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Single cell recordings reveal hydroxydanaidal as the volatile compound attracting insects to pyrrolizidine alkaloids

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Abstract

Employment of electrophysiology in combination with simple chemical techniques elucidated the volatile which permit the localization of sources of pyrrolizidine alkaloids (PAs) by insects exploiting these secondary plant metabolites.

Single cell recordings in *Rhodogastria* moths (Arctiidae) revealed a physiologically clearly separable type of antennal sensilla basiconica ('SB II') which responds to headspace air of certain PAs (*e.g.* monocrotaline), but not to a great variety of 'odorants'. However, stimulus sources of 1 μg were required to elicit responses and the maximum frequency (obtained with stimulus sources of 5 mg) was only 50 imps/s. This suggested the occurrence of small amounts of airborne PA degradation products.

Extraction and hydrolysis experiments in combination with thin-layer chromatography and using the sensory responses of antennal receptor cells as biological detectors eventually demonstrated that the dihydropyrrolizine 'hydroxydanaidal' emanates in small amounts from sources of those PAs which contain retronecine and heliotridine, respectively, as necine moiety. This substance was also implicated as the volatile mediating attraction of the insects to PA-containing plants, as well as to artificial PA-baits. With respect to the high sensitivity and specificity of SB II-receptor cells to hydroxydanaidal, in particular to its R(–)-enantiomer, they are analogous to the well studied receptor cells for sex-attractant pheromones in Lepidoptera. Similar results were obtained with *Danaus* (Danainae) and *Euchromia* (Ctenuchiidae). Initial behavioural tests have proven the attractive power of hydroxydanaidal for PA-insects and thus corroborate our interpretation of the electrophysiological findings.

Introduction

Species of a remarkable diversity of insect groups (Lepidoptera: Arctiidae, Ctenuchiidae, Ithomiinae, Danainae; Coleoptera: Alticinae (*Gabonia*); Orthoptera: Pyrgomorphidae (*Zonocerus*); Diptera: Chloropidae) are known to be attracted to *withered* parts of plants containing certain pyrrolizidine alkaloids (PAs), *e.g.* to species of Boraginaceae, Asteraceae, and Fabaceae. The insects ingest these secondary plant substances and exploit them for various purposes (for review and refs. see Boppré, 1986). Investigations of this phenomenon have left many questions unanswered, amongst which the most ba-

sic concern the specificity of the insects' responses and the chemical nature of the volatile signal(s) which mediate orientation. Because not only plant material but also baits of purified PAs extracted from certain plants (*e.g. Crotalaria scassellatii*) attract the insects in an upwind flight over a long range, there is no doubt that orientation towards PA-sources must be mediated olfactorally and also directly related to PAs and not to any other plant chemical (Boppré, 1986, unpubl.; *cf.* Boppré, 1986; Boppré & Pitkin, 1988; Scherer & Boppré, in prep.): this also emphasizes the peculiarity of this insect-plant relationship (an example of 'pharmacophagy'; Boppré, 1984). However, the high molecular weight of PAs suggests that the insects do not perceive the intact plant products themselves but rather a 'PA-odour' (*i.e.* a volatile emanating from PAs) which could be expected to be a degradation product of PAs.

Our failure to collect airborne chemicals from the headspace air of PA-plants, the unavailability of a reasonable variety of PAs in amounts sufficient for field tests, and difficulties in obtaining quantitative baiting data in the field suggested the use of electrophysiology as a suitable investigative tool. With a sensory physiological approach we hoped to achieve three goals: first to identify the receptor cells responsible for sensing PA-odour, then to make use of these to find volatiles potentially mediating attraction to PA-sources, and finally to develop precise behavioural field tests. In addition, we hoped to gain information on the specificity and sensitivity of the respective receptors for comparison with physiological features of pheromone receptor cells. The chemistry involved was only pursued as far as needed in biological context; most chemical details remained unstudied.

Material and methods

Moths of the genus *Rhodogastria* (Arctiidae) were chosen as main organisms for electrophysiological studies because they can be reared continuously throughout the year and both sexes respond to PAs (Boppré, 1981). Most data reported here were obtained with *Rhodogastria luteibarba* Hampson, however, other species (*R. bubo* (Walker), *R. phaedra* (Weymer/Boppré), *R. vitrea* (Plötz), *R.* sp. C) were also investigated but they showed no difference to *R. luteibarba.*

Cultures of *Rhodogastria* originated from females collected in the field in Kenya, East Africa, at PA-baits (made of 'PA-extract' (*cf.* below); Boppré, unpubl.). Larvae were reared at 20–24 °C and L12:D12 on a semi-artificial diet (Bergomaz & Boppré, 1986). Specimens used for electrophysiological tests were 1–10 days old.

The results obtained with *Rhodogastria* moths were compared with the danaine butterfly *Danaus chrysippus* (L.), in which species attraction to PAs is strongly male-biased and the males use the PA-derivative 'danaidone' as a pheromone component (Meinwald *et al.*, 1971); in addition, therefore, we studied reception of danaidone. Offspring of field-caught females from Kenya were reared on the asclepiad *Asclepias curassavica* L. in a greenhouse. Also, we made comparative tests with the ctenuchiid moth *Euchromia amoena* (Moeschler), both sexes of which visit PA-sources during the day (Boppré, 1981); *Euchromia* were reared on *Ipomoea* sp. (Convolvulaceae).

To aid single cell recordings, the variety of antennal sensilla found in *Rhodogastria* and *Euchromia* was surveyed with a scanning electron microscope using air-dried specimens (the antennae of *Danaus gilippus,* a species very closely related to *D. chrysippus,* have been investigated in detail by Myers, 1968); preparations were kept in a saturated OsO_4 atmosphere for at least 48 h, then gold-coated in a Hummer II (Technics Inc.) sputtering chamber before they were examined using a NOVASCAN 30 (Zeiss) operated at 15 kV.

Electroantennograms (EAGs) and electric activities of single cells were recorded using standard techniques (Schneider, 1957; Boeckh, 1962); the glass-capillary Ag/AgCl electrodes had a tip diameter of 1 μm and were filled with ringer-solution after Kaissling & Thorson (1980). Although illumination with fibre optics made it possible to recognize various sensilla, positioning an electrode at the base of a hair for extracellular single cell recording was a matter of trial and error because the bases were not visible. For registration of spikes, a casette tape

recorder (SONY TC) was used; playback of these recordings onto an oscilloscope allowed filming of selected sections with a 'Recordine' (Tönnies, Freiburg). Spikes were counted over the entire (1 s) stimulus time, *i.e.* 'imps/s'-values given in the following are absolute, not extrapolated from spike counts in the phasic parts of the responses.

