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AGGREGATION PHEROMONE COMPONENTS OF TWO SPECIES OF *PISSODES* WEEVILS (COLEOPTERA: CURCULIONIDAE): Isolation, Identification, and Field Activity[1,2]

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Abstract—Two related volatile compounds were identified from each of two species of *Pissodes* bark weevils and implicated as components of their aggregation pheromones. Grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol), and its corresponding aldehyde, grandisal, were isolated from males of both *P. strobi* and *P. approximatus* and were found in the abdomens and hindguts of the respective species. In field tests synthetic grandisol and grandisal together with odors from cut pine acted synergistically in attracting both sexes of *P. approximatus*. This response was similar to that elicited by male *P. approximatus* feeding on cut pine. Males and females of natural populations of *P. strobi* were more responsive to caged males feeding on leaders of white pine than they were to leaders alone. The combination of grandisol, grandisal, and leaders was less attractive than males on leaders, but more attractive than leaders alone. From isolation of pheromone components at different times of the year, it was

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determined that males of both species produced grandisol and grandisal only at times when cohort females were reproductively mature.

Key Words—*Pissodes strobi*, *Pissodes approximatus*, Coleoptera, Curculionidae, aggregation pheromone, grandisol, grandisal, *Pinus strobus*, white pine weevil.

INTRODUCTION

Bark weevils of the genus *Pissodes* Germar feed on conifer trees in the family Pinaceae and their larvae develop in the inner bark (phloem) of their hosts. *Pissodes strobi* (Peck), the white pine weevil, is notorious among forest insects for its deformation of pines and spruces throughout North America. The biology and habits of *P. strobi* have been extensively documented (Macaloney, 1930; Taylor, 1930; Belyea and Sullivan, 1959). In the spring the adults orient to the healthy terminal leader (top shoot) of a host tree, where they mate, feed, and oviposit. Larval feeding girdles the stem, killing old and new growth; one or more of the remaining lateral branches must assume dominance, resulting in a fork or crook in the stem that may render the tree useless for saw timber. The northern pine weevil, *P. approximatus* Hopkins, is not as economically important as *P. strobi*, although larvae may kill stressed trees and intense feeding by adults may be injurious to small trees (Finnegan, 1958). Adults of *P. approximatus* orient to cut or weakened trees to reproduce, and their larvae develop in the inner bark of this material. *P. approximatus* occurs on most pines and spruces throughout the northeastern and Lake states, and in the Canadian boreal forest west to the Rocky Mountains; *P. strobi* occurs in three somewhat distinct populations across North America, according to the preferred host of the region. In the east, *P. strobi* is found primarily on eastern white pine (*Pinus strobus* L.), in the Rocky Mountains in Engelmann spruce (*Picea engelmannii* Parry), and in the Pacific coastal region on Sitka spruce (*Picea sitchensis* Carr.) (Smith and Sugden, 1969).

Finnegan (1958) reported mass flights of *P. approximatus* in response to suitable host material (moribund pines) in Ontario. *P. strobi* forms aggregations on leaders early in its flight season, prior to increased dispersal and oviposition (Overhulser and Gara, 1975). The first evidence of an aggregation pheromone in *Pissodes* was reported by Booth and Lanier (1974). In that study, *P. approximatus* males confined on host material were more attractive to both sexes than females on host material or host material alone. Interestingly, *P. strobi* males caged on the same host material also attracted local *P. approximatus*. Subsequently, Booth (1978) demonstrated that *P. strobi* males caged on white pine leaders were attractive to conspecific males and females in the field; females feeding on leaders and leaders alone were not attractive. This paper reports the isolation and identification of volatile

pheromone components from males of *P. strobi* and *P. approximatus* and discusses their significance for these species.

METHODS AND MATERIALS

Weevil Populations. Weevils used in experiments were from natural populations within a 100-km radius of Syracuse, New York. *P. strobi* adults were obtained in an 8-year-old eastern white pine plantation in southeastern Cortland County, near Virgil, New York. Overwintered weevils were collected from the upper lateral branches of the previous year's brood trees as daily high temperatures approached 15–20° C, usually in the second or third week of April. *P. strobi* used during winter months were obtained from naturally infested white pine leaders in August. The weevils were sexed according to the method of Harman and Kulman (1966) and maintained on freshly cut white pine branches under ambient lighting in the spring or under a 16:8 (light–dark) photoperiod in winter. *P. approximatus* adults were collected from the undersides of freshly cut red pine logs scattered on the forest floor within a 30-year-old red pine (*Pinus resinosa* Ait.) plantation at Heiberg Memorial Forest, near Tully, New York. Weevils used in the fall were obtained as they emerged from naturally infested pine logs brought into the laboratory.

Collection, Isolation, and Identification of Pheromone Components. Volatiles from living *Pissodes* adults were collected by aeration and absorption on Porapak Q (Waters Assoc., Framingham, Massachusetts) (Byrne et al., 1975). From 50 to 150 weevils of each sex were aerated for 5–10 days in large vacuum desiccators while feeding on fresh cuttings of their respective host material: *P. strobi* on white pine leaders and *P. approximatus* on red pine branches. Aerations were carried out in the laboratory at 21° C under ambient lighting in the spring or under a controlled 16:8 (light–dark) photoperiod in the fall or winter. Prior to aeration, the Porapak was cleaned according to the method of Williams et al. (1981) and, following aeration, the volatiles were extracted with distilled pentane (5 ml/g). The extract was then dried over sodium sulfate and concentrated to 1–4 ml by fractional distillation in preparation for gas chromatography (GC) or bioassay. Hindguts or abdomens were dissected from the weevils and crushed in small amounts of distilled pentane; the slurry was sonicated and centrifuged, and the supernatant was used for GC analysis.

Extracts were fractionated on a Varian 2700 chromatograph equipped with a flame ionization detector, a 99:1 effluent splitter, and a thermal gradient collector (Brownlee and Silverstein, 1968). Fractions were collected in 30.5 cm × 1.7 mm (OD) glass capillary tubes that were flame sealed and stored at −30° until used. A 5.4 m × 4 mm (ID) glass column packed with 4% Carbowax 20M on Chromosorb G 60–80 mesh was used under the following

conditions: 90° for 4 min, raised to 215° at 2°/min, 55 ml He/min, injector 140°, detector 215°. The collected compounds were further fractionated on a 6.0 m × 4 mm (ID) glass column (5% OV-225 on Chromosorb G 60–80 mesh, 80° for 4 min, raised to 215° at 2°/min, 55 ml He/min, injector 140°, detector 215°).

Mass spectral electron impact (70 eV) data were obtained from a Finnegan GLC 9500. Proton nuclear magnetic resonance ([^{1}H]NMR) spectra were recorded (Varian XL-100 instrument) on 80 μg samples in 50 μl C_6D_6 in the inner cell of a concentric tube. Infrared spectra were obtained on a Perkin-Elmer 621 instrument, fitted with a beam condenser, on 60 to 80-μg samples dissolved in CCl_4 in a 4-μl cavity cell.

Synthetic Compounds. Synthetic (racemic) grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol) was obtained from Chemical Samples Co. (division of Albany International, Inc.) and GC-purified for coinjection. The corresponding aldehyde of grandisol (grandisal) was obtained by oxidation with pyridinium chlorochromate in methylene chloride in the presence of sodium acetate (1 equivalent); reaction time was 80 min at room temperature. The reaction mixture was filtered through a 5 × 1.5 cm Florisil column, and after evaporation of the solvents the purity of the aldehyde was determined on a 3 m × 4 mm (ID) glass GC column (7% Carbowax on Chromosorb G 60–80 mesh) at 125° C. The same synthetic procedure was used to provide aldehyde for field experiments.

Field Assays. Bioactivity of putative pheromone components was tested in several trapping experiments in the field. *P. approximatus* experiments took place in mature red pine plantations near Tully, New York, while *P. strobi* tests were conducted in young white pine plantations near Virgil, New York. Two field tests of *P. approximatus* employed hardware cloth sticky traps placed on the ground (Booth and Lanier 1974), arranged in five randomized blocks of six traps each. The six treatments of the first test included: (1) male weevils caged on a red pine bolt, (2) the putative pheromone components grandisol and grandisal with a red pine bolt, (3) the same concentration of these compounds without red pine, (4) higher concentrations of these compounds without red pine, (5) solvent only (hexane), and (6) a red pine bolt alone. The second experiment tested the attractiveness of (1) male weevils on red pine, (2) grandisol and grandisal with red pine, (3) half as much of these compounds with red pine, (4) grandisal only with red pine, (5) grandisol only with red pine, and (6) a red pine bolt alone. The first *P. approximatus* test, conducted in the late summer, assayed those weevils which presumably eclosed in early summer and were currently reproductive (Zerillo et al., undated ms.); the second experiment tested the more active spring population. *P. strobi* tests, conducted in two consecutive spring seasons, employed hardware cloth sticky cylinders which surrounded screen-enclosed

white pine leaders (Phillips 1981). Five replicates each of three different treatments (male *P. strobi* confined on leaders, grandisol and grandisal on a leader, and a leader alone) were established on trees picked at random within a one-acre plantation. In field tests of *Pissodes* species, synthetic pheromone components were released from snap-top polyethylene vials (Cole-Parmer Instrument Co.), and live weevils used in certain treatments were field-collected immediately prior to the tests.

RESULTS

Identification of Pheromone Components. Systematic GC fractionation of the extracts, monitored by laboratory bioassay of the fractions singly and in combination, was attempted (Silverstein, 1977). However, none of the laboratory olfactometers (Booth, 1978) provided consistent, interpretable results. Since the amounts of the fractions isolated from limited numbers of available weevils were too small for field testing, we turned to examining differences between chromatograms of extracts of male and female hindguts or abdomens. Two compounds that were present in the abdomens of *P. strobi* males were absent in abdomens of females (31.5 and 51.0 min on the Carbowax 20M column). Each of these compounds were subsequently found in larger amounts in the more complex chromatogram of the Porapak extract of male *P. strobi*, from which they were collected and refractionated on the OV-225 column (40 and 48 min). The compound with the longer retention time was identified as grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol) (Figure 1a) from the mass, IR, and NMR spectra, and its identity was confirmed by coinjection with an authentic sample and by congruence of spectra (Tumlinson, 1969). We used the same techniques to identify the compound with the shorter retention time as the corresponding aldehyde, referred to here as grandisal (Hedin, 1977) (Figure 1b).

The same compounds were identified from hindgut and Porapak extracts

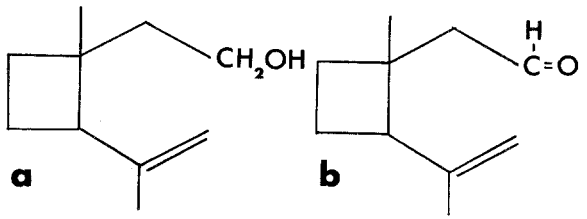

FIG. 1. *Pissodes* pheromone components: (a) grandisol, (b) grandisal.

TABLE 1. OCCURRENCE OF GRANDISOL AND GRANDISAL IN MALE *Pissodes*[a]

Species	Hindgut extracts (μg/individual)		Aerations (μg/weevil-hour)[b]	
	Grandisol	Grandisal	Grandisol	Grandisal
P. strobi	0.200	0.400	0.0033	0.0074
P. approximatus	0.026	0.013	0.0002	0.0006

[a] Presence of compounds determined by coinjection, amounts determined by GC comparison with known standards.
[b] A weevil-hour is equal to the amount produced by one weevil in one hour of aeration.

from *P. approximatus* males. In neither species were the compounds detected in the other body parts of the male or in any of the extracts from females.

The amounts of grandisol and grandisal occurring in the various extracts of male *Pissodes*, as determined by comparative GC, are presented in Table 1; the occurrence of these compounds in males at different times of the year is given in Table 2. One striking difference between the two species of *Pissodes* is that, in both sampling methods (Table 1), *P. strobi* appears to produce both chemicals at a level ten times greater than that of *P. approximatus*. Abdomen and hindgut extracts only reveal the compounds present within the insect at the time of dissection, but the extract from the Porapak aeration should represent the actual amounts and ratios of chemicals emitted by the insects through time. However, the results may not be quantitative because of uncertainties in collection and recovery efficiencies and in the stability of the aldehyde. In the Porapak extracts of the ratio of grandisol to grandisal produced by *P. strobi* is approximately 1:2, and that for *P. approximatus* is 1:3. Attempts to determine enantiomeric composition will be made when larger amounts of isolated grandisol and grandisal become available.

Field Activity. Table 3 presents the data for two field tests of grandisol and grandisal with a population of *P. approximatus*. The highest trap catch in both tests was on the treatment with male *P. approximatus* confined on a red pine bolt. However, the second treatment of each test, involving grandisol, grandisal, and a red pine bolt, attracted almost as many weevils as the male treatment. In the 1976 test, the two treatments with various amounts of grandisol and grandisal that did not include a red pine bolt caught significantly fewer weevils than that which included red pine, indicating synergism between odors from the host material and those from the synthetic chemicals. The two control treatments in this test, solvent (hexane) and a red pine bolt, caught similarly low numbers of weevils. In the 1977 test, the response to the higher levels of grandisol and grandisal was statistically

TABLE 2. SEASONAL OCCURRENCE OF PHEROMONE COMPONENTS IN EXTRACTS OF PORAPAK Q AERATIONS, EXISED HINDGUTS, OR ABDOMENS OF MALE *Pissodes* EXPOSED TO FIELD OR LABORATORY CONDITIONS PRIOR TO EXAMINATION[a]

	Present			Absent		
Species	Date[b]	Extract type	Condition	Date[b]	Extract type	Condition
P. strobi	5 May	Aeration	Field	11 June	Aeration	Field
	10 May	Abdomens	Field	3 Sept.	Aeration	Laboratory
	12 May	Aeration	Field	15 Dec.	Aeration	Laboratory
	26 May	Aeration	Field	24 Feb.	Aeration	Laboratory
				10 March	Aeration	Laboratory
P. approximatus	15 May	Hindgut	Field	18 Oct.	Hindgut	Laboratory
	3 June	Aeration	Field	21 July	Aeration	Field

[a] Occurrence of grandisol and grandisal was determined by GC coinjection with laboratory standards.
[b] Refers to date aeration was completed or hindguts or abdomens dissected.

TABLE 3. RESPONSE OF *Pissodes approximatus* TO GRANDISOL (GOH), GRANDISAL (GCHO), AND NATURAL ATTRACTANTS IN TWO FIELD TESTS

Date of Test	Treatments[a,b]	No. of weevils captured		
		Females	Males	Total[c]
Test 1:	4 Male *P. approximatus* plus red pine	27	18	45a
10 Aug–17 Sept., 1976	8 mg GOH + 4 mg GCHO + red pine	27	10	37a
	8 mg GOH + 4 mg GCHO	7	3	10bc
	14 mg GOH + 5 mg GCHO	14	2	16b
	Solvent (25 μl hexane)	1	6	7bc
	Red pine	3	1	4c
Test 2:	5 Male *P. approximatus* plus red pine	142	21	163a
11 May–6 July, 1977	28 mg GOH + 10 mg GCHO + red pine	109	30	139a
	14 mg GOH + 5 mg GCHO + red pine	54	21	75b
	5 mg GCHO + red pine	22	6	28c
	14 mg GOH + red pine	5	4	9d
	Red pine	8	0	8d

[a] Red pine bolts were freshly cut, 12.7 × 10.2 cm; chemical baits were changed weekly.
[b] Release rate of pheromone components was determined by cold trapping volatiles from a slow airstream and quantifying by GC: 8 mg GOH = 83 μg/day; 14 mg GOH = 174 μg/day; 28 mg GOH = 348 μg/day; 4 mg GCHO = 205 μg/day; 5 mg GCHO = 120 μg/day; 10 mg GCHO = 240 μg/day.
[c] Totals followed by the same letter(s) are not significantly different (chi-square test for independence, $P < 0.05$).

