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BIOASSAY OF COMPOUNDS DERIVED FROM THE HONEYBEE STING[1]

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Abstract—Nine compounds identified from honeybee, *Apis mellifera* L., sting extracts and one compound identified from the honeybee mandibular gland were evaluated in a standardized laboratory test for their effectiveness in eliciting an alarm response from caged honeybees. Two, *n*-decyl acetate and benzyl alcohol, were judged ineffective as alarm pheromones. The remaining eight—2-nonanol, isopentyl acetate, *n*-butyl acetate, *n*-hexyl acetate, benzyl acetate, isopentyl alcohol, and *n*-octyl acetate from the sting and 2-heptanone from the mandibular gland—produced responses of similar frequency and strength.

Key words—Honeybee, *Apis mellifera* L., Hymenoptera, Apidae, alarm pheromone, isopentyl acetate, sting, mandibular gland.

INTRODUCTION

In 1962, Boch et al. identified isopentyl acetate (IPA) as an active component of the sting alarm pheromone of the honeybee, *Apis mellifera* L. When presented on cotton balls at the entrance of a colony, this compound alerted and agitated the guard bees but did not incite them to sting as did an equivalent number of odoriferous stings dissected from live bees and presented on cotton balls at the entrance. Boch et al. then suggested that IPA was only one of several active components of the sting pheromone. Free and Simpson (1968) also reported that targets treated with IPA provoked less stinging than targets treated with stings.

[1]In cooperation with Louisiana Agricultural Experiment Station. Mention of a proprietory product does not constitute an endorsement by the USDA.

In 1965, Shearer and Boch identified 2-heptanone (2HPT) as an alarm pheromone produced in the mandibular glands of the honeybee. This compound also caused the guard bees to become alerted and agitated, issue from the hive, and attack a treated cork. Results of a comparison of 2HPT, IPA, and whole sting extracts also indicated that other sting-derived compounds contribute to the release of alarm behavior (Boch et al., 1970).

In 1978, Blum et al. analyzed extracts of honeybee stings and identified eight previously undetected compounds. We report here results of tests conducted to compare the activity as a chemical releaser of alarm behavior in the honeybee of each of these newly identified compounds and the two previously known compounds (IPA and 2HPT).

METHODS AND MATERIALS

The method of Collins and Rothenbuhler (1978) was used for bioassays. Caged brood from two individual queens (colonies) was emerged in an incubator during a 24-hr period and the young bees placed in glass-fronted wooden cages described by Kulincevic and Rothenbuhler (1973).

Newly emerged bees were used because they have not yet begun to produce alarm pheromone (Boch and Shearer, 1966) which could interfere with the assay. Although bees of this age are not normally involved in colony defense, in cages they respond with the same, but less intensive, behavior (Collins, 1980).

During the tests cages were arranged several inches apart on shelves in a 35° C walk-in incubator. Tests consisted of separate presentation to the bees of each component diluted in paraffin oil 1:9 (v/v). A 0.03-ml sample of this solution was presented under the wire floor of the cage on a small slice of No. 2 cork. The reaction by the bees involved both a flickering of the wings and increased locomotion in the cage. All tests were performed by one observer under double-blind condition; cage numbers were hidden until after testing and the cages were rearranged randomly after each complete sequence of tests.

The characters were measured as follows: (1) initial activity level—the number of bees moving on the floor, sides, and top of the cage prior to presentation of the stimulus; (2) seconds to react—the time until a group reaction was seen; (3) initial intensity of this reaction—graded as a weak, medium, strong, or very strong response based on the number and vigor of responding bees; (4) duration of the reaction; and (5) number of bees engaged in Nasonov fanning behavior at the end of the test. Following testing, a sixth character was calculated—frequency of no reaction—the number of times in which there was no reaction to the test material. Analysis of the data measured in seconds was by least-squares analysis of covariance, with seconds to react and duration being adjusted for initial activity level, and by a least significant

difference test. Intensity of the reaction, frequency of no reaction, and the number of bees fanning were analyzed using chi-square analysis. Spearman's rank correlation procedure was used to test correlations between measures.

The compounds tested were isopentyl acetate (IPA), benzyl acetate (BZA), 2-nonanol (2NL), benzyl alcohol (BZA1), *n*-hexyl acetate (nHA), *n*-butyl acetate (nBA), and isopentyl alcohol (IPA1), all obtained from Aldrich Chemical Co., Milwaukee, Wisconsin; *n*-octyl acetate (nOA) obtained from Matheson, Coleman and Bell, Norwood, Ohio; *n*-decyl acetate (nDA) obtained from Alfred Bader Library of Rare Chemicals, a division of Aldrich Chemical Co.: and 2-heptanone (2HPT) obtained from ICN Pharmaceuticals, Plainview, New York.

RESULTS

Table 1 shows the ranking of intensity, with a rank of 1 given to the compound having the most strong and very strong responses and a rank of 10 to the compound having the greatest number of weak or no reactions. The compounds ranked as the first six were not very different in intensity. IPA1 and nOA, ranked 7 and 8, showed a shift from strong to weaker or no responses.

