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EVIDENCE FOR DIGLYCERIDES AS ATTRACTANTS IN AN ANT-SEED INTERACTION

D.L. MARSHALL,[1] A.J. BEATTIE, and W.E. BOLLENBACHER

Department of Biological Sciences, Northwestern University, Evanston, Illinois 60201

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Abstract—The chemical basis of an ant-seed interaction was investigated for the ant *Aphaenogaster rudis* and the ant-dispersed violet *Viola odorata*. A laboratory behavioral bioassay was developed to chemically identify the attractant responsible for the interaction. The ant attractant, localized in the elaiosome, was classified as a lipid by both field and laboratory bioassays. Assays of partially purified lipid extracts revealed that the principal attractant may be a diglyceride. Gas–liquid chromatography analysis of the hydrolyzed diglyceride fraction revealed oleic acid as the major fatty acid present, suggesting that either 1,2- or 1,3-diolein may be the attractant. Structure-activity correlations for lipid standards demonstrated a clear preference for the diglyceride 1,2-diolein. The data also suggest that ricinoleic acid is not the lipid eliciting the ant response to *Viola odorata*, as had been previously suggested.

Key Words—Hymenoptera, Formicidae, *Aphaenogaster rudis*, ant, behavior, diglyceride, elaiosome, myrmecochory, *Viola odorata*.

INTRODUCTION

The seeds of many plant species bear external appendages which are attractive to ants (Sernander, 1906). The appendages, called elaiosomes, are attached to the outside of the seed coat and may consist of several different types of tissues. Typically the outer tissue, which may be single-layered or multilayered, contains high concentrations of lipids. The inner mass of tissue, distinguished by lower fat content, may be supplied by a vascular bundle which penetrates the seed coat (Bresinsky, 1963; Roth, 1977). When elaiosome-bearing seeds are released into the environment, ants rapidly locate and remove them (Berg, 1966; Beattie, 1978). In the case of most of the

[1]Present address, Department of Botany, University of Texas, Austin, Texas 78712.

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common violets (*Viola*), the seeds are taken back to nests where they are less susceptible to predation and more likely to germinate and to become established (Culver and Beattie, 1978). This interaction clearly has a profound and beneficial effect upon the dispersal and survival of the plant species involved. The adaptive value of the interaction to the ants is much less obvious. We are primarily interested in the effects upon the plant populations, especially in relation to the size and chemical content of the elaiosome. The reason for this is that variation in these characters is likely to affect the type of ant which takes a seed. Since different ant species exhibit different methods of foraging, the pattern of seed dispersal and survival, and hence the plant population structure, is partly dependent upon the chemical properties of the elaiosome.

Although morphological variability exists among elaiosomes from different species of violets, their chemical composition appears to be the critical parameter for recognition and removal by ants. Moreover, the compounds present in elaiosomes from seeds representing a variety of genera have been investigated and, when subjected to bioassay, only the lipid component elicited an attractive response from ants (Bresinsky, 1963). More specifically, for the ant-dispersed violet, *Viola odorata*, which has an elaiosome nearly as large as the seed itself, Bresinsky (1963) suggested that a fatty acid, ricinoleic acid, was the primary attractant. With this apparent identification of the active elaiosome component, we initiated an investigation to obtain a structure–activity correlation for the active lipid components in the elaiosomes of different species of violets. However, initial structure–activity studies with fatty acid standards and extracts from *V. odorata* elaiosomes immediately suggested that ricinoleic acid was not the ant-attracting compound for this violet. This disparity suggested that either the active elaiosome component was another compound or that our initial bioassay did not accurately screen prospective attractants. To resolve this question, it was first necessary that the bioassay method be defined so that the behavior of the ants in the laboratory bioassay closely approximated the normal behavioral response in the field. Therefore, the bioassay methods were first optimized in the field and then simulated in the laboratory so as to attain maximal retention of normal ant behavior. We report here the details of the new bioassay together with a preliminary characterization of the attractive component of *Viola odorata* elaiosomes.