TABLE 4. RESPONSE OF *Pissodes strobi* TO GRANDISOL (GOH) WITH GRANDISAL (GCHO), AND NATURAL ATTRACTANTS IN TWO FIELD TESTS

Treatments[a]	No. weevils captured		
	Females	Males	Total[b]
Test 1: May 1 to June 27, 1980			
3 males + leader	9	14	23a
5 mg GOH + 10 mg GCHO + leader	4	15	19ab
leader	4	5	9b
Test 2: April 30 to June 19, 1981			
5 mg GOH + 10 mg GCHO + leader	10	9	19
leader	0	9	9

[a] Chemical baits were changed biweekly. Release rate of pheromone components was determined gravimetrically: 5 mg GOH = 43 μg/day, 10 mg GCHO = 80 μg/day.
[b] Totals in test 1 followed by the same letter are not significantly different (chi-square test for independence, $P < 0.05$). Differences between totals in test 2 were not significant.

similar to that for the male treatment; the response was nearly halved by halving the concentration of the chemicals. Grandisal with red pine was attractive, but attracted fewer weevils than when grandisol was included, indicating synergism between these two compounds. Grandisol with red pine was not attractive.

Of the three treatments examined in the first *P. strobi* field test (Table 4), the most attractive was that with male *P. strobi* caged on an eastern white pine leader. The response to grandisol plus grandisal on a white pine leader was intermediate between the response to males on a leader and that to a leader control. The second *P. strobi* test was intended to be a repetition of the first; unfortunately, all of the male weevils on the male-baited leaders died early in the experiment and this treatment is not considered here. Responses to grandisol and grandisal on a leader and to the leader control were numerically identical to those of the first test, but not significantly different from each other.

DISCUSSION

Our evidence indicates that two related volatile compounds produced by male *P. approximatus* serve as components of that species' aggregation pheromone and suggests that they serve in the same manner for *P. strobi*. Field response of *P. approximatus* was maximized when grandisol, grandisal, and host-associated odors were deployed together, indicating that the natural attractant is a multicomponent pheromone. The behavioral significance, if any, of each individual component is unknown at this time. As is the case for many bark and timber beetles (Scolytidae) (Borden and Stokkink, 1971) and the boll weevil (Hedin, 1977), pheromone production in *P. approximatus* (and presumably *P. strobi*) appears to be associated with the hindgut, as extracts of male bodies minus hindguts or abdomens revealed no traces of grandisol or grandisal. The different levels of pheromone release (Table 1) may reflect a natural difference between the species, or perhaps simply different responses to the conditions of the aeration procedure.

There are interesting similarities between the pheromone systems of *P. strobi* and *P. approximatus* and those reported for several other circulionid species. Tumlinson et al. (1969) reported that grandisol was one of four components of the male-produced boll weevil (*Anthonomus grandis* Boheman) aggregation pheromone. Although grandisal was never reported to have pheromonal activity for the boll weevil, this aldehyde was found in incubations of macerated male abdomens (Hedin, 1977). In addition, boll weevil pheromone compounds have been reported to be attractive to the pecan weevil *Curculio caryae* (Hedin et al., 1979) and the New Guinea sugarcane weevil, *Rhabdoscelus obscurus* (Chang and Curtis, 1972).

Atkinson (1979) demonstrated field response to the deodar weevil, *P. nemorensis* Germar, to grandisol and grandisal in combination with a pine bolt, in much the same way as we have with *P. approximatus*. Recently, Fontaine and Foltz (1982) have shown that male *P. nemorensis* feeding on slash pine (*Pinus ellioti* Engelm.) were much more attractive to local weevils than feeding females or pine bolts alone. Preliminary studies (unpublished) show that *P. nemorensis* males produce grandisol and grandisal, and we shall investigate their function. *P. approximatus* and *P. nemorensis* are similar ecologically but have different distributions (northeastern and southeastern, respectively) and breed at different times of the year.

Throughout the course of this study, several aerations and hindgut extractions of live weevils were conducted at different times of the year, but not all of the male extracts yielded grandisol and grandisal. It is apparent from Table 2 that males of *P. strobi* and *P. approximatus* that were producing pheromone components were collected in the spring from their respective natural breeding sites. These times coincide well with the periods of peak trap catches for these species, and with the times during which we have observed increased mating and oviposition in the field. In cases where the pheromone components were not found, the weevils were either used soon after eclosion and were not subjected to the same environmental stimuli as those collected in the field, or they were collected late in the breeding season. Those females collected with males that were producing pheromone (Table 2) were reproductively mature and oviposited readily on host material in the laboratory. Females of both *P. strobi* and *P. approximatus* apparently enter reproductive diapause in response to shortening day lengths while they break or avoid reproductive diapause during longer days of spring and early summer (ODell et al., undated ms., Zerillo et al., undated ms.). Male *Pissodes* will complete spermatogenesis within a week of eclosion and do not appear to have special environmental requirements for reproduction. Our data show that pheromone production in males concides with sexual maturity in females and suggest that pheromone production may be influenced by the same environmental stimuli that control reproductive biology in females.

In the field tests of *Pissodes* pheromone activity, the most attractive treatments were the natural pheromone sources: males feeding on host material. We speculate that the natural pheromone of each species may incorporate an optimal blend of enantiomers of grandisol and grandisal. The synthetic grandisol and grandisal used in these experiments were racemic. Documentation now exists of enantiomer-based differences in the pheromone systems of two sympatric species of *Gnathotrichus* ambrosia beetles (Borden et al., 1980) and in various species of *Ips* bark beetles (for example Birch et al., 1980; Lanier et al., 1980). We are approaching the question of enantiomeric

composition of the aggregation pheromones in *Pissodes* by synthesizing the pure enantiomers and bioassaying various ratios.

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DEFENSIVE AND PHEROMONAL SECRETION OF THE TERGAL GLAND OF *Aleochara curtula*[1] II. Release and Inhibition of Male Copulatory Behavior

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Abstract—At high concentrations, the defensive tergal gland secretion (TGS)[3] of the staphylinid beetle, *Aleochara curtula*, inhibits the male copulatory response (grasping with parameres). Inhibitory chemicals, for which a function as alarm substances is assumed, are *n*-undecane, 1-undecene, *n*-dodecanal, toluquinone, and 2-methoxy-3-methyl-1,4-benzoquinone. When emitted in small amounts, however, the TGS releases the male grasping response. The main components with aphrodisiac effect are (*Z*)-4-tridecene, *n*-dodecanal, and (*Z*)-5-tetradecenal. These supplementary mating stimulants, which are not sex specific, work synergistically with the aphrodisiac female sex pheromone from the epicuticular lipids and are discussed as alerting pheromones of short-term effect. Antennal movements of resting males as an indication of the recognition of a female and the approach to the mate are released at somewhat longer distances, when the TGS is additionally present.

Key Words—*Aleochara curtula* (Goeze), Coleoptera, Staphylinidae, tergal gland secretion, defense, mating stimulants, female sex pheromone, hydrocarbons, *n*-aldehydes, substituted 1,4-benzoquinones.

INTRODUCTION

The composition of the defensive secretions of beetles often is very complex and species specific, and the chemical patterns have been used as an additional criterion for exemplary taxonomic investigations (e.g., Dettner, 1980; Eisner et al., 1977; Moore and Wallbank, 1968; Schildknecht et al., 1968). However,

[1]Coleoptera: Staphylinidae (Aleocharinae).
[3]Abbreviations: TGS: tergal gland secretion; FE: female equivalent; ME: male equivalent.

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in the functional interpretation of the chemical complexity, the chief obstacle often is the absence of knowledge of the selective advantage of each of the various compounds (Tschinkel, 1975). Some authors have interpreted the constant occurrence of minor secretion compounds as a subsidiary result of biochemical pathways (Dettner, 1980). Besides the defensive properties and their physical chemical requirements, additional functions in intraspecific communications have been demonstrated for the defensive secretion compounds of some insects (e.g., Ayre and Blum, 1971; Keville and Kannowski, 1975; Löfqvist, 1976; Melber, 1977; Regnier and Wilson, 1968). The results of our present studies of the tergal gland secretion (TGS) of *Aleochara curtula* (Goeze) also indicate the additional use of byproducts of the defensive secretion in communication.

For Aleocharinae, a subfamily of staphylinid beetles, the defensive function of the TGS, as observed in the repulsing of ants, was demonstrated for different species (Hölldobler, 1970; Jordan, 1913; Pasteels, 1968). The composition of the secretion from the gland reservoir has been chemically investigated in *Lomechusa strumosa* (Blum et al., 1971), *Drusilla canaliculata* (Brand et al., 1973), and *A. curtula* (Peschke and Metzler, 1982). 1,4-Benzoquinones and series of aliphatic hydrocarbons and aldehydes have been established as compounds common in these beetles. Quantitative comparisons of males and female secretions of *A. curtula* revealed no sex specificity (Peschke and Metzler, 1982).

Kemner (1923) provided the first evidence that TGS possibly plays a role in the intraspecific communication of *A. curtula*, an abundant species living on carcasses. He reported that the beetles emitted a pungent odor, probably from the tergal gland, when activated at high temperatures in the field. The mating behavior then was released more obviously in males. In our cultures of mixed sexes, copulatory activity was also evidently increased when the beetles were slightly disturbed. Then even homosexual behavior was observed in groups of separated males; this was normally not seen with untroubled males of the same cultures. On the other hand, Peschke (1978a) demonstrated in *A. curtula* the occurrence of an aphrodisiac female sex pheromone, which is spread over the entire surface and trapped by epicuticular waxes. In preliminary experiments, long-chain hydrocarbons, which do not occur in the TGS, have been identified as releasers of the male grasping response; however, TGS compounds have been trapped from the air by Porapak Q (Peschke, in preparation).

In contrast with the observations after slight disturbance, which indicate a releasing or activating effect of the TGS in male copulatory behavior, males did not show sexual responses if the female was badly injured and emitted large amounts of the defensive secretion. The males fled from such females and cleaned the antennae. Other beetles, formerly resting nearby, started to rush around.

In the present paper, experiments on the role of the TGS of *A. curtula* in male copulatory behavior are reported. The preliminary observations have indicated contrary functions depending on concentration. Is the TGS able to release the male copulatory behavior (the grasping with parameres) at low concentrations, and does it inhibit the sexual response at high doses? What are the supplementary functions of the TGS compounds, in contrast to those of the epicuticular pheromone with its short-range and long-lasting effect? To answer these questions, the sex specificity, range, and rate of evaporation of active TGS compounds and their suspected synergism with the epicuticular pheromone in particular were investigated. In addition, experiments on the stimulatory effect on male behavioral patterns prior to the grasping response are reported.

METHODS AND MATERIALS

Rearing. A. curtula was reared in the laboratory according to Fuldner (1968) and Peschke (1978a). Immediately after emergence, sexes were separated, and the beetles were kept in groups of ten. After three weeks they were extracted or used in the bioassay.

Extracts. The tergal gland secretion (TGS) was obtained by inserting a triangle of filter paper between the sixth and seventh abdominal tergites of 20 day-old beetles of separated sexes freshly killed by freezing (45 min at −17° C). The contents of the gland reservoirs were absorbed by the filter papers which were extracted with *n*-hexane (gland contents from 100 beetles in 1 ml). These solutions were used for bioassay without further preparation.

Individual components from the TGS were identified and quantitatively determined by Peschke and Metzler (1982). For the bioassay, *n*-alkanes, 1-alkenes, dodecanal, and toluquinone were purchased from Fluka, Roth, and Sigma in >99.9% purity. (*Z*)-5-Tetradecenal and (*Z*)-4-tridecene were obtained by preparative GLC of the natural secretion, and 2-methoxy-3-methyl-1,4-benzoquinone was synthesized according to Peschke and Metzler (1982). The latter substance was dissolved in acetone; for all other compounds, *n*-hexane was used as the solvent.

The amounts of the total secretion or its single components were specified in terms of female or male equivalents (FE or ME). One FE means the total content of the reservoir of one female obtained by the filter paper method or the total content of individual components. These values represent averages of the gland contents of 100 beetles per preparation. As a control, quantitative GLC gave reproducible results from different extractions.

Extracts of the epicuticular sex pheromone were obtained by washing 100 freshly killed females (20 days old) in 50 ml chloroform–methanol (2:1 v/v) for 15 min at 22° C and evaporation to 1 ml. The concentrations of surface washings were also specified in terms of female equivalents.

Models. The following models for bearing the different extracts were prepared and glued to the tips of glass needles according to Peschke (1978a): (a) acetone extracted beetles (24 hr Soxhlett; male and female extracted beetles alone did not release male copulatory behavior but caused the same response rates after contamination with active agents); (b) 20-day-old males or (c) 20-day-old females kept isolated from each other, freshly killed by freezing (45 min at −17° C).

Samples of 0.01 ml solutions of the TGS, its single compounds, or surface washings were dropped onto the different models. Within 5 min after evaporation of the solvent (the shiny model became dull), the models were tested, then stored at room temperatures and tested for 5 min again at 1 hr, 24 hr, and 48 hr after contamination.

Grasping Reaction. Single males from groups kept in tens were repeatedly tested three weeks after emergence and sexual isolation. A model was held ten times in 1/2-sec intervals before a male running along the margin of a glass dish, the bottom of which was covered with moistened filter paper. The grasping with parameres towards the model was used as the criterion for the release of the male copulatory response (for further details of the test procedure see Peschke, 1978a). The number of males responding sexually at any of the ten encounters was recorded, pooled for identical models, and the response rate was specified in percent males showing the grasping reaction. Significance of differences was established by the chi-square test (fourfold table with original values) or by the exact test of Fisher, if any field of the fourfold table was filled with a value $\leqq 3$ (Sachs, 1969). The exact confidence limits (95%) drawn in the figures were taken from the tables of Hald (1965).

RESULTS

Behavioral Pattern of Grasping Response. Acetone-extracted beetles do not release the male mating behavior ($N_{\delta\delta} = 162$, 20 models). These models were contaminated with 0.01-ml hexane solutions of the TGS separately obtained from male or female beetles and were tested for the release of male sexual behavior 0–5 min after evaporation of the solvent. At certain percentages, the models contaminated with TGS released the same behavioral pattern of the grasping response as shown towards freshly killed females or models contaminated with female surface washings: a few millimeters before contact by the antennae, the males bent their abdomens over the backs of their heads and protruded the genitalia with the tonglike parameres (Figure 5c; Peschke, 1978a,b). In addition, perfect contact orientation along the female abdomen (Peschke, 1979) and attempts to couple the genitalia were also observed, but were not evaluated quantitatively.