Initially, *i.e.* in the course of searching for 'PA-odour' receptors, stimuli used were headspace air* of various 'odorants' (carbonic acids, alcohols, aldehydes, ketones, etc; *cf.* Table 1), and pyrrolizidine alkaloids (either a mixture of axillaridine and axillarine (resembling monocrotaline in structure) extracted from seeds of *Crotalaria scassellatii* Chiov. (Fabaceae) from Kenya (*cf.* Wiedenfeld *et al.*, 1985) and hereafter called 'PA-extract', or the commercially available PAs monocrotaline and heliotrine (Fig. 1A, B) and their N-oxides). Pilot tests were conducted with other PAs (see Table 2) available in small amounts only. PA-extract was used because baits made of it had proved long-lasting and very powerful attractants in the field (Boppré, unpubl.).

Stimuli were applied from an olfactometer (*cf.* Kafka, 1970; Saß, 1976) equipped with 25 ml plastic syringes housing 8 ml glass-vials which contained the chemicals. In the case of the odorants, the vials contained 1 ml of a 10^{-2} dilution in liquid paraffin. In the case of PAs (and other solid chemicals, *cf.* below), the vials were loaded with equivalents of a methanolic solution (1 mg/ml) and the solvent evaporated. Vials containing only paraffin oil or 'evaporated methanol' were used for control stimuli. The headspace air (ca 15 ml) was delivered onto the preparation for 1 s.

Having found the sensilla responding to PA-odour, firstly PA-extract, later monocrotaline and heliotrine were submitted to a variety of treatments in order to characterize and isolate the volatile compound(s) which evidently evaporates from certain PA-sources. Stimuli used in these experiments were

* With many natural products, particularly those from biological sources, the air above the sample might not only contain the volatile components (if any) but volatile derivatives in addition. Thus, we use the term 'headspace air' to refer to the unknown chemical composition of the actual stimulus. Consequently, we talk about 'stimulus loads' because the concentration of volatiles reaching the receptor cell is unknown, too.

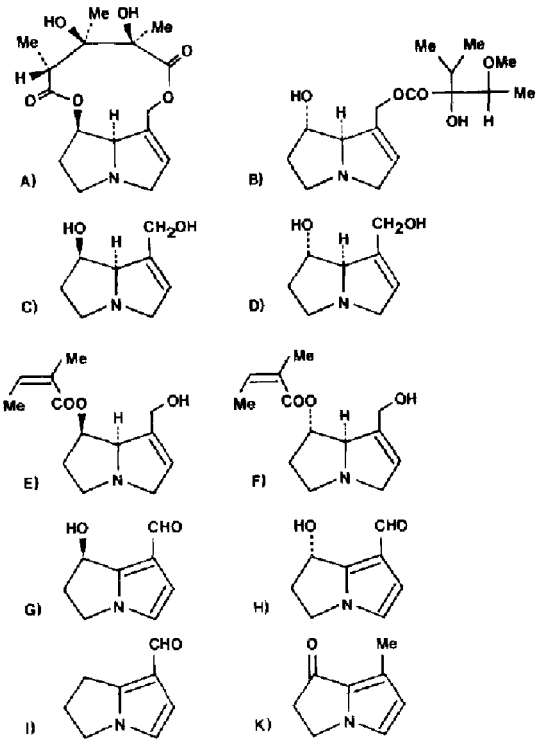

Fig. 1. Molecular structures of principal compounds used in this study. A: monocrotaline, B: heliotrine, C: retronecine, D: heliotridine, E: 7-angelylretronecine, F: 7-angelylheliotridine, G: R(−)-hydroxydanaidal, H: S(+)-hydroxydanaidal, I: danaidal, K: danaidone.

(for experimental rationale *cf.* Discussion):

- PA treated with $Ba(OH)_2$: a saturated, aqueous solution of $Ba(OH)_2$ was added to a methanolic PA solution (1 mg/ml) (vol:vol = 1:1) and stirred under reflux for 1 h in a 60 °C water bath; the supernatant liquid was transferred to a vial, evaporated and then used as a stimulus source (= 'PA + $Ba(OH)_2$').
- PA treated with HCl: 0.1 N HCl was added to a methanolic PA solution to give pH 1, the liquid evaporated and then used as a stimulus source (= 'PA + HCl').
- Extracts of PA + $Ba(OH)_2$ with organic solvents: diethylether or CCl_4 (in pilot tests other solvents including CH_2Cl_2 and hexane) was added to PA + $Ba(OH)_2$ (see above) vol:vol = 1:1 and shaken; both fractions were tested after evaporation of the solvents.
- Washings of PAs with organic solvents: diethylether or CCl_4 was added to methanolic solu-

tions of PA or to the solid alkaloid, respectively; the residue and the liquid phase (after evaporation of the solvents) were tested separately.

Quantitatively, the above treatments were manipulated so that each stimulus vial finally contained 1 mg of PA or the amount resulting from the treatment of 1 mg; 3 vials of each stimulus type were available for testing. Control stimuli were $Ba(OH)_2$, 0.1 N HCl, diethylether, CH_2Cl_2, hexane and CCl_4.

Further stimulus sources were diethylether extracts of individual bands of silica gel from thin layer plates (see below), coremata of *Creatonotos transiens* (which provide a natural source of (R(–)-hydroxydanaidal if the larvae have been fed with PA; *cf.* Discussion) or diethylether extracts thereof. To study the specificity and sensitivity of the receptor cells in more detail, synthetic samples of the PA-derived dihydropyrrolizine pheromones R(–)- and S(+)-'hydroxydanaidal', 'danaidal' and 'danaidone' (Fig. 1G–K) were used.

A recovery period of at least 3 min was generally allowed between stimuli. The preparation was kept under a stream of air humidified by passing through water in a gas-washing bottle, except during stimulation.