METHODS AND MATERIALS

Bioassays. Elaiosome material was separated by a hierarchical series of chemical procedures, in which ants were used to select the most attractive fraction at each step. *Viola odorata* was chosen in part because the elaio-

somes are large, the plants are easily grown in the greenhouse, and because the seeds are extremely attractive to ants in the field (Beattie, unpublished data). The ant *Aphaenogaster rudis* (Crozier, 1977) was selected because it responds preferentially to *Viola* seeds in the field (Culver and Beattie, 1978) and because it can be moved to the laboratory without the loss of normal foraging behavior. The ant colonies were collected in the Monongahela National Forest, West Virginia, where field assays were conducted, and were placed in 2- × 15-cm test tubes covered with aluminum foil. In the laboratory, these colonies were maintained in 30- × 16-cm plastic boxes with a 1-cm-thick plaster-of-paris floor.

The bioassay required mounting test materials on a substrate which was inert and which resembled natural objects (i.e., seeds) as closely as possible. Therefore, precise quantities of test compounds were applied to porous Teflon beads with approximately the same size, weight, and handling characteristics of normal seeds. The beads, although inert, were carefully washed in a range of organic solvents to remove any contaminants. Their light weight and rough texture permitted easy manipulation by ants. Each assay was performed with six seeds (real or artificial) placed in a 1-cm^2 grid, and ant behavior was observed for 30–60 min. The method of scoring was as follows: 1, antennation, a brief touching; 2, examination, exploration of the seed; 4, pick up, holding of the seed; 8, removal, carrying the seed one or more cm from its original position. The scores thus weighted removal behavior more heavily. Following the successful removal of a seed (real or artificial) from the test grid, it was immediately replaced with an identical substitute. It rapidly became clear that contact between the antennae and the seed (real or artificial) was necessary for ant response. Consequently, the scoring of ant behavior in four steps accurately reflected the degree of attraction of a seed or extract. Furthermore, since there was no evidence for the volatile transmission of attractants, ant preferences during bioassays were very clear.

When extracts or standards were assayed, equivalent aliquots were evaporated on the Teflon beads to assure constancy in response. For assays of lipid standards 50 μg of a compound was applied to each seed. For the bioassays performed during attractant purification, an untreated and a solvent-coated Teflon "seed" were run in each assay to establish a behavioral background, the average of which was then subtracted from the scores for the unknowns (extracts) and standards. During an assay the observer was not informed of the treatment of the artificial seed so that assessment of ant behavior would not be biased.

In the field, grids were placed on the forest floor in habitats where the ants would normally encounter violet seeds. They were positioned without reference to the location of ant nests or ant trails. In the laboratory, the grids were placed on the floor of the boxes where the nests were maintained.