Sex Specificity of TGS. The grasping response rate depends on the

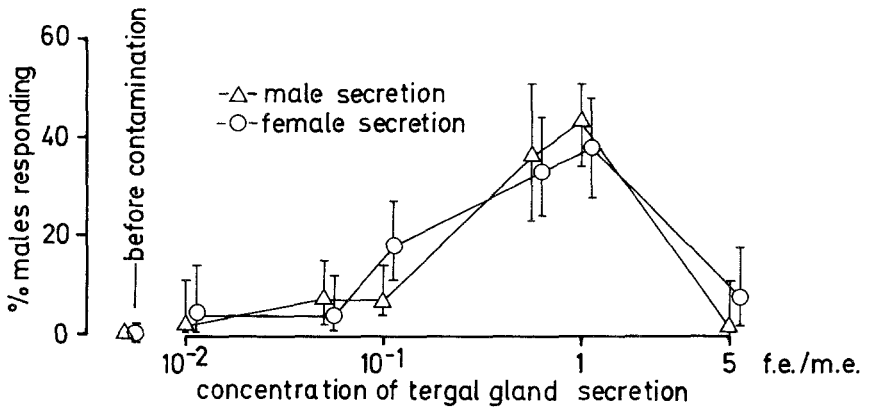

FIG. 1. Copulatory responses of *A. curtula* (% males grasping with parameres) to acetone-extracted beetles 0–5 min after contamination with male or female tergal gland secretion at various concentrations (for each point 50–140 males tested with 2–5 models; vertical lines: 95% confidence limits).

concentration of the TGS (Figure 1); however, significant behavioral differences between male and female secretion could not be established. This was congruent with the chemical comparison which revealed no sex specificity, either qualitatively or quantitatively (Peschke and Metzler, 1982). Therefore, in the subsequent experiments, it was not necessary to test the secretions of both sexes, and the investigations were restricted to the female secretion as releaser of male copulatory behavior.

Release of Grasping Response Depending on Concentration of TGS. The maximum releasing effect of the TGS was observed with 38% of the males responding at a concentration of 1 FE ($N_{\delta\delta}$ = 98, 4 models, Figure 1). This response rate, however, was low in comparison to that obtained with freshly killed females (98%, $N_{\delta\delta}$ = 982, 33 models) or 1 FE surface washings of females spread on acetone-extracted beetles (85%, $N_{\delta\delta}$ = 974, 37 models). At the unnaturally high concentration of 5 FE of TGS, most of the males fled the model and cleaned the antennae; only a few males showed the grasping response (8%, $N_{\delta\delta}$ = 49, 2 models). Dilution to 0.1 FE (18%, $N_{\delta\delta}$ = 101, 4 models) or less caused significant reductions in the response rates in comparison to 1 FE ($P < 0.001$).

Contamination of Freshly Killed Males. In the former experiments, the releasing effect of pure TGS was demonstrated by contamination of acetone-extracted beetles. However, in the natural situation, the secretion is contaminated onto a wax-covered surface. Therefore, freshly killed males (>20 days old, kept isolated) which did not release the male grasping reaction prior to

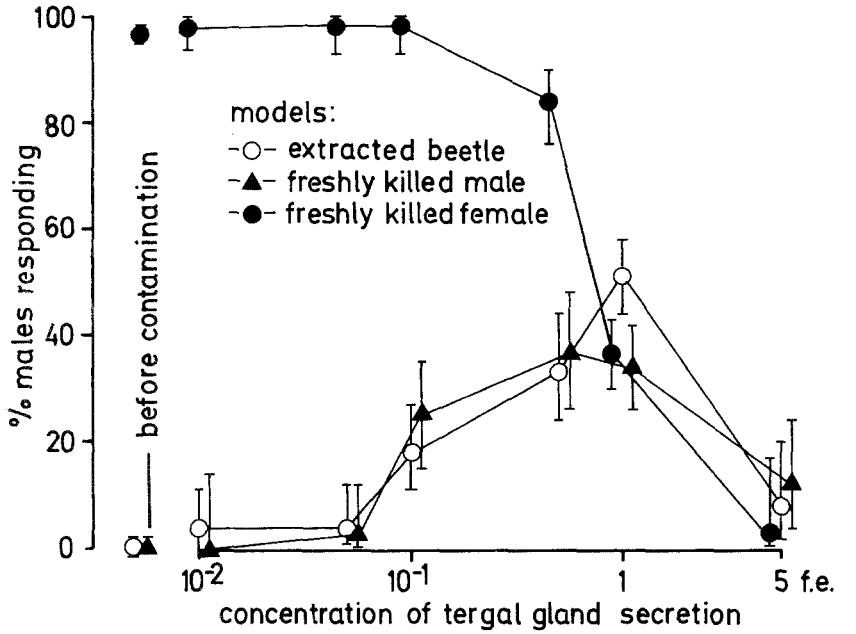

FIG. 2. Copulatory responses of *A. curtula* (% males grasping with parameres) to different models (acetone-extracted beetles, freshly killed males or females) 0–5 min after contamination with the tergal gland secretion at various concentrations (for each point 50–240 males tested with 2–10 models; vertical lines: 95% confidence limits).

contamination (0%, $N_{\delta\delta} = 319$, 17 models) were treated with different concentrations of the TGS and tested 0–5 min after evaporation of the solvent. The response rates were quite similar to those from experiments using extracted beetles as models (Figure 2).

Inhibitory Effect by Contamination of Freshly Killed Females. Freshly killed females released the full male response prior to contamination (98%, $N_{\delta\delta} = 982$, 33 models). In the first 5 min after contamination with the TGS, concentrations up to 0.1 FE caused no significant effects (Figure 2). However, at 0.5 FE a slight but significant decrease to 84% male response was observed ($N_{\delta\delta} = 133$, 3 models; $P < 0.001$). Only 36% or 3% of the males responded sexually to contaminated females at concentrations of 1 and 5 FE, respectively ($N_{\delta\delta} = 234$, 9 models; or $N_{\delta\delta} = 31$, 1 model; $P < 0.001$ in comparison to untreated female).

Synergism of TGS with Female Epicuticular Sex Pheromone. In the former experiment, it was not possible to demonstrate synergism of the TGS with the epicuticular pheromone by contamination of freshly killed females because these already yielded a response rate near 100% prior to contamination. Also a surface washing from females, containing 1 FE of the epicuticular

sex pheromone, had produced a male grasping response rate of 85% when applied to an acetone-extracted beetle. After its dilution to 0.1 FE, however, only 55% of the males responded with the grasping reaction in the first 5 min after evaporation of the solvent ($N_{\delta\delta}$ = 178, 7 models). The TGS was then added at different concentrations to the 0.1 FE surface washing. In comparison to the 55% level, significant increases of the male response rate (Figure 3, $P < 0.001$) were obtained by adding the TGS in concentrations from 1 FE to 10^{-3} FE; at the latter, the TGS alone was not capable of releasing the male reaction. The optimal response rate of 99% was produced by the admixture of 0.05 FE or 0.1 FE of the TGS. ($N_{\delta\delta}$ = 62, 2 models; or $N_{\delta\delta}$ = 66, 2 models). At 10^{-4} FE no further significant synergistic effect could be established (56%; $N_{\delta\delta}$ = 54, 2 models). On the other hand, addition of 5 FE

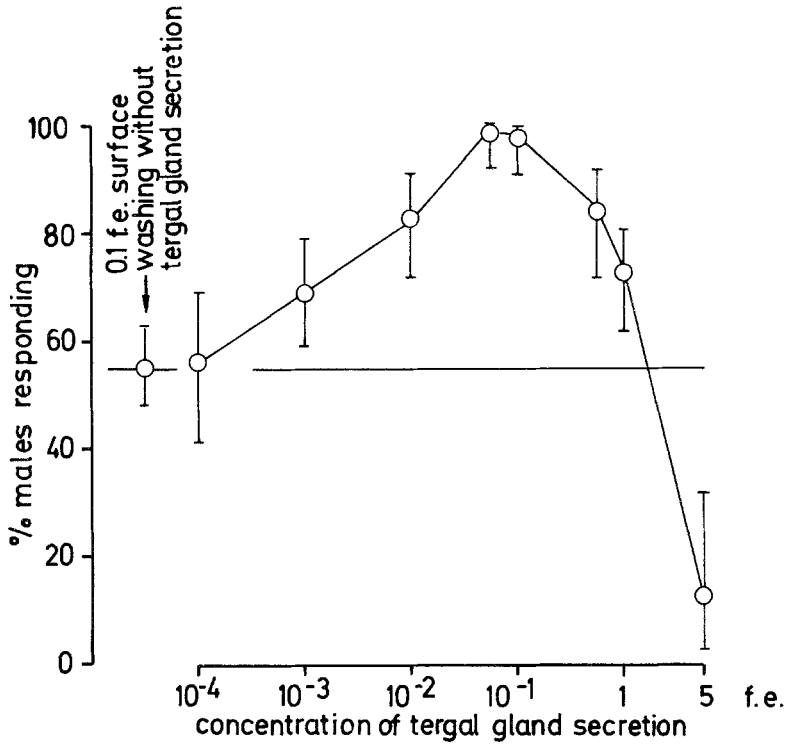

FIG. 3. Copulatory responses of *A. curtula* (% males grasping with parameres) to acetone-extracted beetles 0–5 min after contamination with mixtures of 0.1 FE female surface washing and various concentrations of the tergal gland secretion (for each point 50–100 males tested with 2–3 models; vertical lines: 95% confidence limits; horizontal line: level of response to 0.1 FE female surface washing without admixture of the tergal gland secretion).

caused a striking decrease of the response rate to 13% ($N_{\delta\delta}$ = 24, $P < 0.001$).

Releasing and Inhibitory Effects in Relation to Time. In the former experiments, models contaminated with TGS were used for only 5 min immediately following evaporation of the solvent, because later on the response rate declines according to preliminary observations. However, natural females released the male grasping response at 94% ($N_{\delta\delta}$ = 203, 7 models) even 1 day after being killed by freezing. One FE of a female surface washing spread on an extracted beetle one day earlier still released the male copulatory behavior at a rate of 45% ($N_{\delta\delta}$ = 195, 7 models). Therefore the evaporation of active TGS compounds was evaluated by contamination of different models with 1 FE of the female secretion and tests after 0–5 min at 1 hr, 24 hr, and 48 hr (Figure 4).

In the first 5 min after contamination with 1 FE of the TGS, the extracted beetles produced somewhat higher response rates (56%, $N_{\delta\delta}$ = 144, 6 models) in this new series of experiments than equally contaminated freshly killed males (34%, $N_{\delta\delta}$ = 136, 6 models, $P < 0.001$). However, 1 hr after treatment, the extracted beetles failed to release grasping (0%, $N_{\delta\delta}$ = 95, 3 models), whereas contaminated, unextracted males still yielded response rates of 14%

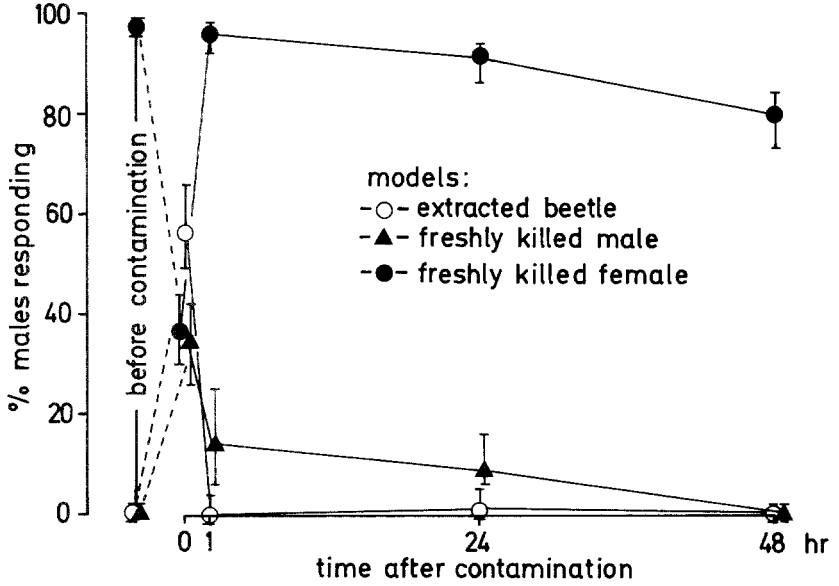

FIG. 4. Copulatory responses of *A. curtula* (% males grasping with parameres) to different models (acetone-extracted beetles, freshly killed males or females) in relation to time after contamination with 1 FE of the tergal gland secretion (for each point 50–240 males tested with 2–9 models; vertical lines: 95% confidence limits).

($N_{\delta\delta}$ = 57, 2 models, $P < 0.001$), and one day later of 9% ($N_{\delta\delta}$ = 175, 6 models, $P < 0.001$). Perhaps this delay of evaporation is caused by the trapping of TGS components in the natural wax cover of the male model. However, this is of little effect when compared to the permanence of the female epicuticular sex pheromone.

Immediately after contamination of freshly killed females with 1 FE of TGS, the grasping response rate was only 36%; ($N_{\delta\delta}$ = 234, 9 models); however, 1 hr after treatment the initial response rate of 96% was reestablished ($N_{\delta\delta}$ = 239, 6 models, $P < 0.001$, Figure 4). Then, 24 or 48 hr later, no significant differences with the very slow decrease of attractiveness of an untreated freshly killed female could be ascertained.

Releasing and Inhibitory Effects of Single Components. In order to determine which single components of the TGS release the male grasping response, the main compounds were tested individually at concentrations of 1 or 0.1 FE, simulating the plentiful and moderate emission of the secretion, respectively. The latter concentration was chosen because it did not reduce the female attractiveness, but gave sufficient response when the complete TGS was tested on acetone-extracted beetles. Acetone-extracted beetles were contaminated and tested for 5 min only, following evaporation of the solvent (Table 1A). Toluquinone and 2-methoxy-3-methyl-1,4-benzoquinone did not release the male copulatory response at either concentration. Also the main hydrocarbons, *n*-undecane and 1-undecene, yielded no response. From the C_{12} hydrocarbons, only *n*-dodecane was available in sufficient amounts; a few males responded sexually to it at a concentration of 1 FE. (*Z*)-4-Tridecene was the most effective hydrocarbon and yielded response rates of 12%. *n*-Pentadecane did not release male grasping at either concentration; however, a few males did respond to *n*-hexadecane at 1 FE. Both aldehydic compounds, *n*-dodecanal and (*Z*)-5-tetradecenal, were quite effective.

