach, *Periplaneta americana*, induced tremors of the body followed by paralysis and death. However, despite the strong convulsive movements of the legs, autotomy was never observed.

The injection of RH-5849 into the hindlegs of newly molted last instar nymphs of *S. americana* induced autotomy of the same hindleg that was injected in 48% of the nymphs. In only one case of twenty a nymph dropped the hindleg that was not injected. Knock-down was absent and mortality at 48 hours very much reduced. Controls injected with 5 µl DMSO dropped the injected hindleg in 18% of the cases. Puncture of the femur, without injection of fluids, induced autotomy in only 2.5% of the nymphs. The injection of RH-5849 into decapitated last instar nymphs induced autotomy and paralysis at similar levels to those found in intact last instar nymphs (data not shown).

The neurotoxic responses induced by RH-5849 were compared with those of other neurotoxins (table 2a, b). Some DDT analogs, pyrethroids, aconitine and 4-AP were able to duplicate the same sequence of neurotoxic responses elicited by RH-5849. Among the DDT analogs, p,p'DDT, p,p'DDD and EDO showed similar activity, whereas o,p'DDT and dicofol lacked activity at the concentrations tested. Pyrethroids were the most active, and were able to induce autotomy at concentrations down to 0.1 µg/g. Even IS-157, the less-active enantiomer of NRDC-257, showed some activity, although only at higher concentrations. The neurotoxins aconitine, veratridine and 4-AP also induced similar responses, but with veratridine only 9% of the cases of autotomy occurred within the first hour, compared to 70% with aconitine, 84% with 4-AP, 78% with pyrethroids, 66% with DDT analogs, and 77% with RH-5849. Furthermore, pyrethroids, aconitine and veratridine were better knock-down than toxic agents, whereas RH-5849 and 4-AP showed similar levels of toxic and knock-down activities, and DDT analogs were slightly better toxicants than knock-down agents. Topical application of NRDC-157 induced the same neurotoxic response as when injected, but the LD50 increased about three times, and the AD30 and KD50 by about one order of magnitude. In addition, the percentage of cases of autotomy within the first hour was reduced to 33%. Topical application of p,p'DDT did not induce any neurotoxic response up to a concentration of 400 µg/g. All other insecticides that were tested induced a different sequence of neurotoxic responses (table 2a, b). Cyclodienes and other chlorinated hydrocarbons did not induce any appreciable response until several hours post-injection, with the exception of lindane that showed knock-down activity. Autotomy sometimes happened, but always in low numbers and after several hours. Organophosphates and carbamates induced tremors within 5–20 minutes, followed by knock-down within the first hour. In 96% of the cases autotomy occurred after several hours. Bee venom and procaine caused knock-down and mortality, but did not induce autotomy. At the higher concentration tested only a small percentage of knock-down and mortality was found with chlordimeform and tetraethyl ammonium chloride (TEA). None of the neurotransmitters and BPUs were able to induce any response at the concentrations tested.

The joint action of mixtures of toxicants that produce the same measured activities may have additive, synergistic or antagonistic effect. We tested the mixtures of RH-5849 with p,p'DDT, 4-AP or procaine, each con-

Table 3. Observed and predicted joint action of the equipotent mixtures of RH-5849 with the neurotoxins p,p'DDT, 4-amino pyridine (4-AP) and procaine.

Mixtures of neurotoxins	Observed		Predicted		Synergistic ratio (Pre/Obs)
		95% CL (μg/g)		95% CL (μg/g)	
4-AP (75%)/ RH-5849 (25%)	LD50 = 312 μg/g	(256, 380)	LD50 = 286 μg/g	(238, 337)	0.92
	KD50 = 296 μg/g	(236, 376)	KD50 = 341 μg/g	(285, 401)	1.15
	AD30 = 336 μg/g	(284, 400)	AD30 = 260 μg/g	(198, 344)	0.77
p,p'DDT (65%)/ RH-5849 (35%)	LD50 = 133 μg/g	(99, 178)	LD50 = 203 μg/g	(171, 236)	1.52
	KD50 = 150 μg/g	(116, 192)	KD50 = 265 μg/g	(235, 316)	1.77
	AD30 = 102 μg/g	(74, 142)	AD30 = 124 μg/g	(93, 166)	1.22
Procaine (92%)/ RH-5849 (8%)	LD50 = 1998 μg/g	(1820, 2199)	LD50 = 899 μg/g	(759, 1048)	0.45
	KD50 = 2326 μg/g	(1972, 2730)	KD50 = 1189 μg/g	(877, 1540)	0.51

The predicted action of the mixtures was calculated based on the ED50s of each neurotoxin and the make-up of the mixture by the equation: 1/ED50(mix) = [proportion toxin-1/ED50(1)] + [proportion toxin-2/ED50(2)].

Table 4. Viability and length of the last instar in mechanically autotomized last instar nymphs of *S. americana*.