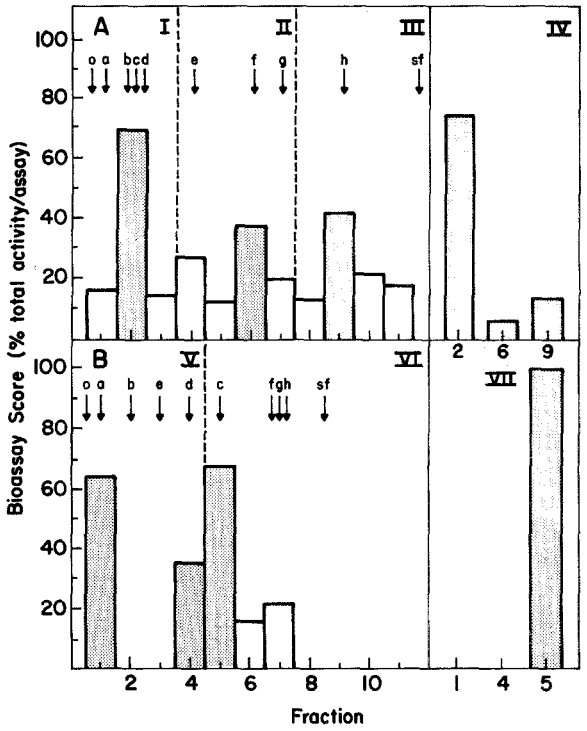

FIG. 1. Thin-layer chromatographic distribution of attractant activity in the nonpolar lipid extract of *V. odorata* elaiosomes separated with the following solvent systems: (A) hexane–diethyl ether–acetic acid, 80:20:2 (v/v/v); and (B) hexane–chloroform–ethanol, 10:10:1 (v/v/v). Fractions from each plate were divided into assay groups (------) and initial bioassays (7-22/group) were run (I, II, III, V, and VI). The most active fractions from each of the initial assays in a given group were then reassayed together (IV and VII, 6 assays each). The activity for each fraction in an assay is expressed as a percentage of the total score for all samples in an assay, corrected for background activity. For the first separation, only fraction 2 contained activity that was significantly higher than all other concurrently tested fractions including controls ($P < 0.01$). In the second TLC separation fraction 5 was unequivocally the most active fraction (VII). The shaded bars denote those fractions from the initial assays that were reassayed (IV and VII). The standards used for the TLC separations are monolein (a), ricinoleic acid (b), 1,2-diolein (c), cholesterol (d), oleic acid (e), triolein (f), methyl oleate (g), and cholesteryl oleate (h). The origin and solvent front for each plate are indicated by o and sf, respectively. Each of these separations was repeated three times with similar bioassay results.

Attractant Purification. Elaiosomes were separated from the seeds and stored in chloroform–methanol, 2:1 (v/v) at −20°C until sufficient tissue was obtained for extraction. Following homogenization in chloroform–methanol, the lipid and nonlipid components were partitioned into nonaqueous and aqueous phases, respectively, by the addition of H_2O to the chloroform–methanol extract, 1:4 (v/v) (Folch et al., 1957).

Separation of the lipids on silicic acid columns yielded nonpolar, chloroform-elutable and polar, methanol-elutable lipid fractions (Borgström, 1952). Simple (nonpolar) lipid extracts were separated by thin-layer chromatography (silica gel 60, F-24, Merck, Darmstadt) using hexane–diethyl ether–acetic acid, 80:20:2 (v/v/v), and hexane–chloroform–ethanol, 10:10:1 (v/v/v), as solvent systems. The plates were fractionated and each fraction eluted with absolute chloroform–ethanol 1:1 (v/v). Fractions from each plate were divided into assay groups and initial bioassays were performed [7–22 assays/group; see Figure 1 (I, II, III, V and VI). The most active fractions from each of the initial assays for a given solvent system were then reassayed together [Figure 1 (IV and VII); 6 assays each]. The activity for each fraction in an assay is expressed as a percentage of the total score for all samples in an assay, corrected for background activity.

Fatty acid methyl esters prepared from the diglyceride fraction of the nonpolar lipids (Ast, 1963) were separated on an F&M gas chromatograph (model 402) equipped with a flame ionization detector. Glass columns, 6 ft × 3 mm, packed with 5% DEGS-PS on 80/100 mesh supelcoport were used at 190°C column temperature, with the carrier gas (N_2) flow rate at 60 ml/min.

RESULTS

Comparison of the number of *Viola odorata* seeds removed relative to the number of other seeds removed demonstrated the reproducibility of ant behavior in the field and in the laboratory. In the field 69 (49%) of 140 removals and in the laboratory 25 (50%) of 50 removals were *V. odorata* seeds, indicating that the ants' seed-removal behavior was not modified under laboratory conditions.

The next step was to determine the reproducibility of behavior toward artificial seeds in the field and in the lab. The first fractionation of the elaiosome extract yielded a lipid and a nonlipid fraction. Ant behavior toward artificial seeds (Teflon beads) coated with these fractions was compared in the field and the laboratory. Field bioassays gave unequivocal results: 99% lipid vs. 1% nonlipid (Table 1). Although the laboratory score was less dramatic, 61% lipid vs. 39% nonlipid, the difference in ant preference under both conditions is highly significant (chi square, $P < 0.005$).

TABLE 1. DISTRIBUTION OF ATTRACTANT ACTIVITY IN LIPID AND NONLIPID EXTRACTS OF *V. odorata* ELAIOSOMES TESTED WITH THE *Aphaenogaster* sp. BIOASSAY UNDER FIELD AND LABORATORY CONDITIONS

Location	Bioassays (number)	Bioassay score		Relative activity	
		Lipid	Nonlipid	Lipid	Nonlipid
Field	13	398[a]	4	0.99	0.01
Laboratory	33	1870[a]	1197	0.61	0.39

[a]Chi square: $P < 0.005$).