In a second test series, freshly killed females were also contaminated with synthetic or purified natural compounds of the TGS (0.1 and 1 FE) and, after evaporation of the solvent, tested 0–5 min for release of the male grasping response (Table 1B). *n*-Hexane and acetone, which were used as solvents, did not reduce the female attractiveness. At a concentration of 1 FE, significantly fewer males responded sexually to females after contamination with toluquinone, 2-methoxy-3-methyl-1,4-benzoquinone, *n*-undecane, 1-undecene, (*Z*)-4-tridecene, and *n*-dodecanal, respectively. At 0.1 FE, the inhibitory effect of all these chemicals was significantly weaker, with more than 80% of the males responding sexually to the models. Only one exception was found: at 0.1 FE of toluquinone, the response rate was still drastically reduced to 39%. Moreover, striking differences of response rates (100% or 5%) were observed with females treated with *n*-dodecanal at concentrations of 0.1 and 1 FE, respectively. These effects demonstrate that the test of individual chemicals could not

TABLE 1. COPULATORY RESPONSES OF *A. curtula* (% MALES GRASPING WITH PARAMERES: %GR) TO ACETONE-EXTRACTED BEETLES (A) OR FRESHLY KILLED FEMALES (B), BOTH CONTAMINATED WITH INDIVIDUAL COMPONENTS OF TERGAL GLAND SECRETION AT CONCENTRATIONS OF 1 OR 0.1 FE

Substance	0.1 FE				1 FE				1 FE (μg/gland)[d]
	%GR	$N_{\delta\delta}$	N_{models}	P^a	%GR	$N_{\delta\delta}$	N_{models}	P^a	
A. *Contamination of acetone-extracted beetles*									
Untreated model	*0*	162	20		*0*	162	20		
Solvents									
n-Hexane	*0*	162	20		*0*	162	20		
Acetone	*0*	42	2		*0*	42	2		
Hydrocarbons									
n-Undecane	*0*	49	2	N.S.[c]	*0*	68	3	N.S.[c]	96.9
1-Undecene	*0*	49	2	N.S.[c]	*0*	67	3	N.S.[c]	10.9
n-Dodecane	*0*	69	3	N.S.[c]	*3*	71	3	N.S.[c]	2.7
(*Z*)-4-Tridecene	*11*	62	3	+++[c]	*12*	59	3	+++[c]	2.9
n-Pentadecane	*0*	45	2	N.S.[c]	*0*	72	3	N.S.[c]	2×10^{-2}
n-Hexadecane	*0*	65	3	N.S.[c]	*6*	67	3	++[c]	2×10^{-2}
Aldehydes									
n-Dodecanal	*38*	48	2	+++[c]	*15*	114	5	+++[c]	21.4
(*Z*)-5-Tetradecenal	*9*	45	2	++[c]	*35*	46	2	+++[c]	4.7
Quinones									
Toluquinone	*0*	75	3	N.S.[c]	*0*	69	2	N.S.[c]	24.0
2-Methoxy-3-methyl-1,4-benzoquinone	*0*	77	3	N.S.[c]	*0*	52	2	N.S.[c]	130.0

B. *Contamination of freshly killed females*									
Untreated model	*97*	982	33		*97*	982	33		
Solvents									
n-Hexane	*93*	406	14	+++[b]	*93*	406	14	+++[b]	
Acetone	*97*	78	3	N.S.[b,c]	*97*	78	3	N.S.[b,c]	
Hydrocarbons									
n-Undecane	*84*	50	2	+	*56*	84	3	+++	96.9
1-Undecene	*90*	62	2	N.S.	*32*	72	3	+++	10.9
n-Dodecane	*85*	59	2	+	*66*	58	2	+++	2.7
(*Z*)-4-Tridecene	*97*	30	1	N.S.[c]	*75*	55	2	+++	2.9
n-Pentadecane	*98*	58	2	N.S.[c]	*97*	60	2	N.S.[c]	2×10^{-2}
n-Hexadecane	*97*	63	2	N.S.[c]	*99*	73	2	N.S.[c]	2×10^{-2}
Aldehydes									
n-Dodecanal	*100*	117	3	+++	*5*	98	4	+++	21.4
(*Z*)-5-Tetradecenal	*96*	113	3	N.S.	*94*	71	2	N.S.	4.7
Quinones									
Toluquinone	*51*	104	4	+++	*62*	124	5	+++	24.0
2-Methoxy-3-methyl-1,4-benzoquinone	*90*	94	3	N.S.[c]	*64*	154	6	+++[c]	130.0

[a] Statistical significances (+++: $P < 0.001$, ++: $P < 0.01$, +: $P < 0.05$, N.S.: not significant) were calculated by chi-square test relative to models contaminated with the solvent, except in *b*)

[b] Models contaminated with the solvent were compared to the untreated model.

[c] Exact test of Fisher used, otherwise normal fourfold table.

[d] Quantitative chemical determination for 20-day-old females after Peschke and Metzler (1982).

exactly simulate the effects of the complex TGS with the concert of compounds of variable functions.

Distance of Release of Antennal Movements and Grasping. Until this point in the study, the only criterion for the degree of sexual excitement was the capability of a female or of a contaminated model to release the male grasping response. The aphrodisiac compounds of the TGS are more volatile than those of the epicuticular sex pheromone. Therefore, it was assumed that the additional mating stimulants of the TGS might act at longer ranges. In the following experiments, we measured the distances from which the grasping response was trigged when the TGS was present or not. When a female ran towards a resting male in our cultures we incidentally observed typical antennal movements of the male just before the grasping response was released. This behavior was apparently a first indication of sexual excitement, and we also measured the distance from which the rapid antennal movements were released.

Special Methods. Single males were allowed to rest at the margin of an 8-cm-diam Petri dish, the bottom of which was covered with moistened filter paper. Directly 180° opposite, a freshly killed female glued to a needle was fixed outside and hung into the dish near the bottom and margin and was preexposed for 1 min in order to allow the development of an odor gradient. Models contaminated with 1 FE of the TGS were used 0–5 min after evaporation of the solvent only. The dish was rotated and thereby moved the marginal male with head first at a speed of 2 cm/sec towards the longitudinal axis of the fixed model. The behavior of the resting male was recorded as it approached by a National video system, and the distance between the freshly killed female and the front of the male's head, when it showed the first indication of antennal movements or grasping with parameres, was measured by analyzing single exposures. Significance of differences between mean distance values was established by t test (Sachs, 1969). In another series of experiments the male beetles were allowed to run freely along the margin of a fixed dish.

RESULTS

At first, freely running males were observed while approaching a freshly killed female. The first indication of the grasping response (opening the genital segment) was observed at a distance of 2.1 ± 0.5 mm ($N = 26$) from the male head to the body of an untreated female. If the female was contaminated with a solution of 0.1 FE of the TGS, the grasping response was released at a distance of 2.2 ± 0.7 mm ($N = 24$). This difference was not significant, and we also could not observe any other change of behavior of the quickly approaching male. With a resting male, however, which was passively advanced to an uncontaminated female, a new male behavioral step prior to

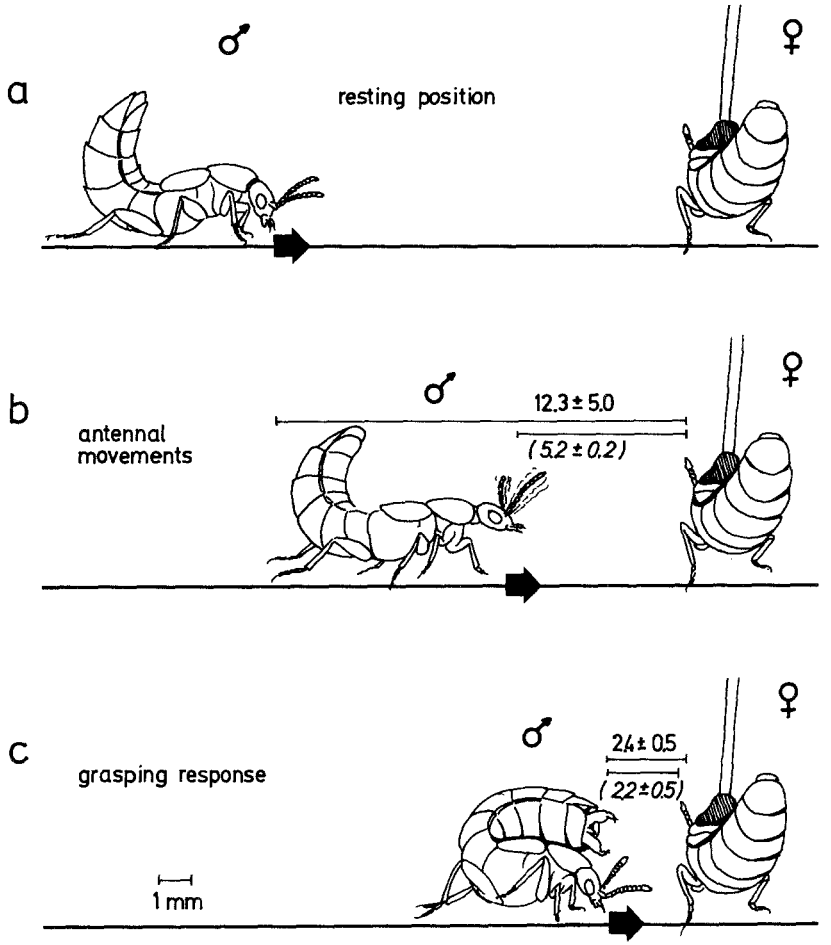

FIG. 5. Behavior of resting *A. curtula* males, passively advanced to a fixed female freshly killed by freezing: (a) resting male with its head bent down and motionless antennae; (b) the male raises its head and vibrates the antennae; (c) grasping with parameres. The distances (mm) to the female, at which the respective male behavior has first been observed, is noted for females 0–5 min after contamination with 0.1 FE of the tergal gland secretion (upper lines) and for untreated females (lower lines, in brackets).

the grasping reaction could be observed. Usually, a resting male bent its head down to the ground and held the antennae horizontal and motionless (Figure 5a). When a female approached to a distance of 5.2 ± 0.2 mm ($N = 29$), the male raised its head and vibrated its antennae (Figure 5b). This behavior was released at a significantly longer distance (12.3 ± 5.0 mm; $N = 18$; $P < 0.001$), if the female had borne 0.1 FE of the TGS. In a separate experiment,

where the approach of the model was stopped at the moment of the release of antennal movements, the males started to walk towards the female within a few seconds. Normally, the male was drawn towards the female at constant speed and the grasping reaction was observed at a distance of 2.4 ± 0.5 mm ($N = 24$) from a control female (Figure 5c) and of 2.2 ± 0.5 mm ($N = 14$) from a female contaminated with 0.1 FE of the TGS. This difference was not significant.

DISCUSSION

Hölldobler (1970), Jordan (1913), and Pasteels (1968) have shown the primary function of the tergal gland secretion (TGS) of different Aleocharinae to be that of defense. This could apply as well for the closely related species *Aleochara curtula*, which has a TGS of quite similar chemical composition (Peschke and Metzler, 1982). One can assume that recognition of the defensive secretion, plentifully emitted by a badly injured beetle could enhance the chances of a conspecific individual escaping a predator. In these situations, copulatory behavior would be inappropriate. Indeed, at high concentrations of TGS, the male grasping response to females is inhibited in our experiments, even though this is a rather brief effect.

The aphrodisiac sex pheromone from the epicuticular hydrocarbons of *A. curtula* females alone is capable of releasing the male grasping response, since components of the TGS could not be detected in these preparations (Peschke, 1978a). However, TGS compounds were trapped from air currents over a group of beetles by Porapak absorption (Peschke, in preparation). At present, the actual concentration of the secretion cannot be measured in the immediate surroundings of the beetles in different short-term behavioral situations. However, male copulatory behavior of *A. curtula* is enhanced when the odor of the TGS is present, thus confirming former observations of Kemner (1923) in the field. The stimulatory effect of the TGS alone is rather weak in comparison to the epicuticular pheromone, but it works synergistically to that sex pheromone even at very low concentrations.

Comparative chemical investigations revealed no sex specificity of the composition of the TGS of *A. curtula* (Peschke and Metzler, 1982), and male or female secretions alone release equivalent copulatory responses. However, the homosexual reactions to these substances are rare when the essential information from the epicuticular sex pheromone is lacking. In a combat situation, where the defensive secretion is emitted, it was occasionally observed that the males switch from fighting to grasping with the parameres.

The additional function of the TGS of *A. curtula* when emitted at low concentration would fit the definition of a supplementary mating stimulant or aphrodisiac. The information of the female sex pheromone from the

epicuticular hydrocarbons is very permanent and therefore difficult to alter. In contrast, the volatile secretion from the tergal gland can be used in sending short-term information, because the releasing as well as the inhibitory effects decline within minutes. In addition, it is believed that males perceive the volatile releasing compounds of TGS from distances somewhat greater than those observed for the epicuticular pheromone alone. The distance from which the male grasping response is triggered by a female was not influenced by the TGS; however, the male antennal movements as a first sign of excitement and the approach to a female then is released at a 2.4 times greater distance, when the odor of the TGS is additionally present. In the first instance, this extension of range might seem to be unimportant; however the beetles live in small irregular cavities under carcasses, particularly those of mammals, and the probability of encounters of males and females of *A. curtula* in those labyrinths would be increased after emission of the TGS. Long-distance attractants emitted by the beetles themselves have not yet been demonstrated; it is, however, assumed that odors from the carcasses bring both sexes of the abundant species together in the small habitat.

For the repellent TGS compounds, which inhibit the male copulatory response at high concentrations, the definition of a "pheromone" seems not to be appropriate, since the responding beetles only secondarily use information apparently not selected for them, and the advantage for the signaler is not established (Burghardt, 1970). However, according to Atema (1977) the neutral term "alarm substance" can be used without any implication of communicative or social function. The capability of the receiver to perceive TGS compounds, however, presupposes the use of the secretion as a pheromone as a mating stimulant when emitted at low concentrations. Then the female is able to send a distinct and obviously adaptive signal to the mate. Perhaps the essential function of the TGS in the male mating behavior of *A. curtula* is generally a stimulatory or activating one, alerting the male to receive the essential information from the female epicuticular pheromone. In further investigations the alerting function of the TGS compounds in intraspecific communication, other than that of mating, will be analyzed in connection with chemical measurements of the actual emission of the secretion.

The gradual emission of the TGS is accomplished by the morphology of the gland reservoir, an invagination of the intersegmental membrane between the sixth and seventh abdominal tergites (Araujo, 1978; Peschke and Metzler, 1982). The morphology of the abdominal musculature was described by Peschke (1978b): supported by a fold of the intersegmental membrane, the opening of the reservoir is closed when the sixth and seventh tergites are pressed together by the strong tergosternal muscle of tergite VI (M8). This closure is so tight that compounds of the TGS could not be detected by GLC in

surface washings of beetles carefully killed by freezing. The gradual opening of the reservoir could be accomplished by tilting the seventh tergite in its transverse axis; this motion is produced by the action of modified tergosternal muscles upon the anterior rim of tergite VII (M8). An extensive ejection of the TGS could be supported by a small muscle, which inserts at the reservoir itself (derived from the tergal muscle M2), and by increasing the pressure of the abdominal hemolymph.

In many arthropods 1,4-benzoquinones have evolved independently to essential defensive agents with deterrent or toxic effects towards a proposed enemy (Eisner and Meinwald, 1966; Schildknecht et al., 1968; Weatherston and Percy, 1970). Conforming with this obvious function, toluquinone and 2-methoxy-3-methyl-1,4-benzoquinone of *A. curtula* do not release the male copulatory behavior; on the other hand, when a female is badly injured, the plentiful emission of quinones diminishes the releasing effect of the epicuticular sex pheromone. However, the inhibitory effect seems not to be restricted to the quinones, because *n*-undecane, 1-undecene, (*Z*)-4-tridecene, and *n*-dodecanal also reduce the female attractiveness at high concentrations. On the other hand, at low concentrations simulating the moderate emission of the TGS, some minor hydrocarbons, such as (*Z*)-4-tridecene, *n*-dodecane, and even hexadecane, as well as *n*-dodecanal and (*Z*)-5-tetradecenal, can individually release the male grasping response.