Thin-layer chromatography (TLC) was employed in the course of trials to identify the volatile token(s). Samples of stimuli (see above) in amounts of equivalents to 1 mg PA were spotted onto Polygram SIL G/UV sheets (Machery & Nagel) and run with methanol:chloroform:ammonia (14.5:85:0.5). For detection of pyrrolizidine alkaloids and dihydropyrrolizines, the Ehrlich pyrrole reaction was used (*cf.* Mattocks, 1986; plates were kept in a saturated iodine atmosphere for 15 min and then sprayed with 10% 4-dimethylamino-benzaldehyde in concentrated HCl, diluted 1:4 with acetone just before use; for a more selective detection of dihydropyrrolizines, treatment of the sheets with iodine was omitted (J. A. Edgar, pers. comm. to M.B.)).

Results

Antennal sensilla in Rhodogastria. The filiform antennae of *R. luteibarba* are about 15 mm long and 200 μm wide tapering to 130 μm at the tips. In both sexes they consist of 65–70 segments all of which have a basically similar distribution of four types of hairs (Fig. 2A, B): i) 150 μm or so long bristles, arranged on a spiral line; ii) numerous s. basiconica, distributed on the entire antennal surface; iii) s. trichodea, occurring on a 150 μm wide longitudinal band directed forward when the antennae are held upright; iv) 1–3 sensilla styloconica are found distally on all segments except the basal 10. The antennae are entirely covered by microtrichia, amongst which several s. coeloconica and a few s. campaniforma are scattered. The fine-structure of antennal hairs in *Rhodogastria* has not been investigated, but sensilla types seem to conform with those already well known from other taxa (Altner & Prillinger, 1980; Keil & Steinbrecht, 1984); sensilla trichodea and s. basiconica appeared as the most likely candidates for PA-odour reception and we concentrated single cell recordings on these.

Antennal sensilla in Euchromia. The antennal segments of *E. amoena* possess prominent lateral projections which are bend and thus form a ridge housing many sensilla trichodea and some s. basiconica (Fig. 2C). The outer lateral sides of the projections (which taper and end with a bristle; Fig. 2C, D) exhibit s. basiconica exclusively (Fig. 2D). The entire antennal surface is densely covered by microtrichia, and there is no apparent difference between the sexes.

Electroantennograms (Rhodogastria). Testing the excitatory effects of PAs and 75 odorants proved the antennae capable of perceiving PA-odour and revealed a number of stimulative odorants. Table 1 lists examples of responses to 34 odorants. The maximum amplitudes were in the range of 1.4 mV and could be obtained, for example, by stimulation with 10^{-2} concentrations of butanol or octanol. However, high amounts of PAs were required as stimulus loads to elicit responses (Fig. 3) and even these had comparatively weak effects: loads of 1 mg of monocrotaline were needed to elicit responses different from controls, and as much as 5 mg to obtain maximal amplitudes. PA-extract and monocrotaline

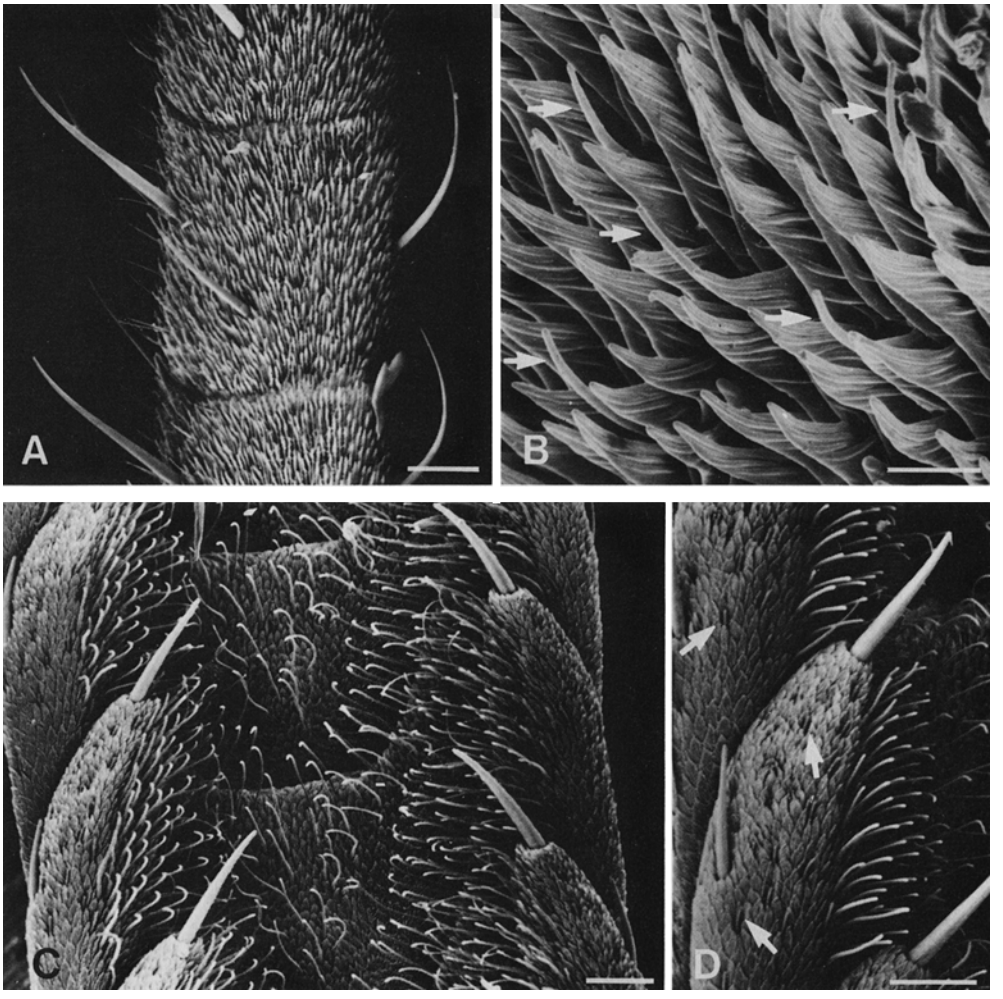

Fig. 2. Scanning electron micrographs. A, B: An antennal segment (A) and a part of it (B) of a male *Rhodogastria luteibarba*. C, D: Part of antenna (C) and lateral view of projection (D) of a male *Euchromia amoena*. Arrows point to s. basiconica. Scale bars: A, C, D: 50 μm, B: 10 μm.

proved to be 5–6 times more powerful as a stimulus than heliotrine. EAGs showed no difference between the sexes.