Group	N	Dead during last instar	Dead during ecdysis	Normal adults	Adults with curled wings	Adults with tip of wings folded	Length of last instar (days) (mean ± SE)
No hind legs	50	0	2	36	4	8	8.29 ± 0.10
1 hind leg	50	1	2	42	0	5	8.45 ± 0.11
Control	50	1	0	48	0	1	8.49 ± 0.12

taining equipotent concentrations of these neurotoxins, as calculated by the ratio of their respective LD50s (table 2). We then considered whether the observed responses to the mixtures were significantly more or less active than expected on the basis of additive action (table 3). When two toxins have additive effects we can predict the joint action of the mixture (ED50mix) based on the ED50s of the individual components and the make up of the mixture by the following equation: 1/ED50(mix) = [proportion toxin-1/ED50(1)] + [proportion toxin-2/ED50(2)]. We found that the mixture of 4-AP (75%) with RH-5849 (25%) showed similar activities to those predicted on the basis of additive action. On the other hand, the mixture of p,p'DDT (65%) with RH-5849 (35%) was more active than predicted, suggesting a synergistic effect. The difference between the observed and predicted action was significant for the KD50, with a synergistic ratio of 1.77, but not for the AD30. The difference for the LD50 was in the limit of significance (95% confidence limits slightly overlap), with a synergistic ratio of 1.52. Finally, the mixture of procaine (92%) and RH-5849 (8%) was half as active as predicted, suggesting an antagonistic effect. In this case the differences were significant for both LD50 and KD50, with synergistic ratios of 0.45 and 0.51 respectively (table 3).

The mechanically induced autotomy of one or both hind legs in last instar nymphs of *S. americana* did not affect the survivorship during the last instar and only reduced it by 4% at the time of adult ecdysis (table 4). However, whereas 98% of the control nymphs that molted to adults were normal, only 75% of nymphs lacking both hind legs and 89% of nymphs with one hind leg were able to molt successfully to normal adults. The rest of the resulting adults possessed curled front wings or wings with their tips folded (table 4). The length of the last instar was not significantly affected by autotomy (table 4).

Discussion

The ecdysone agonist RH-5849 was discovered to be an effective neuropoison when injected in nymphs and adults of the grasshopper *S. americana*. Treated grasshoppers became immediately hyperactive, exhibiting tremors of the body and convulsive movements of the hind legs, followed by loss of coordination, paralysis and eventually death. These neurotoxic symptoms are similar to those reported for the American cockroach[17] and some species of Coleoptera[7]. We have corroborated the symptoms observed in the American cockroach, and showed that similar neurotoxic responses also occurred in the milkweed bug, and the house cricket. However, during the first minutes of the excitatory reaction the grasshoppers may drop one or both hind legs (chemically induced autotomy).

We did not find any evidence of ecdysonergic or morphogenetic activities of RH-5849 in grasshoppers. There was no induction of premature molting at any concentration tested, and the nymphs completed development to normal adults in about the same time as controls, except for those survivors of the injection of 200 and

100 µg/g who required one more day to molt to adults. It has been reported that sublethal doses of 20-hydroxyecdysone[21] and RH-5849[5,14] delay molting, probably because at low concentrations these compounds, though unable to induce molting, may induce the metabolism of both exogenous and endogenous hormones. However, the delay observed in the molting of grasshoppers appears to be a consequence of the neurotoxic activity of RH-5849. Following treatment at these high concentrations, all nymphs demonstrated a neurotoxic response and even the survivors became prostrate for a couple of days without feeding before they started to recover, which could account for the delay in the length of the instar. Furthermore, it is known that in cockroaches and crickets the regeneration of amputated legs and other appendages increases the duration of the intermolt period[22]. Since autotomy of hindlegs was common at these concentrations, it might be argued that this could be an alternative reason for the delay observed in the molt. Nevertheless, we found that this is not the case either, since grasshoppers never regenerate autotomized hindlegs, and the length of last instar was not significantly affected in mechanically autotomized grasshoppers. The lack of any type of activity following the injection of 20-hydroxyecdysone might be due to its inactivation before it reached the target tissues. It has been reported that 20-hydroxyecdysone injected into the African migratory locust, *Locusta migratoria*, is rapidly metabolized and excreted at stages when the titers of endogenous hormone are low[23,24].