While not assessed in each case experimentally, the function of additional lipid compounds of defensive secretions, especially hydrocarbons, as solvents or spreading the penetration agents is discussed by many authors (Blum et al., 1968; Calam and Youdeowei, 1968; Regnier and Wilson, 1968; Remold, 1962; Tschinkel, 1975; von Endt and Wheeler, 1972; Waterhouse and Gilby, 1964; Wilson and Regnier, 1971). Supplementary to their proposed physico chemical function, the significance in chemical intraspecific communication of the hydrocarbons from defensive secretions has been proved with some insects (e.g., Ayre and Blum, 1971; Löfqvist, 1976; Melber, 1977; Regnier and Wilson, 1968). Similar to *A. curtula*, an additional aphrodisiac function of hydrocarbons has been demonstrated for the defensive secretion of the pygidial gland of *Tribolium confusum* (Tenebrionidae) by Keville and Kannowski (1975).

Because of their lipid character and their pungent odor, the aldehydes from the TGS of *A. curtula* might also act as solvents and deterrents. Long-chain aliphatic aldehydes occurring in arthropod defensive secretions have been found previously in other Aleocharinae (Brand et al., 1973) and Myriapoda (Wheeler et al., 1964). Similar aldehydes have been identified as male or female sex pheromones of moths, but they are produced in quite different glands and are not connected with a defensive function (Dahm et al., 1971; Leyrer and Monroe, 1973; Röller et al., 1968; Roelofs et al., 1974; Underhill et al., 1977; Weatherston et al., 1971).

In summary, minor compounds of the TGS of *A. curtula*, which were formerly considered as byproducts of the biochemical pathway of defensive agents only (Peschke and Metzler, 1981), appear to have additional functions in intraspecific communication. These findings provide new insight into the selective advantage of the complexity and species specificity of the defensive secretion of these beetles.

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APPARENT LONG-TERM BODILY CONTAMINATION BY DISPARLURE, THE GYPSY MOTH (*Lymantria dispar*[1]) ATTRACTANT[2]

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Abstract—Positive attraction of male gypsy moths to the body of the author, now four years after his last known direct contact with disparlure, the synthetic pheromone, is documented. A designed test showed that moths responded to him in highly significantly greater numbers than to others who had had less or no previous exposure to the insect and/or disparlure. The data present persuasive evidence of bodily contamination with disparlure and suggest that the material is very persistent.

Key Words—Disparlure, *Lymantria dispar*, Lepidoptera, Lymantriidae, gypsy moth, pheromone persistence.

INTRODUCTION

From 1971 to 1977, I was exposed to large quantities of racemic disparlure (*cis*-7,8-epoxy-2-methyloctadecane), the synthetic pheromone of the gypsy moth [*Lymantria dispar* (L.) (Lepidoptera: Lymantriidae)] (Bierl et al., 1970), during a variety of laboratory and field tests. Based on observations made in Italy during 1979, two years after my last known direct contact with disparlure, I recently reported (Cameron, 1981) the apparent contamination of my body by this attractant. Casual observation in Pennsylvania during the male flight period in 1980, and again early in the flight period in 1981, suggested that, even though I have not resumed studies using disparlure, I

[1] Lepidoptera: Lymantriidae.

[2] Authorized for publication as paper No. 6284 in The Journal Series of The Pennsylvania Agricultural Experiment Station. Mention of commercial products is for information purposes only.

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remain more attractive to male moths than are other people. Consequently, an experiment was designed to test the hypothesis that I am not differentially attractive to male gypsy moth adults.

METHODS AND MATERIALS

During tests conducted from July 13 to 15, 1981, six subjects stood 20 m apart on a 100 m line established along the edge of Old Main Lawn at The Pennsylvania State University. Each subject provided a vertical silhouette which plays an important role in aiding males to orient to a location (such as the bole of a tree) where female moths are likely to be found (Richerson et al., 1976a). The line was aligned at approximately right angles to the predominant direction of the wind during observations. Each subject was assigned positions on the line such that all occupied each station exactly once during each test. The order and position were determined by hypergeometric sampling using the roll of a die. All tests were conducted between 1420 and 1540 hr. Weather conditions were similar on each of the three days of observation. Recorded temperatures were between 25° and 26.5° C; there was a light overcast on July 13 (first replicate), clear sky on July 14 (second and third replicates), and high scattered cloud on July 15 (fourth and fifth replicates). Wind was fairly steady at 8–13 kph on July 13, but erratic, intermittent, and swirling with speeds of 0–11 kph on both July 14 and 15.

In addition to myself, test subjects included my wife, son, and daughter, all of whom were present when observations were made in 1979 in Italy (Cameron, 1981), and two graduate student volunteers, one male and one female. In an attempt to eliminate as many extraneous stimuli as possible, all participants showered and washed their hair immediately prior to each day's tests, using Ivory soap and Flex shampoo. Each day, all clothing (including undergarments) had been washed simultaneously, using Tide® detergent, and placed in sealed plastic bags until donned following the shower. Towels were also washed in the same load. Each subject wore a white cotton T-shirt, blue gym shorts, and white socks. All wore jogging or tennis shoes, which included the colors white, blue, and yellow; the shoes were not washed or otherwise treated prior to the tests.

Each male gypsy moth making contact with a subject was recorded during six successive synchronous 5-min periods of time. (Each subject acted as his or her own observer and recorder.) Contacts were recorded separately for the head, torso (including arms and hands), and legs and feet. An individual moth was counted only once, regardless of the number of individual contacts with the subject, unless it clearly broke off searching behavior and left the vicinity of the subject.

On both July 14 and 15, successive tests (replicates) were conducted with

TABLE 1. ANALYSIS OF VARIANCE TABLE SHOWING SIGNIFICANT FACTORS IN GYPSY MOTH ATTRACTION TESTS

Source of variation	*df*	Mean square	*F* value[a]
Replicate	4	76.66	10.28***
Subject	5	138.49	18.56***
Body part[b]	2	1180.82	158.29***
Subject by body part	10	94.32	12.64***
Error	518	7.46	
Total	539	15.15	

[a] Ratios indicated by *** are very highly significant ($P < 0.0001$).
[b] Head, torso, legs/feet.

subjects wearing both shoes and socks during the first test but barefoot during the second. No differences in response, attributable to footwear, were recorded; consequently, data from all five tests could be analyzed together.

RESULTS AND DISCUSSION

An analysis of variance of the data (Table 1) indicate very highly significant differences among the replicates, the body part first contacted, the subjects (Table 2), and the subject by body part interaction term. Differences among all of the other factors tested (date, presence or absence of footwear, successive 5-min intervals during each repetition of the test, and spot along the line of observers), and other interactions tested, were not significant. (Significance levels ranged from 48 to 99+% for these factors.)

TABLE 2. AVERAGE NUMBERS OF MALE GYPSY MOTH CONTACTS PER BODY PART[a] PER 5 MIN

Subject	N[b]	Mean numbers of contacts[c]
5	90	4.200a
1	90	2.111b
2	90	1.867bc
4	90	1.656bc
3	90	0.944bc
6	90	0.722c

[a] Head, torso, legs/feet.
[b] $N = 30$ five-minute intervals times 3 body parts per interval.
[c] Numbers followed by the same letter are not significantly different ($\alpha = 0.01$) (Duncan's Multiple-Range Test).

Six commercial gypsy moth traps baited with disparlure, located approximately 2 km from the campus and operated continuously and independently of this test, captured 227 (46.6% of the total), 193 (39.6%), and 67 (13.8%) male gypsy moths on July 13, 14, and 15, respectively. Moth contacts per test replicate for all observers combined were 343 (50.1%), 212 (average of two replicates, 30.9%) and 130 (average of two replicates, 19.0%) on these same dates. The significant difference among replicates is thus reasonably explained by the pool of moths available to respond on successive days. During all tests, 11 (1.1%) of the 1027 first contacts were recorded on the head, 150 (14.6%) on the torso, and 866 (84.3%) on the legs and feet. Most frequent initial contact was on the lower leg or foot, often followed by searching activity up the legs toward the torso. The orientation of male moths to a vertical silhouette, and their subsequent searching behavior, were consistent with that reported for males stimulated by the pheromone under field conditions (Richerson et al., 1976a,b). The great majority of moths responding to all subjects was noted to be flying within 1 m of the ground, explaining this difference. The high significance of the subject by body part interaction mean square is attributed to the single subject (No. 5) who is most attractive, and the large difference between legs/feet and the rest of the body. No effort is made to explain this further because subject 5 did not differ from the others in body part first contacted.

Based on the data presented, it is reasonable to conclude that I (subject 5) retain a positive attraction to male gypsy moths, now four years after last working with disparlure.

All of my family members were present with me at the time of the observations in Italy; none of them attracted any moths at that time. In the 1981 tests, moths did respond to them. Subject 1 (a student) had worked with laboratory-reared gypsy moth adults during a period of approximately 3–5 months prior to these tests. My son (subject 4) has had some incidental contact with disparlure during the early and mid-1970s; on occasion, my daughter, wife (subjects 2 and 3), and son have assisted me in both laboratory and field phases of research, including the handling of female moths, during that same time. Subject 6 (a student) has never previously worked with the gypsy moth.

It remains to be determined whether or not traces of disparlure can be detected in my body. The gypsy moth responds far more readily to the (+)-enantiomer of disparlure than to the racemic mixture (Plimmer et al., 1977; Cardé et al., 1977; Miller et al., 1977; *inter alia*), although at low rates (1.0 μg bait) (+)-disparlure was only slightly more attractive than racemic disparlure (Plimmer et al., 1977). All my experience and exposure during earlier studies has been with and to racemic disparlure, never the (+)-enantiomer. If my body is contaminated, either this contamination has persisted for a period of at least four years, or I am apparently synthesizing the

material. It has been speculated that disparlure may be rather less susceptible to metabolic alteration than pheromones containing long-chain alkenes or alkanes that terminate in an aldehyde or alcohol function (J.R. Plimmer,[3] personal communication).

These results reinforce the cautions I raised (Cameron, 1981) concerning the conduct of critical behavioral experiments by researchers who have been exposed directly to the pheromone in operations such as synthesis or field tests. Further, on the assumption that disparlure has been sequestered in my body, it must be very stable under certain conditions, the emission rates must be very very low, and the behavioral response threshold of a male moth must be exceedingly low. Acceptance of such a low response threshold must force reevaluation of the assumptions made over the years concerning "female equivalents" or other comparative measures of attraction.

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DRYWOOD TERMITE FEEDING DETERRENTS IN SUGAR PINE AND ANTITERMITIC ACTIVITY OF RELATED COMPOUNDS

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Abstract—The feeding deterrent activity of sapwood extracts of sugar pine, *Pinus lambertiana* Dougl., and related compounds was determined against immatures of the western drywood termite, *Incisitermes minor* (Hagen). A bioassay was designed to quantify reductions in termite feeding caused by deterrent chemicals. Crude extracts and isolated fractions of sugar pine were deterrent and not preferred by *I. minor* at 0.5 mg/cm^2. Fatty acids occurring in sugar pine extracts had a broad range of deterrent activity. Long-chain saturated fatty acids were deterrent at 0.25 and 0.05 mg/cm^2. Unsaturated or intermediate length (C_8–C_{14}) acids, many not found in sugar pine wood, were less active than long-chain saturated acids. Related alpha-halogenated compounds were highly deterrent at 0.05 mg/cm^2 regardless of chain length or presence of a carboxylic acid moiety. Deposits of 2-iodooctadecanoic acid reduced termite feeding at 5 μg/cm^2, while 2-bromooctadecanoic acid had deterrent activity comparable to commercial wood preservatives. None of the halogenated compounds tested were termiticidal.

Key Words—Feeding deterrents, drywood termites, *Incisitermes minor*, Isoptera, Kalotermitidae, sugar pine, *Pinus lambertiana*, fatty acids α-halogenated acids, esters.

INTRODUCTION

The search for naturally occurring termite feeding deterrents, termiticides, and substances detrimental to termites and other xylophagous organisms has encompassed a wide variety of woods from different habitats. Studies of tropical hardwoods have yielded numerous compounds which contribute to the wood's natural resistance to termite attack such as tectoquinone from *Tectona*

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grandis and chlorophorin from *Chlorophora excelsa* (Wolcott, 1958), hydroxy- and dihydroxyoleanolic acids from *Manilkara zapota* (Sandermann and Funke, 1970), and the dalbergiones from *Dalbergia* spp. (Dietrichs and Hausen, 1971). Such timbers grow in highly competitive and diverse ecosystems where the necessity to evolve protective adaptations for survival is acute. Certain hardwoods of temperate regions are known to be resistant to termites (Carter, 1976); however, the factors conferring this antitermitic activity are unknown. A notable exception is *Diospyros virginiana* from which the potent termiticide 7-methyljuglone was isolated by Carter et al. (1978).

Species of the order Coniferales (Gymnospermae) comprise the vast bulk of building lumber throughout temperate zones, but only a few conifers, such as the *Callitris* species, are rated as termite resistant. Nevertheless, a number of investigators have reported that some chemical constituents of coniferous woods have varying degrees of antitermitic activity including the pinosylvins (Wolcott, 1955) and furfural (Becker et al., 1971) from *Pinus sylvestris*, taxifolin from *Pseudotsuga menziesii* (Wolcott, 1955), chamaecynone from *Chamaecyparis pisifera* (Saeki et al., 1973) and *l*-citronellic acid from *Callitris* spp. isolated by Baker and Smith (1910) and identified by Trikojus and White (1932). Carter (1976) found that several native American conifers are suitable candidates as sources of yet unidentified antitermitics.

The objectives undertaken in the current research were initiated from findings of two termite feeding-preference studies. Rust and Reierson (1977) showed that the western drywood termite, *Incisitermes minor* (Hagen), avoided feeding on paper treated with methanol extracts of sugar pine, *Pinus labertiana* Dougl., when given a choice to feed on paper treated with other wood extracts. Rust et al. (1979) found that both *I. minor* and *Incisitermes fruticavus* Rust preferred redwood and Douglas-fir over sugar pine even though it was the least dense of the woods tested. Both termite species fed heavily on Douglas-fir, a wood used extensively for construction in California. *I. minor* fed only lightly on sugar pine while *I. fruticavus* did not measurably feed on this wood. We again assumed that chemical deterrents in sugar pine were acting against the drywood termites. Sugar pine, a minor species of western forests, occupies only 1.6% of the commercial timber growth in the United States (USDA, 1980). It is the largest member of its genus but does not form pure stands and usually occupies less than 25% of mixed stands (Harlow and Harrar, 1968).

In order to identify deterrent compounds in sugar pine, we developed a feeding bioassay for use with drywood termites. Various solvent extractives and fractions from an acetone extract of sugar pine and additional pure compounds, some occurring in sugar pine, were bioassayed and evaluated for their effectiveness as feeding deterrents of *I. minor*.

METHODS AND MATERIALS

Wood Extraction. A sample cut from a large sugar pine sapwood board obtained from a local lumberyard was verified by Dr. R.C. Koeppen at the USDA Forest Products Laboratory in Madison, Wisconsin, as *Pinus lambertiana* Dougl. A section of board was ground with a Wiley mill into a coarse meal passed through a 2-mm exit screen. The moisture content of the wood meal was measured by comparing weights before and after heating a sample (ca. 4 g) at 115° C for 2 hr.