Specific receptor cells for perception of 'PA-odour' (Rhodogastria). In most preparations, the activity of one single cell could be recorded for several hours. In recordings from sensilla trichodea two or three spike amplitudes (0.5–3 mV) were evident, but stimulation with PA-odour never yielded responses. We therefore here ignore this type of receptor cells.

Recordings from sensilla basiconica revealed at least two distinct types of reaction spectra without any overlap, but the respective hairs were outwardly indistinguishable (*cf.* Bogner & Boppré, 1985). We obtained either up to three spike amplitudes (0.8/1.2/2 mV) with an overall spontaneous spike frequency of 10–15 imps/s (type 'SB I'; Fig. 4A, left), or only spikes of 1 mV with a spontaneous activity of 4–8 imps/s (type 'SB II'; Fig. 4A, right). In recordings of SB I, a variety of odorants (*cf.* Table 1) yielded excitatory effects (on one or the other spike class, not evaluated in detail) but PA-odour was not responded to (Fig. 4B, C *vs.* D, E, left); maximum spike frequencies during stimulation were 180 imps/s. In recordings of SB II, none of the odorants

which had been effective in EAG-recordings increased the spontaneous activity, but stimulation with headspace air of several PAs was excitatory (Fig. 4B, C *vs.* 4D, E, right). This latter finding indicated that SB II-receptor cells might be specific for reception of PA-odour. The responses were found to be dose-dependent, however, samples of pure PA peaked at 50 imps/s (*i.e.* below the expected maximum), even though excessive amounts of stimulus loads (5 mg) were used. Consistent with the EAG data, PA-extract and monocrotaline were much better stimuli than heliotrine.

Characterization of volatile stimulus component (Rhodogastria). Having found that SB II-receptor cells responded to PA-odour, we restricted further recordings to those sensilla basiconica bearing this receptor type and tested the stimulatory effects of various other pyrrolizidine alkaloids, of differently treated monocrotaline and heliotrine, and eventually of PA-derived lepidopteran pheromones.

Table 2 lists the reactions various PAs caused on SB II-receptors. The data show that in general all stimulatory PAs contain the amino alcohols retronecine or heliotridine (structures: Fig. 1C, D), while

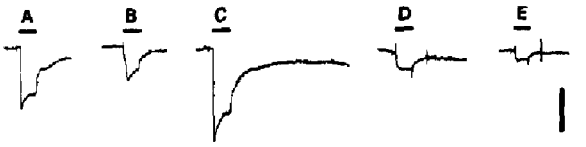

Fig. 3. EAGs from an isolated antenna of a male *Rhodogastria luteibarba* after stimulation with octanol (A, 10^{-2} dilution), monocrotaline (B, 0.5 mg; C, 5 mg), heliotrine (D, 5 mg); E: control stimulus ('evaporated methanol'). Bars: stimulus duration (=1 s), 1 mV.

PAs composed of the necines otonecine or platynecine were – like N-oxides of monocrotaline and heliotrine – not excitatory. Significantly different responses to 7-angelylretronecine and to 7-angelylheliotridine, two PAs with identical acid but slightly different in the structure of their alcohol moieties (Fig. 1E, F), matched the responses to monocrotaline and heliotrine, the latter sharing the alcohol moieties with 7-angelylretronecine and 7-angelylheliotridine, respectively. These findings indicated that the alcohol rather than the acid moiety is a key for determining the stimulatory power of the ester-loaded stimulus sources. This idea was substantiated by finding senecic acid and viridifloric

Table 1. EAG-responses of male *Rhodogastria luteibarba* to stimulation with various odorants (10^{-2} dilution) and the PAs monocrotaline and heliotrine (5 mg). o = no response; + = 0.3 mV; + + = 0.6 mV; + + + = 0.9 mV; + + + + = 1.2 mV.

Test compound	EAG-resp.	Test compound	EAG-resp.
monocrotaline	+ + +	heliotrine	+
allylacetate	+ +	heptanoic acid	+ +
benzylbenzoate	+	hexanal	+ +
benzylalcohol	+ + +	n-hexanol	+ + +
n-butanol	+ + + +	hexylacetate	o
butylacetate	+ +	α-ionone	o
(+)-carvone	o	β-ionone	o
cit-trans-citral	+	linalool	o
citronellal	o	menthon	o
citronellol	o	6-methyl-5-hepten-2-one	+
citronellylacetate	+	nonanal	+
citronellyformiate	o	n-nonalol	o
decanal	o	nonanoic acid	o
diphenylether	o	octanal	o
dodecanal	+	n-octanol	+ + + +
n-dodecanol	+	4-phenylbutanone-2	+
geraniol	+ +	propylacetate	+
n-heptanol	+ +	terpineol	+ + + +