Table 2 presents a summary of the measured responses to each of the 10

TABLE 1. INTENSITY OF RESPONSE BY CAGED HONEYBEES TO 10 COMPOUNDS TESTED AS ALARM PHEROMONES

		Intensity					
Rank[a]	Chemical	No response	Weak	Medium	Strong	Very strong	Total responses[b]
1	2-Nonanol	2	1	29	32	8	72
2	Isopentyl acetate	1	4	61	68	10	144
3	2-Heptanone	2	2	22	42	4	72
4	*n*-Hexyl acetate	1	3	33	31	4	72
5	*n*-Butyl acetate	5	5	31	27	4	72
6	Benzyl acetate	5	7	34	25	1	72
7	Isopentyl alcohol	14	10	35	13	0	72
8	*n*-Octyl acetate	17	12	28	15	0	72
9	*n*-Decyl acetate	35	5	28	4	0	72
10	Benzyl alcohol	37	12	19	4	0	72

[a]Rank was determined by relative number of observations in each category with 1 being the group with the greatest number of strong responses and 10 the group with the greatest number of weak or no responses.
[b]Eight cages with 30 bees each were tested 3 times a day for 3 days.

TABLE 2. MEASURES OF RESPONSE OF CAGED HONEYBEES TO 9 COMPOUNDS ASSOCIATED WITH HONEYBEE STING AND 1 FROM HONEYBEE MANDIBULAR GLAND

	% of total sting pheromone extract	No. nonreactors/total	$\bar{X}$ seconds to react	Intensity (rank)[a]	$\bar{X}$ duration of reactions (s)	No. of bees fanning
2-Nonanol (2NL)	8.6 mandibular gland	2/72	3.7 a	1	72.5 a[c]	203
2-Heptanone (2HPT)		2/72	4.1 a	3	52.8 bcd	91
Isopentyl acetate (IPA)	27.2	1/144	4.3 ab	2	51.0 bcd	86
n-Butyl acetate (nBA)	1.2	5/72	4.9 ab	5	45.7 bcd	21
n-Hexyl acetate (nHA)	3.4	1/72	5.0 ab	4	57.6 d	225
Benzyl acetate (BZA)	13.3	5/72	5.3 ab	6	76.3 a	432
Isopentyl alcohol (IPAl)	12.3	14/72	5.3 ab	7	43.8 bcd	8
n-Octyl acetate (nOA)	14.3	17/72	6.2 abc	8	45.2 bcd	42
n-Decyl acetate (nDA)[b]	1.2	35/72	6.9 bc	9	39.2 d	4
Benzyl alcohol (BZAl)[b]	3.4	37/72	8.6 c	10	43.5 cd	60

[a]Rank determined by the relative intensity of responses in repeated tests. Rank 1 had the most vigorous responses, rank 10 the weakest or no responses.
[b]These chemicals probably do not function as alarm pheromones based on the frequency of no reaction.
[c]Means within a column followed by the same letter(s) are not significantly different ($P < 0.01$).

compounds. The percentages of the total string pheromone are taken from the samples used for identification by Blum et al. (1978).

The two compounds, nDA and BZA1, which caused no response more than half the time, were judged to be ineffective in stimulating alarm behavior. When the bees did react to these compounds, the responses were slow, weak, and brief. For the remaining eight compounds, mean seconds to react were not significantly different, although they ranged from 3.7 to 6.2 sec. These results indicate that acetates with alcoholic moieties in the range C_4 (*n*-butyl) to C_8 (*n*-octyl) are of relatively equivalent activity. Furthermore, some secondary alcohols (e.g., 2-nonanol) appear to be as active as esters in releasing alarm behavior in worker bees.

The duration of the reaction was more variable than the speed of the reaction. Significantly longer mean responses were seen for BZA and 2NL, as well as larger numbers of fanning bees, as compared to the other compounds.

The correlations between these characters are presented in Table 3. The percentage of the total pheromone extract was not significantly related to any of the measures of response. The frequency of no reaction, the speed of the reaction, and the intensity of the reaction were all highly correlated ($P < 0.01$) with each other. The duration of the reaction was slightly less ($P < 0.05$) but still significantly correlated to those three measures. The only significant correlation for number of bees fanning was with the duration of the reaction.

Not only were there differences in the overall response to each of the compounds, but there were also colony differences. Table 4 shows a difference

TABLE 3. SPEARMAN'S RANK CORRELATION COEFFICIENTS FOR PROPORTION OF TOTAL PHEROMONE EXTRACT OF 9 COMPOUNDS ASSOCIATED WITH HONEYBEE STING AND 5 MEASURES OF RESPONSE BY CAGED HONEYBEES TO THESE COMPOUNDS

	Frequency of no reaction	Speed of reaction	Intensity of reaction	Duration of reaction	No. of bees fanning
Percent of pheromone extract	−0.176	0.069	−0.073	0.194	0.194
Frequency of no reaction		0.852**[a]	0.936**	−0.736*	−0.603
Speed of reaction			0.973**	−0.676*	−0.439
Intensity of reaction				−0.745*	−0.515
Duration of reaction					0.903**

[a] ** Significant at $P < 0.01$; * significant at $P < 0.05$.