Four separate 8-hr extractions with four different solvents, hexanes, diethyl ether, acetone, or methanol, were performed in a soxhlet apparatus with 1 liter of solvent for each of four 100-g wood meal batches. Finished extracts were filtered and yields were measured after removing solvents in vacuo.

Fractionation of Acetone Extract. Exactly 1.500 g of the nonvolatiles from the acetone extract were dissolved in about 10 ml of acetone and mixed with ca. 20 g of sand. The acetone was evaporated and the extract-coated sand was placed atop a 2-cm ID × 75-cm long glass column packed with 60 g of silica gel (60/200 mesh) in benzene. A 500-ml aliquot of diethyl ether, passed through the column, yielded a fraction of 1.240 g. This was followed with 500 ml of methanol to remove the remaining solubles.

The ether-eluted fraction, redissolved in ca. 250 ml of diethyl ether, was extracted three times with 100-ml aliquots of 3% NaOH. The aqueous layers containing sodium salts of acids were combined, acidified with 3% aqueous HCl, and then extracted three times with 100-ml aliquots of ether. The combined ether layers were washed once with ca. 250 ml of water and then dried over anhydrous Na_2SO_4. This dried acid fraction was filtered, concentrated in vacuo, and the yield determined. After extraction with alkali, the original ether layer was extracted with 3% HCl. The aqueous layer was neutralized with base and then extracted with ether. No extractable components (bases) were detected in this layer. The neutral components, which remained in the ether phase after base and acid extractions, were dried, concentrated in vacuo and their yield determined. Chemical identification of the free acids in the acid fraction of the ether eluate and a quantitative analysis of an ether extract of this sugar pine sample are reported elsewhere (Scheffrahn, 1981).

Termite Collection. Sycamore and walnut logs infested with native field populations of *I. minor* were collected locally in Riverside, California, and stored outside the laboratory. When termites were needed, the logs were sawed into ca. 30-cm sections and split with the grain. These wedges were lightly tapped to dislodge the adhering termites into a shallow pan. Late-instar pseudergates and nymphs (8–9 mm in length, 10–13 mg in weight),

separated from the colony, were placed in plastic tubs (12 cm high × 12 cm diam.) provisioned with sycamore wafers. Termites injured during removal from logs died at the bottom of the tubs, while healthy ones clung to the wafers and were used in bioassays.

Bioassay Procedure. Crown Contract Natural® paper towels cut into disks, 9.6 cm diam. (72 cm^2, 360 mg average weight), were individually placed in glass Petri dish covers (9.7 cm ID). Predetermined amounts of wood extracts or pure compounds which would yield a specific mass per unit area of paper (mg/cm^2) were weighed in glass shell vials. For example, 36 mg of a compound applied to the disk provided a deposit of 0.5 mg/cm^2. The test compound, dissolved in 2 ml of an appropriate solvent (usually diethyl ether) was poured from the vial evenly onto the disk resting inside a Petri dish cover. The cover was then placed on a warm hotplate to evaporate the solvent. Trial tests using a ether-soluble dye confirmed that, with practice, a uniform extract deposit could be obtained in this manner. Each treated disk was cut into four smaller disks (3.9 cm diam.) and held overnight at room conditions before testing.

The termites were separated into groups of 10, weighed, and placed into small Stendor dish bottoms (3.7 cm OD). Each small treated disk was weighed and placed into Petri dish bottoms in groups of four. The Stendor dishes containing the 10 nymphs were inverted over each small disk, confining the nymphs to the treated paper. For each compound to be bioassayed, four replicates were constructed from the original large paper disk. The completed bioassay units were held in the dark at room conditions (24° C, 40% relative humidity), allowing the nymphs to feed until moderate consumption was visible on the solvent-treated control disks. This period lasted 4–7 days, at which time termite mortality was recorded and paper disks were again weighed. Survivors were confined to untreated filter paper in closed Petri dishes to monitor any latent effects from chemical exposure. In the deterrency tests, weight loss due to feeding was averaged for each treatment and a statistical comparison of these means was determined with an analysis of variance and Duncan's new multiple-range test.

Bioassay Specifications and Modifications. The deterrency bioassay for deposits of the four different solvent extracts of sugar pine and the fractions from the acetone extract applied at 0.5 mg/cm^2 was conducted for 3 days. A choice test in which four disks were constructed from two half disks, one treated with an extract (acetone, acid portion of acetone extract, or ether) applied at 0.5 mg/cm^2 and the other half a solvent blank, was conducted for 4 days to demonstrate termite feeding preference. The half disks were joined with a thin strip of cellophane tape on the surface not exposed to the termites.

The amount of chemical deposited for the four deterrency tests with pure compounds ranged from 0.005 to 0.25 mg/cm^2. The first test involved 13

compounds, including eight sugar pine acids deposited at 0.05 mg/cm^2 and exposed to termites for 6 days. A serial dilution of 2-iodooctadecanoic acid applied from 0.25 to 0.005 mg/cm^2 was bioassayed for 6 days. In the final test, three commercial grade wood preservatives were compared to 2-bromo-octadecanoic acid and selected compounds. After a 7-day exposure, termites from each treatment in the final bioassay were removed and pooled together in Petri dishes containing filter paper. Mortality was recorded immediately after termites were removed from the treatments and periodically thereafter for 71 days.

Simple fatty acids were obtained in the purest available forms from chemical suppliers. The 14-methylhexadecanoic acid (anteisomargaric acid) was synthesized following the procedure outlined by Scheffrahn (1981) and the *d*-citronellic, halogenated, and other branched-chain acids were synthesized by Dr. Yih-Shen Hwang, Department of Entomology, University of California, Riverside. The CCA (chromated copper arsenate), type C, formulated on a metal oxide basis (American Wood Preservers Institute, 1977) was a mixture of 6.98 g $K_2Cr_2O_7$, 5.77 g $CuSO_4 \cdot 5H_2O$, and 3.4 g arsenic as As_2O_5, yielding a 10-g equivalent of active oxides dissolved in water to a total volume of 100 ml (10% w/v). This stock solution was diluted to 0.2% and 1.8 ml were pipetted on a 72-cm^2 disk of paper toweling to provide 0.05 mg active ingredient/cm^2. Stadco® 97% coal tar creosote (3% water) graded #1 by the American Wood Preservers Association and reagent grade pentachlorophenol (99%) were applied to the paper toweling in 2 ml of acetone at 0.05 mg/cm^2.

RESULTS

The incidence of termite mortality, recorded immediately after termination of each bioassay, was $\leqslant$0.5% for any given test except among immatures exposed to commercial preservatives. The average weight of each group of termites ($N = 40$) used in tests between treatments within any bioassay did not vary more than 8% (usually much less). Furthermore, there was no association between the average weights of termite groups and the amount of feeding on a given treatment. The natural paper toweling proved to be an excellent substrate for the feeding bioassay with *I. minor*. The toweling was nearly as uniform in texture and thickness and more preferred than filter paper and white or brown wrapping paper (Scheffrahn, unpublished). The consumption rate by *I. minor* of untreated control disks was 0.25 mg paper/termite/day from combined control feeding rates in Table 4. This rate closely approximates the natural consumption of wood (0.26 mg wood/termite/day at 24° C) by *Paraneotermes simplicicornis* (Banks) which we calculated from the results published by Haverty and Nutting (1974).

Each of the four solvent extracts of sugar pine produced deterrent activity at 0.5 mg/cm^2 (Table 1). Methanol removed the most material from the wood, but this extract was the least active. All three choice tests showed feeding by *I. minor* to be confined to the untreated half. Representative paper disks fed upon by termites in the feeding deterrency and preference bioassays are shown in Figure 1.

The different fractions of the acetone extract showed varying degrees of deterrency (Table 2). The ether eluate was more active than the original acetone extract. Further fractionation of the ether eluate produced a large acidic portion (57%) and a lesser neutral fraction (28%). The remaining 15% was rendered as an interphase emulsion. Compared to the control, termite feeding was reduced over sixfold by the neutral fraction. The acids remained as deterrent as the original acetone extract; however, preliminary bioassay results with pure fatty acids identified in sugar pine (Scheffrahn, 1981) indicated that some acids bioassayed at the same concentration were much more deterrent than the original acid fraction.

Table 3 gives the amount of feeding by *I. minor* immatures confined to treatments of 12 fatty acids and one resin acid mixture deposited at 0.25 mg/cm^2. The mean values (mg) were derived from four replicates per acid exposed to termites for 7 days. The saturated straight-chain fatty acids from C_{16} to C_{19}, including 2-bromooctadecanoic acid, were significantly more active in inhibiting termite feeding than the abietic, 14-methylhexadecanoic, and the unsaturated acids. The two intermediate-length saturated acids, lauric (C_{12}) and myristic (C_{14}), had activity levels between these two groups at this concentration. The three most active fatty acids occurring in sugar pine were palmitic (C_{16}), margaric (C_{17}), and stearic (C_{18}) acids which constituted ca. 0.25% w/w of the sugar pine wood (Scheffrahn, 1981). The C_{18} un-

TABLE 1. CHEMICAL YIELD AND BIOASSAY RESULTS OF SUGAR PINE EXTRACTS

Solvent	Extractive yield (g)[a]	Mean paper weight loss due to feeding (mg)[b]
Methanol	10.10	4.68a
Acetone	4.60	3.33a
Diethyl ether	2.85	3.95a
Hexanes	2.08	3.15a
Control[c]		9.95b

[a] Grams extracted from 100 g wood meal (7.2% moisture content) with 1 liter solvent.
[b] At 0.5 mg/cm^2, 3-day exposure. Means followed by same letter not significantly different at $P < 0.05$ (Duncan's NMRT).
[c] Paper treated with reagent acetone only.

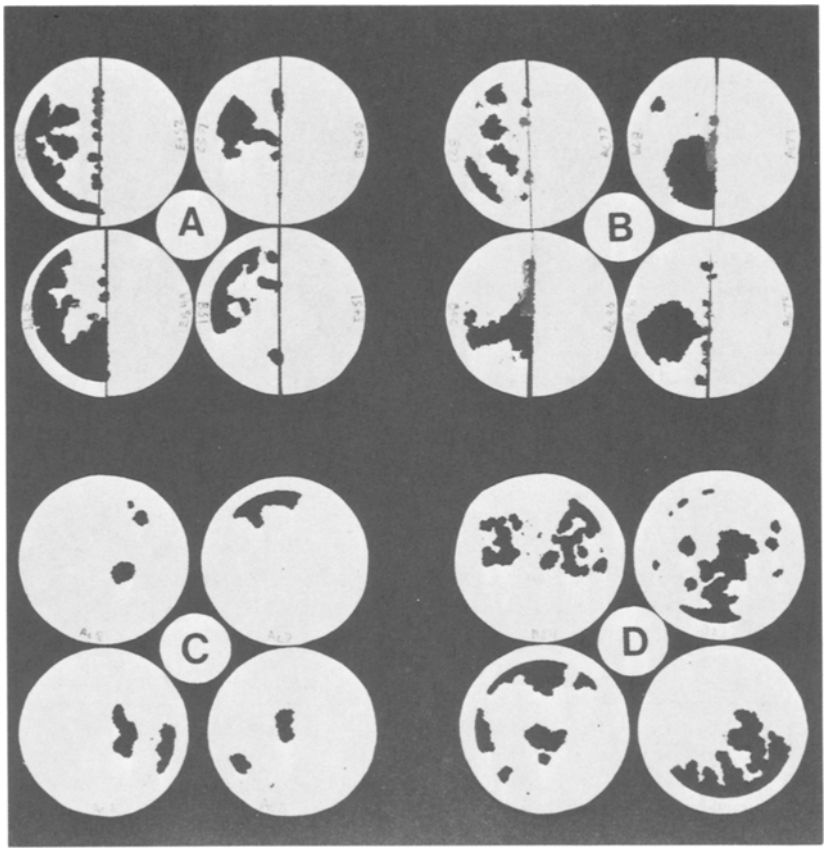

FIG. 1. Choice tests (A and B) show feeding limited to solvent-treated half disks on left half of each replicate. The ether extract of sugar pine is on the right half in each of four replicates in A and the acetone extract is on the right halves in B. Standard feeding tests (C and D) show reduced feeding on four replicates of whole disks treated with the acetone extract of sugar pine in C as compared to acetone treated controls in D. All extracts were deposited at 0.5 mg/cm^2 and exposed to termites for 3–4 days.

saturates, all identified as wood constituents, were increasingly more deterrent with increasing levels of unsaturation. Feeding on the oleic acid ($C_{18:1}$) treatments, the second most prevalent fatty acid in sugar pine, was not significantly different from the control group, so oleic acid could not be considered truly deterrent. Total mortality for all the treatments over the exposure period was negligible as only 2 of 560 nymphs died, each on a separate treatment.

In a second more comprehensive test, 35 compounds were bioassayed at a

TABLE 2. CHEMICAL YIELD AND BIOASSAY RESULTS OF FRACTIONS FROM ACETONE EXTRACT OF SUGAR PINE

Fraction	Yield (g)	Mean paper weight loss due to feeding (mg)[a]
Acetone	1.500[b]	2.65cd
Et_2O elution	1.240	1.90c–e
Et_2O elution acids	0.615[c]	2.70c
Et_2O elution neutrals	0.306[c]	1.33e
MeOH strip	0.196	6.93b
Control[d]		8.53a

[a] At 0.5 mg/cm^2, 4-day exposure. Means followed by same letter not significantly different at $P < 0.05$ (Duncan's NMRT).
[b] Initial quantity of extractive before separation.
[c] From 1.084 g Et_2O elution.
[d] Paper treated with reagent ether only.

TABLE 3. CONSUMPTION BY *I. minor* OF PAPER TREATED WITH VARIOUS ACIDS AT 0.25 mg/cm^2 AFTER 7-DAY EXPOSURE

Compound	Mean consumption (mg)[b]
Margaric acid[a]	0.45a
2-Bromooctadecanoic acid	0.73a
Palmitic acid[a]	0.93a
Nonadecanoic acid	1.08a
Stearic acid[a]	1.38ab
Myristic acid	2.33a–c
Lauric acid	3.13bc
Linolenic acid[a]	3.50cd
Linoleic acid[a]	3.65cd
Abietic acid[a,c]	4.13c–e
14-Methylhexadecanoic acid[a]	4.48de
9-Hexadecenoic acid	5.83e
Oleic acid[a]	7.85f
Control	9.25f

[a] Acids occurring in sugar pine (Scheffrahn, 1981).
[b] Means ($N = 4$) using 10 immature termites per replicate. Numbers followed by the same letter are not significantly different at $P < 0.05$ (Duncan's NMRT).
[c] The abietic acid (Sigma Chemical Co.) was a commercial mixture of coniferous resin acids.