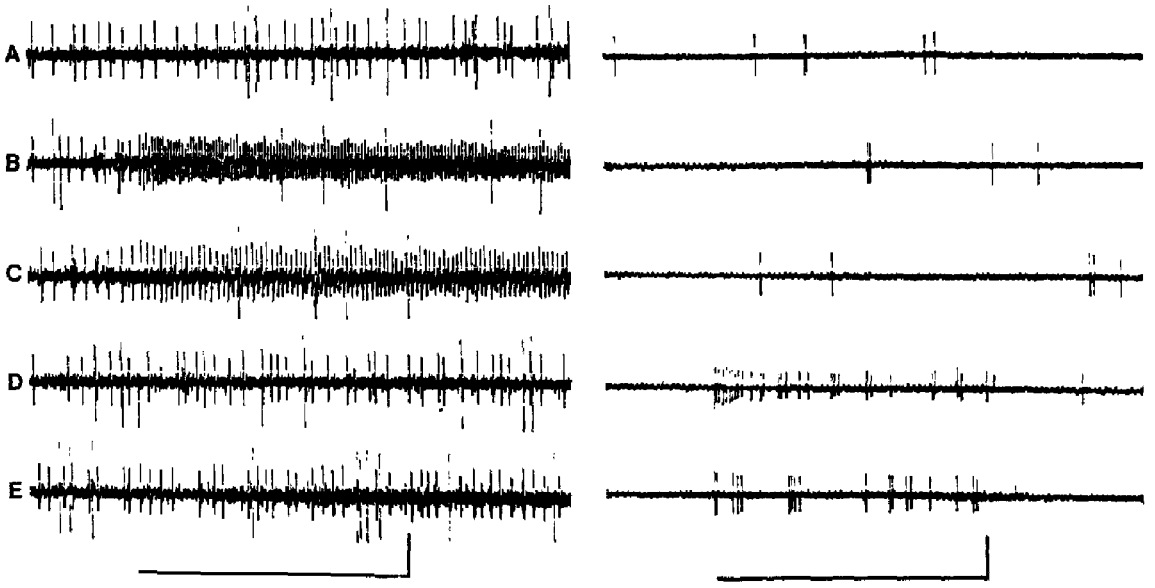

Fig. 4. Recordings from 'SB I'- (left) and 'SB II'- (right) receptor cells of a male *Rhodogastria luteibarba.* A: control stimulus (paraffin and 'evaporated methanol', respectively). SB I responds to stimulation with various 'odorants' (B: 10^{-2} octanol, C: 10^{-2} geraniol), but not to 'PA-odour' (D: 5 mg monocrotaline, E: 5 mg heliotrine), SB II is not affected by 'odorants' but by 'PA-odour'. Bars: stimulus duration (=1 s), 1 mV.

Table 2. Activity of 'SB II'-receptor cells of a male *Rhodogastria luteibarba* stimulated with various pyrrolizidine alkaloids. Responses expressed by average values and s.d.

Stimulus (1 mg)	Response (n = 16)
heliotrine (= heliotridine + heliotric acid)	10 ± 3 imps/s
monocrotaline (= retronecine + monocrotalic acid)	36 ± 9 imps/s
spektabiline (= retronecine + acetyl-monocrotalic acid)	15 ± 5 imps/s
lycopsamine (= retronecine + (−)-viridofloric acid)	16 ± 5 imps/s
senecionine (= retronecine + senecic acid)	15 ± 6 imps/s
platyphylline (= platynecine + senecic acid)	5 ± 2 imps/s
senkirkine (= otonecine + senecic acid)	3 ± 2 imps/s
florosenine (= otonecine + acetyl jacobinecic acid)	4 ± 2 imps/s
monocrotaline-N-oxid	6 ± 3 imps/s
heliotrine-N-oxid	5 ± 3 imps/s
7-angelylretronecine (= retronecine + angelic acid)	29 ± 5 imps/s
7-angelylheliotridine (= heliotridine + angelic acid)	11 ± 5 imps/s
retronecine	39 ± 6 imps/s
heliotridine	8 ± 3 imps/s
control ('evaporated methanol')	4 ± 3 imps/s

Table 3. Activity of 'SB-II'-receptor cells of male *Rhodogastria luteibarba* stimulated with monocrotaline and heliotrine and various treatments of these PAs. Responses expressed by average values and s.d.

Stimulus (1 mg)	Response (n = 9)
monocrotaline	23 ± 3 imps/s
monocrotaline + $Ba(OH)_2$	35 ± 7 imps/s
monocrotaline + $Ba(OH)_2$ + HCl	10 ± 1 imps/s
monocrotaline + $Ba(OH)_2$ + HCl + $Ba(OH)_2$	32 ± 5 imps/s
monocrotaline + HCl	7 ± 1 imps/s
heliotrine	14 ± 3 imps/s
heliotrine + $Ba(OH)_2$	27 ± 4 imps/s
heliotrine + $Ba(OH)_2$ + HCl	10 ± 3 imps/s
heliotrine + $Ba(OH)_2$ + HCl + $Ba(OH)_2$	23 ± 3 imps/s
heliotrine + HCl	6 ± 1 imps/s
control ('evaporated methanol')	6 ± 3 imps/s

acid to be ineffective stimuli.

Since hydrolysis seemed to be an important step in the degradation of PAs (*cf.* Discussion), we put PAs under hydrolytic conditions. Table 3 shows that $Ba(OH)_2$-treatment increased the stimulatory power of both monocrotaline and heliotrine although in different strength. In contrast, addition of HCl abolished the stimulatory power of previously excitatory stimulus sources (Table 3), an effect which could be reversed by subsequent treatment with $Ba(OH)_2$.

Based on considerations on the solubility of possible hydrolysis products of PAs, we fractionated $Ba(OH)_2$-treated PAs with organic solvents of different polarity and found that polar solvents like diethylether took up the stimulatory volatile(s) but nonpolar ones (*e.g.* CCl_4, hexane) did not; the residues of either fractions had most weak excitatory effects on the SB II-receptor cells while those from extraction with the nonpolar solvents were stimulatory. Corresponding results were obtained by washing untreated PAs with the above mentioned solvents, however, the excitatory effects were much weaker than with $Ba(OH)_2$-treated PAs.

TLC of diethylether fractions of PA + $Ba(OH)_2$ revealed a spot which appeared without iodine treatment as is the case with dihydropyrrolizines. R_f and colour of this spot, which neither showed up in washings with CH_2Cl_2, CCl_4, or hexane nor in samples of PA treated with HCl, was indistinguishable from those of hydroxydanaidal and was – as is hydroxydanaidal – reducible by $NaBH_4$. TLCs of residues from extraction with diethylether lacked the respective spot, but it appeared in those of residues from extraction with the other solvents. In tests with heliotrine the assumed hydroxydanaidal spot was much weaker than in those involving PA-extract or monocrotaline. If untreated PAs were applied, the respective spot did show up very weakly and only if the sheets were overloaded.

Thus, we stimulated SB II-receptors with coremata of *Creatonotos transiens* as well as with synthetic hydroxydanaidal and revealed strong responses (Fig. 5). Dose response curves of reactions to R(–) and S(+)-hydroxydanaidal as well as to danaidal and danaidone are provided in Fig. 6. They demon-

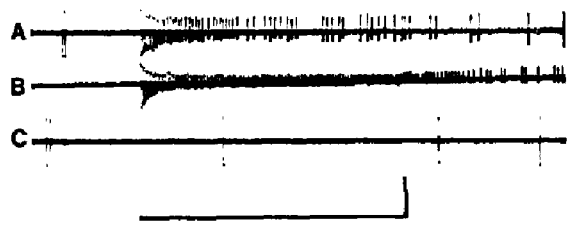

Fig. 5. Activity of 'SB II'-receptor cell of a male *Rhodogastria luteibarba* stimulated with 10 mg of monocrotaline (A) and 10 µg of R(–)-hydroxydanaidal (B), showing maximum activity of this cell type. C: control stimulus ('evaporated methanol'). Bars: stimulus duration (=1 s), 1 mV.