TABLE 4. SIGNIFICANT COLONY DIFFERENCES IN RESPONSE OF CAGED HONEYBEES TO *n*-OCTYL ACETATE[a]

	Colony number	
	1	2
No. nonreactors/total	4/36	13/36*[b]
$\bar{X}$ seconds to react ± SD	4.5 ± 4.0	8.2 ± 6.8*
Intensity		
Weak	2	10**
Medium	17	11
Strong	13	2
Very strong	0	0
$\bar{X}$ duration of reaction (s) ± SD	55.0 ± 19.7	25.6 ± 21.5**
No. of bees fanning	23	19 NS

[a]Four cages of 30 bees each from each colony tested 3 times a day for 3 days.
[b]* Significant at $P < 0.05$; ** significant at $P < 0.01$; NS-not significant.

in response by the two colonies to nOA. Colony 2 showed a greater frequency of no response and responded more slowly, for a shorter period of time, and with less intensity, when it did show a response, than did colony 1.

In a preliminary experiment of the same design where a slightly different group of compounds was tested, three colonies were used. The responses of these colonies to three of the compounds in the test are shown in Table 5. Colony 7 responded equally to all three compounds. Colony 8 responded more slowly to IPA than the other two colonies. Colony 9 responded more slowly to IPA and BZA. Clearly, these results imply genetic differences in response to alarm pheromones.

TABLE 5. COLONY DIFFERENCES IN SPEED OF RESPONSE BY CAGED HONEYBEES TO 3 ALARM PHEROMONES[a]

	Chemical		
Colony No.[b]	IPA	IPAl	BZA
7	5.08	5.63	5.65
8	4.20	7.58[c]	5.56
9	7.54[c]	4.93	11.17[c]

[a]Values are mean seconds to react.
[b]Four cages of 30 bees each per colony were tested 3 times a day for 3 days.
[c]Significantly different from other values in column and row at $P < 0.05$.

DISCUSSION

No striking differences were observed in the effects of nine compounds identified from extracts of the honeybee sting and bioassayed for their possible function as alarm pheromones. Rather, the response to these compounds varied along a continuous scale. An observation was made, based on the frequency of nonresponse, that two of the compounds (nDA and BZA) were not functioning as alarm pheromones under the cage test conditions. Among the remaining seven and 2HPT, response varied gradually. Boch et al. (1970) reported that 2HPT was significantly less effective as an alarm pheromone than was IPA. However, results of our tests seem to indicate that, at least under the test conditions outlined, 2HPT is as effective an alarm releaser as several of the most active sting-derived compounds.

Boch and Shearer (1971) also compared six of the compounds tested here to IPA. Responses were ranked from 5 (equivalent to IPA) to 0 (not effective) based on the concentration required to attract a similar number of guard bees to a cork at the colony entrance. 2HPT and nBA were scored as 4, nHA as 2, IPA1 as 1, and nOA and BZA as 0. None of these were significantly different in our laboratory cage test. The difference in results may be explained by the fact that the cage test measures only alarm, whereas Boch and Shearer's test required attraction to the source and a higher level of activity. In addition, the bees used in the two experiments may have different reactivities such as the colonies mentioned earlier.

Why is this array of compounds produced by the honeybee if they all produce a similar reaction? Several investigators comparing IPA and whole stings (Boch et al., 1962; Free and Simpson, 1968) reported that, whereas IPA-marked targets elicited no stinging response, previously stung targets were stung again frequently. Some of the other chemicals tested probably mark objects, causing further stinging, or simply incite stinging by alerted bees, a behavior not measured by the cage test. It is also possible that some of the sting-derived compounds provide longer lasting alarm signals than IPA (i.e., 2NL and BZA). Further research is underway to examine these hypotheses and to determine whether additive or synergistic effects may occur when these compounds are used in combination.

It is not surprising that there are colony differences in the response to these pheromones. With such a complex array of possible alarm pheromones, it is not unexpected to find that some bees may respond more effectively to certain components than to others. Two inbred lines examined by Collins (1979) showed strikingly different responses to the same chemical, IPA, in cage tests as well as quite different extremes of defensive and stinging behavior in the field. It is possible that, in addition to the difference in response, a more careful analysis of sting extracts from genetically different colonies may show variation in the type and quantity of alarm pheromones present.

The lack of correlation between the relative amount of a compound in the extract and the resulting behavioral response is consistent with the results obtained by Boch and Rothenbuhler (1974), who found no correlations in behavior resulting from manipulation of field colonies and quantitative measures of IPA production. However, correlations between defensive behavior of field colonies and 2HPT was reported by Brazilian investigators (Kerr et al., 1974).

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