TABLE 4. CONSUMPTION BY *I. minor* OF PAPER TREATED WITH SELECTED COMPOUNDS AT 0.05 mg/cm^2 AFTER 6-DAY EXPOSURE

Compound	Mean feeding (mg)[a]
2-Iodooctadecanoic acid	3.43a
2-Bromoeicosanoic acid	4.20ab
2-Bromooctadecanoic acid	4.35ab
2-Bromodecanoic acid	4.48ab
Methyl 2-bromooctadecanoate	5.08a–c
2-Bromotetradecanoic acid	5.33a–c
2-Chlorooctadecanoic acid	5.95a–d
Eicosanoic acid	6.25b–e
Nonadecanoic acid	6.28b–e
Palmitic acid	7.25c–f
Methyl abietate[b]	7.48c–f
Stearic acid	7.78c–g
13-Eicosenoic acid	8.40d–h
trans-Cinnamic acid	8.95e–i
2-Methyloctadecanoic acid	9.08f–i
Linolenic acid	9.20f–i
Nonanoic acid	10.25g–j
14-Methylhexadecanoic acid	10.38g–j
Lauric acid	10.43g–j
Abietic acid[b]	10.65h–k
Octanoic acid	11.08h–k
3-Methyloctadecanoic acid	11.15i–k
Linoleic acid	11.23i–k
11-Octadecenoic acid	11.30i–k
10-Undecenoic acid	11.35i–k
Decanoic acid	11.43i–k
Undecanoic acid	11.45i–k
Phthalic acid	11.50i–k
d-Citronellic acid	11.50i–k
Myristic acid	11.88j–k
9-Hexadecenoic acid	11.90j–k
Sodium nonanoate	11.98j–l
Oleic acid	12.23j–l
Methyloctadecanoate	12.33j–l
1-Octadecene	12.50j–l
Control 1	13.18k–l
Control 2	14.45l

[a] Means ($N = 4$) using 10 immature termites per replicate. Numbers followed by the same letter are not significantly different at $P < 0.05$ (Duncan's NMRT).

[b] The abietic acid (Sigma Chemical Co.) and the methyl abietate (Pfaltz & Baver) were commercial mixtures of coniferous resin acids and methyl esters, respectively.

lower concentration (0.05 mg/cm^2) to separate the specific activities of the numerous treatments since overall feeding would, therefore, increase. Table 4 gives the results of this 6-day bioassay in which the total mortality was 4 of 1480 individuals. Two control groups were tested and statistically analyzed as separate treatments. Results showed that the C_9–C_{11} aliphatic acids, including *d*-citronellic acid, were only slightly deterrent when compared to some of the other compounds tested, even though they were previously found to be toxic at higher concentrations (Scheffrahn, 1981). The unsaturated fatty acids had a somewhat broader range of activity than did other structurally related groups tested, but most of them were only slightly deterrent. The 13-eicosenoic acid, a notable exception to the unsaturates, produced feeding inhibition in the saturated range. The long-chain saturated acids including palmitic, stearic, and nonadecanoic remained active feeding deterrents when tested at 0.05 mg/cm^2. Eicosanoic acid (C_{20}) also had a similar level of activity. All seven alpha-halogenated compounds bioassayed, consisting of a group having a 10-to 20-carbon chain, produced the greatest feeding deterrency, regardless of the chain length.

Figure 2 visually represents the structure–activity relationship of compounds bioassayed in Table 4. The diameter of each circle is derived from the milligram difference in weight loss due to feeding between control group 1 (13.18 mg) and the particular compound depicted by the circle. The larger the circle (i.e., the greater the difference between treatment and control), the more deterrent the compound. Relative comparisons in activity can, therefore, be made on the basis of circle size. The black circles denote compounds identified in sugar pine of which the C_{16}–C_{20} group is represented by palmitic and stearic acids. Open circles indicate synthetic chemicals. The short- to medium-chain fatty acids (C_8–C_{14}) were only marginally deterrent even though a 10-fold increase in concentration of the C_9–C_{11} acids is toxic to *I. minor* (Scheffrahn, 1981). The sodium salt of nonanoic acid was neither deterrent nor toxic. However, singular alpha-bromination transformed decanoic acid (C_{10}) into a potent feeding inhibitor. As previously noted, the saturated long-chain fatty acids (C_{16}–C_{20}) were active feeding deterrents. The effects of structural modification of such compounds can be best illustrated in Figure 2 by examining the results of tests with 12 compounds, each containing an 18-carbon backbone, included in the large bioassay. 1-Octadecene ($C_{18}H_{36}$), a simple hydrocarbon olefin, was the least deterrent compound tested, resulting in a feeding reduction of only 0.68 mg. The methyl ester of stearic acid (methyl octadecanoate) was also inactive compared to its free acid. An alpha-bromine addition to the stearate resulted in a sharp increase in activity, in contrast to the more modest difference in activity between the acid and its brominated analog. The specific activities of the halogenated octadecanoic acids were dependent on the halogen substituted in the alpha position. The deterrency of these compounds was related to the atomic size of the halogen as follows:

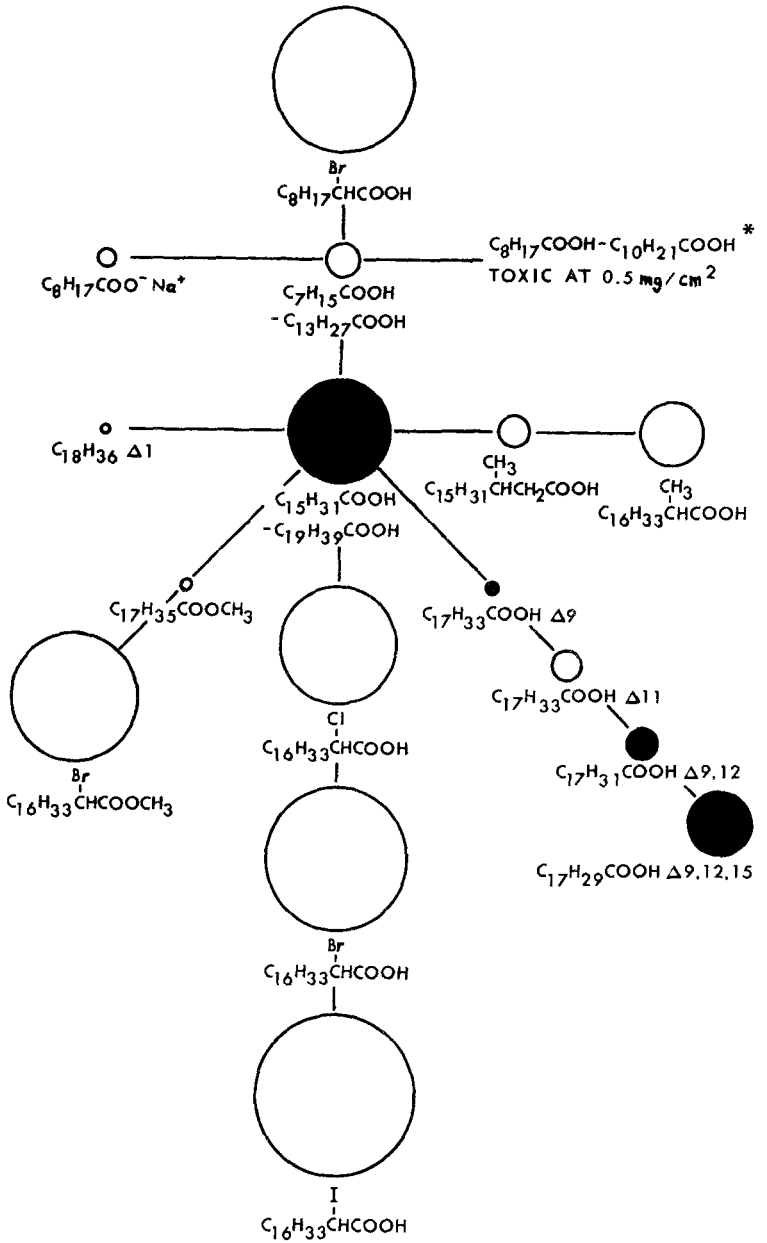

FIG. 2. The relative feeding inhibition of *I. minor* by selected compounds deposited at 0.05 mg/cm^2. Circle diameters are derived from the difference (mg) between the control group and the compound representing the circle, so that larger circles indicate proportionally greater deterrency. Circles in black are compounds identified in the acid fractions of sugar pine sapwood extracts (Scheffrahn, 1981). *Scheffrahn, 1981.

2-chlorooctadecanoic < 2-bromooctadecanoic < 2-iodooctadecanoic acid. Based on this relationship, the molar concentration of these compounds on the substrate decreased with increasing activity. When the alpha-positioned halogen of octadecanoic acid was replaced by a methyl group, activity decreased below that of the free acid. A further reduction in deterrency occurred when the methyl branch was located at the beta carbon. All C_{18} unsaturated acids were less deterrent than their saturated forms, with oleic acid being the least deterrent acid tested. As the unsaturation number in creased, deterrency also increased, producing a deterrency hierarchy of oleic < linoleic < linolenic acid. Deposits of 11-octadecenoic acid were more deterrent than the 9-octadecenoic (oleic) acid but less active than the 9,12 diene, linoleic acid.

The most deterrent compound tested in this study, 2-iodooctadecanoic acid, was separately bioassayed from 5 to 250 $\mu g/cm^2$, along with an untreated control group (Figure 3). A significant negative linear regression plotted from the equation $y = -5.54\ (\log_{10}X) + 12.3$ ($F = 66.64$, $P < 0.01$, $N = 4$) was calculated for the amount of feeding (mg) in relation to the $\log_{10}$ of the six

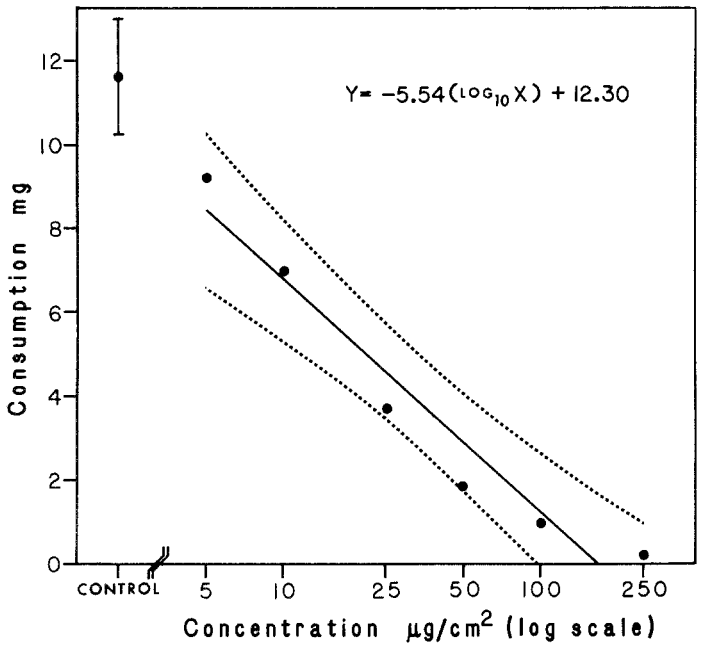

FIG. 3. The negative linear relationship of paper consumption by *I. minor* and the concentration $\log_{10}$ of 2-iodooctadecanoic acid. Confidence limits (95%) are shown for the regression line and the untreated control. The untreated control is not graphed on the abscissa scale. Points are average values ($N = 4$) at each concentration.

TABLE 5. COMSUMPTION BY *I. minor* OF PAPER TREATED WITH VARIOUS COMPOUNDS AT 0.05 mg/cm^2 AND SUBSEQUENT MORTALITY AFTER 7-DAY EXPOSURE

Compound	Mean consumption (mg)[a]	Mortality, %[b] (at days after exposure)							
		0	12	20	29	34	41	62	71
Pentachlorophenol	0.43a	55	62.5	67.5	75	77.5	80	90	90
CCA type I	0.68a	2.5	40	45	50	62.5	72.5	82.5	82.5
Creosote	1.35a	2.5	5	10	22.5	35	45	60	60
2-Bromooctadecanoic acid	1.83a	2.5	10	12.5	20	25	30	80	82.5
Stearic acid	4.15b	0	2.5	5	10	15	30	67.5	72.5
Control	7.48c	0	0	5	10	17.5	22.5	57.5	60
Mineral oil	9.40cd	0	2.5	12.5	15	17.5	22.5	57.5	57.5
Nonanoic acid	9.53d	0	0	0	5	17.5	17.5	40	47.5

[a]Means ($N = 4$) using 10 immature termites per replicate. Numbers followed by same letter are not significantly different at $P < 0.05$ (Duncan's NMRT).

[b]Termites removed from deposits after 7 days and placed on untreated filter paper. Days indicate time elapsed after removal from treatments.

deposit concentrations of 2-iodooctadecanoic acid ($\mu g/cm^2$). At 250 $\mu g/cm^2$, only trace amounts of feeding were evident while, at the other extreme, the threshold of feeding inhibition was near 5 $\mu g/cm^2$. No mortality or any signs of adverse effects from exposure to 2-iodooctadecanoic acid occurred among the 280 termites used during the 6-day bioassay period.

Table 5 lists the mean consumption of paper treated at 0.05 mg/cm^2 with three major types of wood preservatives as well as 2-bromooctadecanoic, stearic, and nonanoic acids and mineral oil. Little feeding occurred on paper toweling treated with the commercial preservatives. There was, however, no significant difference in the amount of feeding of these and the 2-bromooctadecanoic acid treatments. Pentachlorophenol killed 55% of the termites within the 7 days, although some of the nymphs made a recovery from their intoxication after removal from the deposits as suggested by the decrease in the mortality rate. The arsenical treatment (CCA type C) had more latent toxic effects. The percentage of mortality of termites exposed to CCA increased pronouncedly during the 12 days subsequent to exposure—from 2.5 to 40%. Thereafter, some immatures also recovered and survived the entire recording period. Creosote was deterrent, but not toxic at this concentration. The mineral oil and nonanoic acid were not deterrent, with feeding levels above the control value. Incidental mortality was higher than normal, especially among termites exposed to 2-bromooctadecanoic acid, and overall survival rates were lower than expected. This may have been the result of using termites from an older laboratory colony which was experiencing deleterious effects of being removed from their original log (Scheffrahn, 1981).

DISCUSSION

Deterrent activity would have been limited to a specific fraction if only a single compound or class of compounds was inhibiting termite feeding. However, both neutral and acidic isolates from the acetone extract were active, implying that two or more chemically different constituents contributed to the extract's deterrency. The carboxylic acid fraction was of interest since the only previous naturally occurring acid shown to possess antitermitic qualities is *l*-citronellic acid (Rudman, 1965). It is possible that some of the neutral extractives could be identical to antitermitics previously identified in coniferous woods. Mirov (1967) states that some of the aromatic compounds, such as the pinosylvins, are characteristic of many pine species. Triglycerides may also be potentially active neutral compounds (Rudman and Gay, 1967).

Based on our results, fatty acids probably contribute to the level of nonpreference demonstrated by *I. minor* for sugar pine wood (Rust et al., 1979). Furthermore, when the acidic complement of other wood species is at a

sufficient level, greater resistance to termite attack and infestation may be anticipated, especially if saturated long-chain fatty acids such as palmitic or stearic acids are major components of the wood extracts. Such conditions may exist in other *Pinus* species not yet examined. Rudman and Gay (1967) associated termite resistance with lipid content in tallowwood, *Eucalyptus microrcorys* F. Muell. They tested the deterrent nature of filter paper treated with various fatty acids and esters by surrounding the papers with termite mound material and then adding the subterranean termites, *Coptotermes lacteus* (Frogg). Saturated fatty acids and their ethyl esters from C_{12} to C_{20} were bioassayed. Results showed the C_{18} and C_{19} acids rendered the maximum deterrent activity. The ethyl esters were less active than the free acids, but the difference was not as great as that between stearic acid and its methyl ester as reported in our results. Unsaturated acids were found by Rudman and Gay (1967) to be relatively inactive. Oleic acid was less deterrent than linoleic acid; however, contrary to our results with *I. minor*, the feeding of *C. lacteus* was essentially undeterred by the C_{18} triene, linolenic acid. A noteworthy finding by Rudman and Gay (1967) was that the triglyceride of palmitic acid was as active as the free form, suggesting that hydrolysis of the glyceride fraction of wood lipids is not a requisite for conferring increased wood resistance. Triglycerides may likely constitute at least some of the deterrent components of the neutral phase of the sugar pine extract.