Autotomy is a common predator escape response mechanism in grasshoppers[25] and crickets[26,27]. A grasshopper held by one of its legs can escape by voluntarily discarding it. Autotomy is achieved by a rupture between the base of the femur and the trochanter as a result of violent muscular contractions. In the process no muscles are damaged, and a membrane between the trochanter and the femur acts as a diaphragm to prevent blood loss after autotomy[28]. Once lost, the hind legs never regenerate. Autotomy in grasshoppers and crickets can be induced by physical stimulus applied to the hind femur, such as mechanical pressure, temperature or an electrical shock[25,28]. Chemically induced autotomy has been also described. Brousse-Gaury[28] showed that the injection of potassium cyanide or the inhalation of formaldehyde vapors by the house cricket induced autotomy, but not sulfur dioxide or tobacco vapors. Uvarov[25] mentioned that autotomy occurs when grasshoppers are put into a killing bottle. We have found that RH-5849 was able to induce autotomy in different species of grasshoppers, but not in the house cricket. On learning of our work with *S. americana*, Pener (Hebrew Univ of Jerusalem) found that RH-5849 would also induce autotomy in the African migratory locust, and the desert locust, *Schistocerca gregaria*, but not in the cricket *Gryllus bimaculatus* (personal communication). A variety of neurotoxins with different modes of action were also able to induce autotomy in *S. americana*. This suggests that chemically induced autotomy in grasshoppers is a non-specific response to many neuropoisons that are able to produce violent muscular contractions. We have found, however, two exceptions in the carbamate aldicarb and bee venom, which were able to produce tremors and spasms but failed to induce autotomy. The case of bee venom is of particular interest, since it has been reported to induce autotomy in the orb-weaving spider, *Argiope spp.*[29]. In this species, autotomy occurs at the coxa-trochanter joint when a spider is stung in a leg by a poisonous predator.

Brousse-Caury[28] showed that in the house cricket autotomy disappears if the nervous connection between the hindlegs and methathoracic ganglia were cut. On the other hand, decapitation of crickets did not prevent the occurrence of autotomy, suggesting that autotomy is a reflexive action controlled by the methathoracic ganglia. Our results support this contention, since chemically induced autotomy in grasshoppers was not prevented by decapitation. Salgado[17] showed that the convulsive leg movements induced by the injection of RH-5849 into adults of the American cockroach were not affected when the connective nerves between thoracic ganglia were cut. However, when a thoracic ganglia was severed from the leg nerve, the movement of that leg stopped, suggesting that RH-5849 may stimulate activity in motor circuits by affecting the central nervous system. We have found that injection of RH-5849 into the abdomen of grasshoppers induced bilateral autotomy, whereas injection into the femur of a methathoracic leg induced autotomy of only the injected leg in most cases. This unilateral autotomy cannot be totally explained by physical stimulus, since injection with DMSO or puncture with the microsyringe induced autotomy of the treated leg at a much lower rate. Therefore, chemically induced autotomy in grasshoppers appears to be the result of the activity of RH-5849 in the peripheral nervous system or muscle itself, in addition to the central nervous system.

Since only DDT analogs, pyrethroids, aconitine and 4-AP were able to match the sequence of neurotoxic symptoms elucidated by RH-5849, they may possess a similar mode of action. Pyrethroids, DDT analogs and aconitine are known to prolong the action potential of nerve membranes by retaining depolarizing voltage-dependent sodium channels in an open configuration[30,31]. On the other hand, 4-AP prolongs the action potential of muscle and nerve membranes by blocking the potassium channels that restore the resting potential[32]. If RH-5849 selectivity blocks voltage dependent potassium channels, we expect that it will synergize with a neurotoxin that selectivity opens sodium channels (p,p'DDT), and have and additive effect with a neurotoxin that blocks potassium channels (4-AP). On the other hand, if RH-5849 retains sodium channels in an open configuration, it will synergize with 4-AP and have

an additive effect with p,p'DDT. In both cases it should be antagonized by a neurotoxin that specifically blocks sodium channels (procaine)[33,34]. We discovered that RH-5849 synergizes with p,p'DDT, antagonizes with procaine and has an additive effect with 4-AP, suggesting that RH-5849 has a mechanism of action similar to 4-AP, by blocking potassium channels. During the course of our investigations, Salgado[17,19] arrived at the same conclusion, establishing that the selective block of potassium channels in neurons and muscles is the primary neurotoxic mechanism of action of RH-5849.

Because of the important role of the hind legs during ecdysis, Uvarov[25] suggested that autotomy in nymphs may interfere with the molting process. We have found that autotomy of one or both hind legs did not reduce significantly the survivorship rate at the time of ecdysis, but 11 and 25% of the resulting adults respectively presented some type of morphological abnormalities in the wings. Most autotomized nymphs were observed to complete the molting process successfully. However, some fell from the support at the end of ecdysis, and the resulting adults were forced to stretch their wings under unnatural conditions. This may explain why some nymphs lacking one or both hind legs molted to adults possessing morphological abnormalities in their wings. Antotomy and the other neurotoxic symptoms are also induced in the desert locust and the African migratory locust (Pener, personal communication), opening a new approach to locust and grasshopper control. Lethal doses of RH-5849 would kill most of the population in a brief period of 2–3 days, preventing the consumption of vegetation immediately after treatment. Autotomy of survivors would prevent marching in nymphs and interfere with the last ecdysis to produce adults with limited capacity to fly. Affected nymphs and adults without the capacity to migrate will die by starvation. Unfortunately, our results have revealed that RH-5849 is only effective when injected, and is without any activity by contact or topical application. In addition, lethal doses were reached at relatively high concentrations, while sublethal doses induced very low levels of autotomy. The optimization of RH-5849 to increase its neurotoxicity and suitable formulations for ingestion or topical application would be required before growth regulators acting, like RH-5849 could be considered as any practical alternative for locust control.

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