Silicic acid chromatography of the lipid fraction chosen by the ants as the most attractive was followed by bioassay of the nonpolar and polar lipids. Sixteen independent laboratory bioassays indicated that 80% of the activity could be coeluted with the nonpolar lipids while only 20% of the ant activity was associated with the polar lipids (chi square for polar vs. nonpolar lipids, $P < 0.005$). Attractant content of the polar lipids was assumed to be minimal since the 20% response to that fraction was less than laboratory background activity shown toward the nonlipids previously bioassayed.

Bioassays of fractions obtained from thin-layer separations of the nonpolar lipids with hexane–diethyl ether–acetic acid, 80:20:2 (v/v/v), demonstrated that the majority of the activity separated into a single band near the origin (Figure 1A). Three classes of standards migrate with R_fs comparable to the active fraction: hydroxy fatty acids (ricinoleic acid), sterols (cholesterol), and diglycerides (1,2-diolein). The original solvent system had been chosen to give a wide range of separation of elaiosome components, so a second solvent system [hexane–chloroform–ethanol, 10:10:1 (v/v/v)] was selected to separate the active fraction more completely. Ricinoleic acid clearly differentiated from the other kinds of compounds in the active fraction, but the elaiosome material migrating along with ricinoleic acid elicited little positive bioassay activity (Figure 1B). Therefore, ricinoleic acid probably is not an attractant in *V. odorata* elaiosomes. In the final series of bioassays [Figure 1B(VII)], the ants showed a dramatic preference for the band migrating with 1,2-diolein. No other class of standard migrates with a similar R_f, and all other fractions generated minimal, if any, bioassay response.

Gas–liquid chromatography of the fatty acid methyl esters of the hydrolyzed TLC diglyceride fraction showed that a range of fatty acids is present (Figure 2). The chromatographic profile was characterized by a

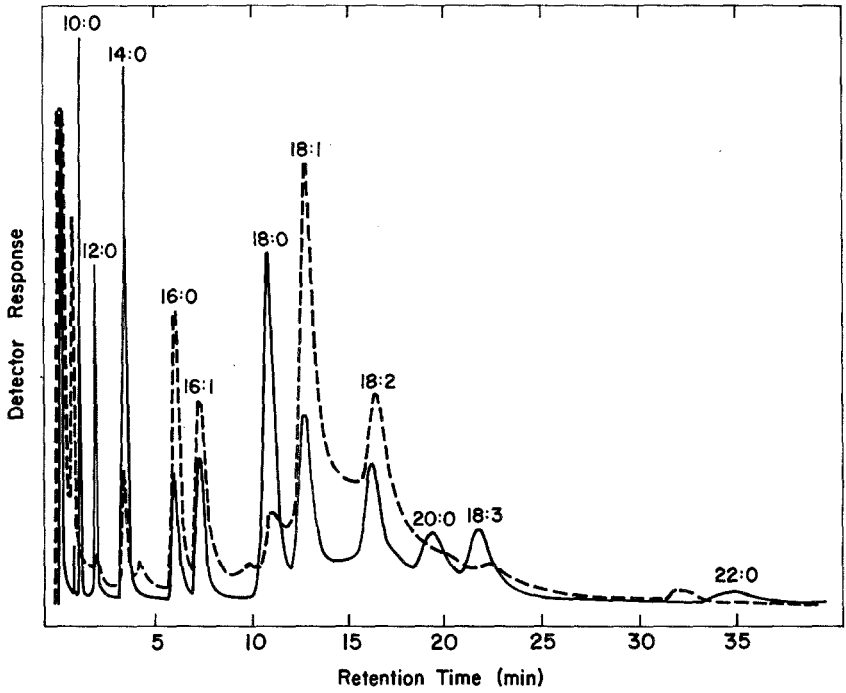

FIG. 2. Gas–liquid chromatography of methyl esters of fatty acids from the diglyceride fraction of elaiosomes (------) and of standards (———). The retention times for standards are denoted by their chain length and degree of unsaturation.