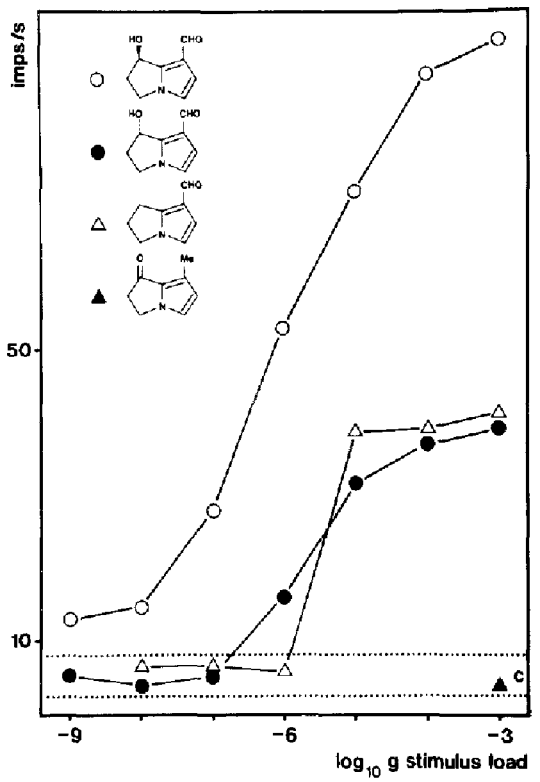

Fig. 6. Dose-response curves of responses of 'SB II'-receptor cells of male *Rhodogastria luteibarba* (n = 10) to stimulation with R(−)-hydroxydanaidal, S(+)-hydroxydanaidal, danaidal, and danaidone; c: range of activity to control stimululi ('evaporated methanol').

strate a high sensitivity of SB II-receptor cells to (R−)-hydroxydanaidal, significant differences of the responses to the two enantiomers of hydroxydanaidal, and the potency of these receptor cells to produce spike frequencies of about 100 imps/s. The strong specificity of SB II-receptors is not only shown by the lack of response to stimulation with odorants but also by much weaker reactions to S(+)-hydroxydanaidal and to danaidal as well as by the complete lack of response to danaidone (Fig. 6). It was also noted that addition of HCl to hydroxydanaidal erased the stimulatory effect completely (*cf.* above PA + HCl).

Reception of PAs and hydroxydanaidal in Danaus chrysippus. Physiologically, the short sensilla basiconica in males of *Danaus chrysippus* belong to two distinct physiological types, one ('SSB I') responding to the PA-derived male pheromone component danaidone, the second ('SSB II') is insensitive to this compound but is excited by stimulation with monocrotaline and R(−)-hydroxydanaidal (Table 4; the S(+)-form was not available for experiments with danaines). S. basiconica were never found to respond to the odorants tested on *Rhodogastria* (see above). In contrast to SSB I, however, SSB II also respond to danaidal. To date, in females we failed to find receptors responding to hydroxydanaidal.

Reception of PAs and hydroxydanaidal in Euchromia amoena. Single cell recordings of males and females of *E. amoena* fully corroborate the findings obtained with *Rhodogastria:* there are two physiological types of sensilla basiconica, one responds to various odorants, the other to PAs but best to R(−)-hydroxydanaidal (Fig. 7).

Table 4. Activity of two cell types ('SSB I', 'SSB II') within short sensilla basiconica of male *Danaus chrysippus* stimulated with dihydropyrrolizines and monocrotaline. Responses expressed by average values and s.d.

Stimulus	Response	
	SSB I (n = 6)	SSB II (n = 4)
100 µg danaidone	110 ± 13	6 ± 4 imps/s
100 µg danaidal	12 ± 8	31 ± 10 imps/s
100 µg R(−)-hydroxydanaidal	10 ± 6	117 ± 14 imps/s
1 mg monocrotaline	14 ± 4	40 ± 10 imps/s
control ('evaporated methanol')	7 ± 3	6 ± 3 imps/s

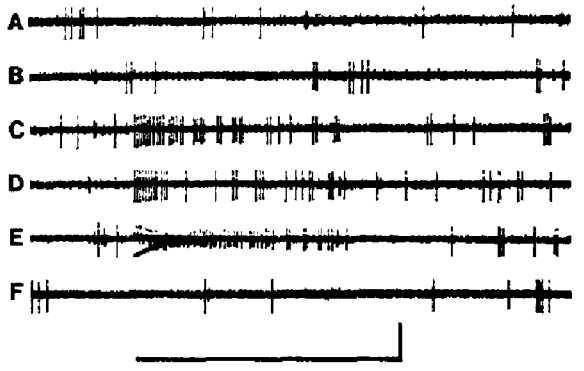

Fig. 7. Recordings from 'SB II'-receptor cell of a female *Euchromia amoena* stimulated with 'odorants' (A: 10^{-2} n-butanol, B: 10^{-2} heptanoic acid), 'PA-odour' (C: 1 mg monocrotaline, D: 1 mg heliotrine) and R(−)-hydroxydanaidal (E, 100 µg). F: control stimulus ('evaporated methanol'). Bars: stimulus duration (=1 s), 1 mV.

Discussion

When C. M. Woodford in 1890 originally discovered males of *Euploea* ssp. congregating and sucking at broken twigs of *Tournefortia argentea* (Boraginaceae), he was quite unable to account for the biological significance of this strange behaviour. We now know that other butterflies and moths as well as insects of several other orders exhibit a corresponding behaviour, and further, that visits of insects at withered plants and damaged parts of certain species of plants are due to their possession of pyrrolizidine alkaloids. These PAs are utilized by the insects in various ways (review: Boppré, 1986).

Prior to this paper, an important unresolved problem in the 'PA-story' has been the identity of the volatile principle responsible for olfactory detection of PA-sources by the insects; it has so far received little attention. Schneider *et al.* (1975) showed by electroantennograms that antennal receptors of *Danaus chrysippus* are excitable by air blown over withered *Heliotropium* plant material, particularly after it had been moistened (*cf.* below). Pliske *et al.* (1976) stated that a volatile product derived from esterifying acids of PAs would attract male Ithomiinae to PA-plants, where the intact alkaloids act as phagostimulants. We believe this report to be inconclusive: firstly, the numbers of specimens attracted to esteryfying acids were very low compared to the high number visiting withered PA-plant material (*cf.* Pliske, 1975a, b); secondly, the chemicals used were apparently unstable and not pure when tested in the field (*cf.* Pliske *et al.*, 1976).

We considered it necessary to avoid trial and error tests with all kinds of potential PA derivatives in the field, and difficulties of making quantitative evaluations of field data. We therefore adopted sensory physiological techniques as well as simple chemical procedures first for screening candidate PA breakdown products, then to isolate, obtain quantified responses to, and identify whatever stimulatory chemical(s) we had found, and finally to use them for behavioural tests.

Experimental rationale. Recording electroantennograms was the routine initial step to demonstrate that receptor cells responsible for perception of PA-odour are located on the antennae. The high stimulus loads of monocrotaline required to elicit EAG responses supported the idea that it is a derivative, present in tiny amounts, which actually stimulates the chemosensilla.

Responses of SB II-receptor cells to stimulation with a variety of structurally different PAs revealed that all those PAs possessing retronecine or heliotridine as heterocyclic moieties had excitatory effects, seemingly independent of the type of the necic acid. This finding was consistent with the data on plants attractive for PA-insects. PAs have been found in a great variety of plant species (Mattocks, 1986), but insects visiting withered PA-containing plants have been recorded only from a limited number of species (mainly of *Heliotropium, Crotalaria, Parsonsia, Ageratum, Senecio, Eupatorium; cf.* Pliske, 1975a; Boppré, 1978, 1986, and refs. therein). The common factor in the PAs of these plants appears to be the necines retronecine and heliotridine, respectively; there is no record of attraction of insects to withered parts of plants which contain PAs with other necine types (*e.g.* otonecine, platynecine). Results from tests with 7-angelylretronecine and 7-angelylheliotridine as well as with senecic and viridifloric acid, provided further evidence that it was not the structures of the acid moieties but rather aminoalcohols which influence the stimulatory powers of PAs.

Therefore, we assumed that hydrolysis might be a necessary step to make a given PA(-plant) attractive. The great instability of PAs under alkaline conditions is well known, heliotrine being amongst the most stable, while monocrotaline is hydrolysed quite quickly (refs. in Bull *et al.*, 1968; Mattocks, 1986). In addition, various reports state that PA-plants have to be withered and eventually remoistened to become attractive baits for insects (Schneider *et al.*, 1975; Edgar *et al.*, 1973). Notably, dishes containing PA-extract are particularly good lures if they have been exposed in humid climatic conditions (Boppré, unpubl.). In consequence of this and the results discussed above, we treated PAs with $Ba(OH)_2$ (or HCl) to force hydrolysis, hoping to simulate the conditions PAs might be exposed to in wilting and decaying plant material, or in withered plants after remoistening or in humid conditions.

As anticipated, $Ba(OH)_2$-treatment increased the stimulatory effects of PAs on the responses of SB II-receptor cells while HCl abolished the responses completely – but its effect could be reversed by subsequent treatment with $Ba(OH)_2$. This supports the idea that $Ba(OH)_2$-treatment promotes the appearance of airborne chemicals which might be volatile token(s) for insects to recognize sources of PAs. The role of HCl on the esters is, however, not clear; it might cause hydrolysis but in addition protonation of the nitrogen in the necine moiety or dehydration, reactions which apparently diminished the stimulatory power of the emanations for the insects' receptors.

Extraction of PA + $Ba(OH)_2$ with a variety of solvents revealed only polar phases to be stimulatory for SB II-receptor cells (*i.e.* to contain the volatile token). In TLC, only the very one samples yielded a spot which in various ways resembled the one appearing from extracts of coremata of *Creatonotos transiens* (Arctiidae). These androconia contain R(–)-hydroxydanaidal exclusively (Schneider *et al.*, 1982; Bell *et al.*, 1984; Bell & Meinwald, 1986) which encouraged us to test the organs, as well as synthetic hydroxydanaidal.

Behavioural tests. Extensive studies on the biological significance of the electrophysiological findings are still in progress. However, for discussing the results reported here it is necessary to mention that coremata of *Creatonotos transiens* exposed in the field attracted both sexes of all the species of *Rhodogastria* moths available in the area during the testing period. These coremata – like hydroxydanaidal – also lured *Gabonia* beetles and chloropid flies, and it seems that hydroxydanaidal is the only volatile involved in insect-PA relationships. In confinement, *Rhodogastria* preferred R(–)-hydroxydanaidal to S(+)-hydroxydanaidal.

General discussion. Hydroxydanaidal, previously known as a male pheromone component in various Lepidoptera (refs. in Boppré, 1989), turned out to occur in tiny amounts as a spontaneous degradation product of those pyrrolizidine alkaloids which contain the aminoalcohols retronecine or heliotridine. It thus acts as a kind of kairomone for various insects, enabling them to detect and localize sources of pyrrolizidine alkaloids. This unexpected finding results from employment of electrophysiological techniques and simple chemical procedures, and underlines the value of combination of chemical, sensory-physiological and behavioural approaches in the study of insect-plant relationships. Although we have succeeded with the biological task, it needs to be stressed that the chemical reactions involved are not fully understood and require detailed studies.

Our results are compatible with the previously reported information on attractivity of PA-plants. (1) Plants have to be withered or damaged to be attractive, even though the intact plants also contain PAs: the milieu of PAs in the living tissue prevents degradation. (2) Remoistening of withered plants increases their attractive power: moisture is a requirement for hydrolysis (Edgar (1973) found hydroxydanaidal (plus minor amounts of other Ehrlich-positive substances) in chloroform washings of *Heliotropium* plants which had been moistened). (3) Plants containing PAs with aminoalcohols other than retronecine or heliotridine are unattractive: because of their structure, these PAs cannot degrade to hydroxydanaidal. (4) Plant parts containing PAs (mainly) in the form of N-oxides are less attractive than plants containing tertiary bases: N-oxides are unsuited as precursors for hydroxydanaidal. (5) The attractive power of withered PA-

plants declines through time: the limited amount of hydroxydanaidal generated from the usually small amount of available alkaloid simply evaporates little by little.