TABLE 2. STRUCTURE–ACTIVITY CORRELATION FOR SIMPLE LIPID STANDARDS AND THE BEHAVIORAL RESPONSE OF *Aphaenogaster* sp. IN THE LABORATORY BIOASSAY

Standard	Antennation	Bioassay score			Total
		Examination	Pick Up	Removal	
1,2-Diolein	25 (25)	6 (3)	28 (7)	80 (10)	139
1,3-Diolein	10 (10)		4 (1)		14
Monolein	17 (17)				17
Oleic acid	18 (18)	4 (2)	4 (1)	16 (2)	42
Ricinoleic acid	29 (29)	2 (1)	4 (1)		35
Chloroform	12 (12)				12
Untreated	12 (12)				12

large peak having a retention time identical to that of oleic acid (18:1). This suggested that 1,2- and/or 1,3-diolein may function as an attractant.

Bioassays in which 1,2- and 1,3-diolein were compared to monolein, oleic acid, and ricinoleic acid were conducted to confirm the observed ant preference for diglycerides. Ants strongly preferred 1,2-diolein over 1,3-diolein and over monolein and oleic acid (Table 2). Ricinoleic acid standards elicited little ant response. Twelve additional fatty acid standards were also bioassayed: linoleic, stearic, palmitic, palmitoleic, nervonic, elaidic, vaccenic, lignoceric, myristic, behenic, 12-hydroxystearic, and α-hydroxypalmitic acid. These each yielded scores below that of ricinoleic acid. 1,2-Diolein induced ants to attempt to pick up and remove artificial seeds as if they were real seeds, while the other standards elicited surprisingly little of this type of activity.

DISCUSSION

The active portion of the elaiosome has been isolated in the diglyceride fraction. 1,2-Diolein standards elicit strong ant response and oleic acid is the major fatty acid constituent in the diglyceride sample. Therefore, while other diglycerides certainly are present, 1,2-diolein may be an important attractant in *V. odorata* elaiosomes.

Free ricinoleic acid is clearly not the active component of *V. odorata* elaiosomes. Furthermore, this fatty acid is only present as a minor component, if at all, of the total diglyceride sample (ricinoleic acid has the same retention time as C20 on 5% DEGS-PS). The data presented do not support Bresinsky's (1963) conclusion that ricinoleic acid is the ant attractant in *Viola* elaiosomes.

Lipids might act to provoke ant response in several ways, perhaps functioning as nutrients or behavioral releasers. Insects in general require a sterol and a polyunsaturated fatty acid in their diets (Dadd, 1973) and some ants respond to polyunsaturated fatty acids as phagostimulants (Vinson et al., 1967). Diglycerides are known to be the major class of neutral lipid in hemolymph of some insect species which use the compounds to transport lipids throughout the body (Gilbert and Chino, 1974). Thus, ants might respond to diglycerides as nutrients.

Lipids also include attractants, arrestants, and aggregating pheromones (Gilbert, 1967; Dethier, 1947), so that elaiosome components might act to release behaviors which are essential to the ants in their usual context. One kind of carrying behavior, removal of dead ants to the refuse pile, has been elicited by a lipid, probably oleic acid (Wilson et al., 1958). Ants also carry brood into the nest. Brood-carrying has been used in some orientation experiments with ants because the workers carry brood back to the nest when-

ever the brood are outside, while food-carrying stops with colony satiation (Carthy, 1951). Thus, elaiosome components releasing a behavior mimicking brood or corpse-carrying might be a very reliable means of inducing seed removal. Diglycerides do sometimes act as pheromones. Starrat and Osgood (1972) report that a diglyceride acts as an inducer of ovipositing in the mosquito *Culex tarsalis*.

Viola odorata may, therefore, manipulate ants in either of these two ways. If the ants respond to the diglycerides as nutrients, a wide range of diglycerides should be active. If, however, the diglycerides are behavior releasers, only a few closely related compounds should elicit a response. The striking difference in ant response to 1,2- as opposed to 1,3-diolein suggests that the active fraction may have some behavior-releasing function.

Since all violet seeds do not exhibit parity with regard to the frequency and intensity of seed removals by ants, it is possible that different attractants in the elaiosomes of different violet species determine the species-specificity of the ant–seed interaction. Further work is in progress aimed at correlating the quantity and quality of elaiosome components with ant preferences and with various parameters of plant dispersal and survivorship. In the herbaceous flora of temperate forests, many species besides violets are dispersed by ants. Consequently, the comparative biochemistry of elaiosome attractants is likely to provide much information on the abundance and distribution of a wide variety of plant and ant species.

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