The field observation that monocrotaline is a far better attractant than heliotrine (Boppré, unpubl.) is consistent with the observed differences in the neurophysiological effects of PA-extract, monocrotaline and heliotrine (with or without $Ba(OH)_2$-treatment) and is comparable with the responses of SB II-receptor cells to stimulation with 7-angelylretronecine and 7-angelylheliotridine (see above). Retronecine and heliotridine have identical structure except the stereochemistry of the OH-group at C-7 (Fig. 1C, D), and according to X-ray analyses by Wiedenfeld & Röder (1984), this stereochemical difference influences the bond lengths and thus the stability of the heterocycles. Therefore, we assumed a significant difference in the susceptibility of monocrotaline and heliotrine to hydrolysis, *i.e.* in the amount of the resulting volatile. However, the potency of SB II-receptors to discriminate between enantiomers demonstrates that differences in the attractivity of different PAs are not necessarily due the quantity of adequate metabolites released but to the specificity of the receptor cells. Again, detailed chemical studies are required for a final interpretation.

It is conceivable that the receptor is tuned to R(−)-hydroxydanaidal because its precursor (retronecine) is the most common aminoalcohol moiety found in PA-plants. While insect chemoreceptors capable of discriminating between enantiomeric pheromones generally seem to perceive either form exclusively (*cf., e.g.,* Hansen, 1984; Mustaparta *et al.,* 1984), in the case of *Rhodogastria*'s relation to PA plants, such discrimination is not vital – the moths do not have to avoid sources of the opposite enantiomer because they can also utilize PAs with heliotridine moieties. Apart from this aspect, the specificity as well as the sensitivity of this kairomone receptor compares closely with the well-studied female pheromone receptors found in moths (*e.g.,* Kaissling, 1972; Mustaparta, 1984).

At present, the lack of discrimination between S(+)-hydroxydanaidal and danaidal (*cf.* Fig. 6) cannot be explained ecologically; we have no idea if danaidal naturally occurs as a degradation product of certain PAs and could thus be a key-stimulus for PA-insects.

In the course of preparing this report we received a manuscript by S. B. Krasnoff & D. E. Dussourd (1988) who dealt with the same subject in a quite different approach. Based on the finding that coremata of *Pyrrharctia isabella* (Arctiidae) as well as *Eupatorium* plants (Asteraceae) attract adults of three other arctiid species (*Cisseps fulvicollis, Ctenucha virginica* and *Halysidota tessellaris*), Krasnoff & Dussourd assumed both *Pyrrharctia* and *Eupatorium* might contain similar attractants. The authors found hydroxydanaidal in *Pyrrharctia* but failed to detect this substance in *Eupatorium*. Nevertheless, experiments with *Eupatorium* roots, PAs, aminoalcohols, necic acids and hydroxydanaidal used as baits in sticky traps basicaly revealed that *Cisseps, Ctenucha* and *Halysidota* were strongly attracted by hydroxydanaidal. At first sight these data match our findings reported above. However, Krasnoff & Dussourd found the S(+)- significantly more potent than the R(−)-enantiomer – just the opposite of our results. We find it difficult to believe that the American species are so strikingly different to the African insects which we have investigated. As discussed above, S(+)-hydroxydanaidal must be derived from PAs with an aminoalcohol of the heliotridine type which, in fact, is quite rare. Also, the finding by Krasnoff & Dussourd that in *Cisseps* the percentage of males attracted by hydroxydanaidal was significantly lower than the one by *Eupatorium* roots is inexplicable to us.

A preliminary report by Kittmann & Schneider (1989) states two types of receptor cells in *Danaus gilippus,* one responding to danaidone but not to hydroxydanaidal and danaidal, the other responding equally to danaidal and R(−)-hydroxydanaidal but not to danaidone. Some of the cells are said to give good, others only weak reactions to the S(+)-enantiomer; neither receptor type responded to stimulation with the PAs lycopsamine and heliotrine. Due to lack of details, the findings of Kittmann & Schneider (1989) cannot be properly compared with our results; strikingly, these authors have gained quite low absolute numbers of spikes.

Zusammenfassung

Einzelzellableitungen identifizierten Hydroxydanaidal als das volatile Prinzip für die Anlockung von Insekten zu Pyrrolizidin-Alkaloiden

Elektrophysiologische und einfache chemische Verfahren erlaubten die Eingrenzung und Identifikation des reizwirksamen Prinzips, welches Insekten, die Pyrrolizidin-Alkaloide (PA) nutzen, die Orientierung zu PA-Quellen ermöglicht.

Einzelzellableitungen von antennalen Sensilla basiconica bei *Rhodogastria* (Lepidoptera: Arctiidae) zeigten einen morphologisch nicht unterscheidbaren physiologischen Typ, dessen Rezeptorzellen sich durch eine sehr geringe Spontanaktivität auszeichneten und ausschließlich auf 'PA-Duft' reagierten ('SB II'). Allerdings wurden (z. B. mit Monocrotalin) Reizquellen-Beladungen von 1 μg benötigt, um überschwellige Antworten auszulösen, und die maximal erreichte Aktivität (bei Reizung mit 5 mg) betrug lediglich 50 Imp./s; dies deutete auf das Auftreten von kleinen Mengen eines volatilen Abbauprodukts von PA hin.

SB II wurden als biologische Detektoren verwendet, um – kombiniert mit Dünnschichtchromatographie – die Reizwirksamkeit von Produkten von Hydrolyse- und Extraktions-Experimenten mit PA zu testen. Auf diese Weise konnte schließlich gezeigt werden, daß von PA mit Retronecin bzw. Heliotridin als Necin geringe Mengen des Dihydropyrrolizins Hydroxydanaidal (Fig. 1G, H) ausgehen. Synthetisches Hydroxydanaidal stellte sich als der bestwirksame Reiz für SB II-Rezeptorzellen heraus, die bzgl. Spezifität und Sensitivität (insbesondere für R(–)-Hydroxydanaidal) den gut untersuchten Rezeptoren für weibliche Sexualpheromone bei Nachtfaltern vergleichbar sind. Entsprechende Ergebnisse wurden auch mit *Danaus* (Danainae) und *Euchromia* (Ctenuchiidae) gewonnen. Erste Verhaltensversuche beweisen die Lockwirkung von Hydroxydanaidal für PA-Insekten und bestätigen die Interpretation der elektrophysiologischen Befunde.

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