When comparing the relative efficacy of halogenated fatty acids with well-known antitermitic compounds reported in previous studies, one must consider the variability in results due to the differences in termite species and bioassay procedures used. Becker et al. (1972) reported on the deterrent activity of four frequently cited "heartwood extractives," tectoquinone, lapachol, lapachonone, and pinosylvinmonomethylether, against 14 termite species including four genera of drywood termites. When tested against the drywood termites *Kalotermes flavicollis* (Fabr.), *Incisitermes tabogae* (Snyder), and *Cryptotermes dudleyi* Banks, tectoquinone deposits of ca. 0.22 mg/cm^2 performed similarly to 2-iodooctadecanoic acid at 0.25 mg/cm^2 against *I. minor*—yielding only minute traces of feeding. Tectoquinone-treated filter paper disks were heavily fed upon when exposed to *Neotermes jouteli* (Banks). The remaining three compounds, tested at 0.33 mg/cm^2, were overall less deterrent to all four kalotermitids than were the halogenated compounds deposited at 0.05 mg/cm^2 against *I. minor*.

Drywood termites are usually more tolerant of toxic substances, feeding deterrents, and repellents than are subterranean species. Becker et al. (1972) found that lapachonone treatments were heavily fed upon by three of the four drywood termite species, but none of the other 11 termite species attacked these deposits above low levels. Becker (1975) also found drywood termites to be less sensitive to the toxic effects of short-chain fatty acids, pentachloro-

phenol, DDT, and other chlorinated pesticides than were either rhinotermitid or termitid species used in his bioassays. Becker et al. (1971) showed that furfural was highly repellent to *Heterotermes indicola* (Wasmann) but was at best slightly repellent against species of Kalotermitidae. Concerning the activity of *l*-citronellic acid, Rudman (1965) reports "Even a small quantity of *l*-citronellic acid is sufficient to deter termites . . . ." His study shows that the feeding of *Nasutitermes exitiosus* is inhibited by deposits as low as ca. 0.0125 mg/cm^2. Our results showed that at 0.05 mg/cm^2, *d*-citronellic acid was not very deterrent to *I. minor*, even though the dextro isomer is reported to be more toxic to *Reticulitermes flavipes* Kollar than the levo form (Weissmann and Dietrichs, 1975). Phthalic acid was also inactive against *I. minor*, although Rudman and Gay (1963) mention that its derivatives are well-known termite deterrents. Lund (1965) states that methyl cinnamate is toxic to *Reticulitermes* spp., but this compound is too volatile to test in our bioassay. Deposits of *trans*-cinnamic acid were not toxic to *I. minor*, but acted as mild feeding inhibitors.

Unlike other compounds in this study, our results would indicate that pentachlorophenol and chromated copper arsenate were both toxic and deterrent. Since the physiological stress imposed by the toxic action of these materials would likely impair normal functions, including feeding behavior, the termites may not feed because they are moribund and not because of a separate inhibitory response to feeding. One must be cautious when interpreting feeding results if toxic compounds are involved. The creosote, on the other hand, acted as a feeding deterrent, a finding supported by Rudman and Gay (1963), who reported that anthracene derivatives are deterrent but not toxic to *Nasutitermes exitiosus*.

The search for behavior-modifying chemicals such as feeding deterrents may provide a source of novel wood preservatives. Mixtures of such termite suppressant chemicals formulated with fungicides may prove to be suitable replacements of the nonspecific toxicants currently used for wood preservation. Further research on the structure–activity relationships of the compounds reported in this study and field trials with the more promising antitermitics are warranted.

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ALARM RESPONSES CAUSED BY NEWLY IDENTIFIED COMPOUNDS DERIVED FROM THE HONEYBEE STING[1,2]

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Abstract—Twelve compounds identified from honeybee, *Apis mellifera* L., sting extracts were evaluated in a standardized laboratory test for their effectiveness in eliciting an alarm response from caged worker honeybees. Two—1-decanol and phenol—were judged ineffective as alarm pheromones. The other ten—1-butanol, isopentyl acetate, isopentyl alcohol, 1-hexanol, 2-heptyl acetate, 2-heptanol, 1-octanol, 1-acetoxy-2-octene, 2-nonyl acetate, and 1-acetoxy-2-nonene—produced alarm responses of similar speed and intensity. Three non-sting-derived compounds—β-ionone, methyl benzoate, and *trans*-cinnamaldehyde—caused weak or no responses, indicating that the responses were not simply a reaction to concentrated odoriferous substances.

Key Words—Honeybee, *Apis mellifera*, alarm pheromone, acetate, alcohol, Hymenoptera, Apidae, sting.

INTRODUCTION

In 1978, Blum et al. analyzed extracts of honeybee, *Apis mellifera* L., stings and identified eight previously unreported compounds associated with this structure. The only other sting-derived compound identified prior to that time was isopentyl acetate (IPA) (Boch et al., 1962). These eight compounds,

[1]In cooperation with Louisiana Agricultural Experiment Station.
[2]Mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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n-butyl acetate (nBA), isopentyl alcohol (IPA1), *n*-hexyl acetate (nHA), *n*-octyl acetate (nOA), 2-nonanol (2NL), *n*-decyl acetate (nDA), benzyl acetate (BZA), and benzyl alcohol (BZA1), as well as IPA and 2-heptanone (2HPT), a compound derived from the mandibular glands (Shearer and Boch, 1965), were bioassayed for their effectiveness as alarm pheromones by Collins and Blum (1982). All but nDA and BZA1 were effective in producing alarm response in caged honeybee workers.

Continuing analyses of extracts of honeybee stings have resulted in the identification of 10 more short-chain compounds that can be regarded as potential alarm pheromones (Blum et al., unpublished data). The results of bioassays of the activities of these compounds as alarm pheromones are reported here.

METHODS AND MATERIALS

The method of Collins and Rothenbuhler (1978) was used for bioassays. Caged brood from 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 Kulinčević and Rothenbuhler, 1973) in single-colony groups of 30 workers.

Newly emerged bees are 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 intense, behavior as guard bees (Collins, 1980).

During the tests, cages were arranged 15–20 cm 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 conditions; cage numbers were hidden until after testing, and the cages were rearranged randomly within a set after each complete sequence of tests. A set of eight cages was exposed only to one chemical during the experiment.

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) time to react—the time, in seconds, until a group reaction was seen including flickering of the wings and increased locomotion; (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; and (4) number of bees engaged in fanning behavior at the end of the test.

Occasionally this fanning included exposure of the Nasonov gland. Following testing, a fifth character was calculated—frequency of no reaction—the number of times in which there was no reaction to the test material. Analysis of the time to react was done on square-root transformed data by least-squares analysis of covariance, adjusted for initial activity level, and by a least significant difference test (LSD). The adjustment of time to react was necessary because bees that are already active tend to respond more quickly and strongly. Spearman's rank correlation coefficients were calculated for all pairs of measures of response (Hollander and Wolfe, 1973). Intensity of the response was analyzed by chi-square and fanning by the t test of equality of two means.

The sting-derived compounds tested included 2-heptanol (2HPA1), 1-octanol (OA1), 1-butanol (BA1), and phenol purchased from Aldrich Chemical Co., Milwaukee, Wisconsin; and 1-acetoxy-2-octene (1AO), 1-acetoxy-2-nonene (1AN), 2-heptyl acetate (2HPA), and 2-nonyl acetate (2NA) which were synthesized and purified by preparative gas chromatography. The assays were done during two 6-day periods, with half of the compounds tested each period, due to space limitation. Bees from two colonies were used for testing. 2-Nonyl acetate was tested during both periods as a control. IPA, also from Aldrich Chemical Co., was used as a control so that comparisons could be made with the group of compounds previously tested (Collins and Blum, 1982).

In addition, three aromatic compounds not produced by worker honeybees, methyl benzoate (MB), *trans*-cinnamaldehyde (tCNM), and β-ionone (βI) (Aldrich Chemical Co.) were included in the study. This was done in order to distinguish between alarm behavior and simple aversive responses to volatile compounds introduced into test cages.

At a later date, two compounds not found in sting extracts, 1-hexanol (HA1) and 1-decanol (DA1) (Aldrich Chemical Co.) were assayed to complete the series of acetates and their alcoholic moieties in the range C_4 (*n*-butyl) to C_{10} (*n*-decyl). 2NA was the control and IPA1 (Aldrich Chemical Co.) was the comparison control with this set, rather than IPA. It was necessary to use three colonies to provide sufficient bees, as brood rearing was reduced at this time.

RESULTS

The three test sequences using 2NA were not significantly different in the LSD test, so the data from the three testing periods are presented together. The responses to IPA and IPA1 were not significantly different from those during the 1982 (Collins and Blum) assays, so comparisons were made including all chemicals tested to date.

Table 1 shows the distribution of observations by intensity of response. Two of the sting-derived compounds, DA1 and phenol, and the three foreign compounds, tCNM, MB, and βI, elicited response from the caged bees less than half the time. When a response to these compounds was seen, it was usually a weak one. Responses to the remaining compounds occurred more often, although the intensity levels varied. The compounds were ranked based on the numbers of observations in each category, with a rank of 1 indicating the greatest number of strong or very strong responses and 15 the fewest responses.

The time to react, intensity ranking based on Table 1, and number of bees fanning for each compound tested are shown in Table 2. Mean times to react varied on a continuum from 3.4 to 14.4 sec with only the most extreme values being significantly different from each other. The five chemicals with low levels of response had the slowest mean times to react. The three measures of alarm response (frequency of no response, time to react, and intensity) were significantly correlated at $P < 0.01$ using Spearman's rank correlation (Table 3). Compounds eliciting frequent response got reactions that were faster and

TABLE 1. INTENSITY OF RESPONSE BY CAGED HONEYBEES TO 15 COMPOUNDS TESTED AS ALARM PHEROMONES

Rank[a]	Chemical	No response	Weak	Medium	Strong	Very strong	Total observations[b]
1a	1-hexanol	1	7	31	32	10	81
2b	isopentyl acetate	1	9	41	21	0	72
3b	2-heptanol	0	16	43	13	0	72
4b	isopentyl alcohol	3	19	35	20	4	81
5c	1-acetoxy-2-nonene	0	32	29	11	0	72
6d	1-butanol	6	24	35	7	0	72
7d	1-octanol	6	24	30	3	0	63
8de	2-heptyl acetate	10	26	30	6	0	72
9e	2-nonyl acetate	53	96	65	9	2	144
10f	1-acetoxy-2-octene	14	43	15	0	0	72
11g	phenol	41	24	7	0	0	72
12g	*trans*-cinnamaldehyde	40	28	4	0	0	72
13g	methyl benzoate	46	23	3	0	0	72
14h	1-decanol	57	11	14	7	2	81
15g	β-ionone	61	11	0	0	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 15 the group with the greatest number of weak or no responses. Chemicals with the same letter are not significantly different by contingency chi-square.

[b]Eight cages with 30 bees each were tested 3 times a day for 3 days. 2-Nonyl acetate was tested three times to serve as a control for different test dates.

TABLE 2. MEASURES OF RESPONSE OF CAGED HONEYBEE WORKERS TO 12 COMPOUNDS ASSOCIATED WITH STING AND 3 UNRELATED AROMATIC COMPOUNDS[a]

	No. non-reactors total	Least squares $\overline{X}$ time to react (s)[b]	Intensity (rank)[c]	No. of bees fanning[d]
Isopentyl acetate (IPA)	1/72	3.8 a	2	25
2-Heptanol (2HPA1)	0/72	4.4 a	3	141
1-Hexanol (HA1)	1/81	4.5 a	1	319
Isopentyl alcohol (IPA1)	3/81	5.1 ab	4	119
1-Octanol (OA1)	6/63	5.6 ab	7	41
1-Acetoxy-2-octene (1AO)	14/72	5.8 ab	10	127
1-Acetoxy-2-nonene (1AN)	0/72	6.0 ab	5	41
2-Heptyl acetate (2HPA)	10/72	6.2 ab	8	155
1-Butanol (BA1)	6/72	6.5 abc	6	135
2-Nonyl acetate (2NA)	34/144	7.0 bc	9	111
Methyl benzoate[a,e] (MB)	46/72	7.1 bc	13	351
Phenol[e] (P)	41/72	8.3 bcd	11	291
trans-Cinnamaldehyde[a,e] (tCNM)	40/72	9.5 cd	12	138
1-Decanol[e] (DA1)	57/81	10.4 cd	14	22
β-Ionone[a,e] (βI)	61/72	14.4 d	15	259

[a]Methyl benzoate, *trans*-Cinnamaldehyde and β-ionone not associated with sting.
[b]Means followed by the same letter(s) are not significantly different ($P < 0.01$).
[c]Rank taken from Table 1.
[d]Total of all observations from nine tests with each chemical.
[e]These chemicals probably do not function as alarm pheromones based on the frequency of nonreactors.

TABLE 3. SPEARMAN'S RANK CORRELATION COEFFICIENT AMONG 4 MEASURES OF ALARM RESPONSE[a]

	Time to react	Intensity	Fanning
Frequency of no reaction	0.88[b]	0.88[b]	0.07
Time to react		0.94[b]	0.16
Intensity			0.01

[a]Calculations done using data from this paper and Collins and Blum (1982).
[b]Correlation is significant at $P < 0.01$, $df = 10$.

TABLE 4. HONEYBEE COLONY DIFFERENCES[a] IN TIME TO REACT TO VARIOUS ALARM PHEROMONES (VALUES ARE COLONY MEANS IN SECONDS)

Pheromone	Colony	
	A	B
Isopentyl acetate	3.9	4.2
2-Heptanol	3.1	5.6
1-Octanol	3.9	7.3
1-Acetoxy-2-octene	4.7	7.2
1-Acetoxy-2-nonene	5.4	6.7
2-Heptyl acetate	5.4	7.0
1-Butanol	5.6	6.6
2-Nonyl acetate	6.1	8.7

[a]Differences significant at $P < 0.01$ ($F = 37.23$; df 1, 12).

more intense. Fanning level differed by chemical tested, but was not significantly correlated with the other three measures.

In addition, there were significant colony differences. Bees from colony B reacted more slowly to each of the compounds (Table 4) and overall with less sensitivity (Table 5). The heterogeneity chi-square for the intensity was not significant, so the data were pooled. Colony A had a mean of 58 bees seen fanning in response to a compound during the test period, colony B had a mean of 97.3 bees, significantly ($t = 5.18$, $P < 0.01$) more.

DISCUSSION

The evaluation of *trans*-cinnamaldehyde, methyl benzoate, and β-ionone as alarm pheromones was considered to be of critical importance in order to establish unequivocally that the response by the small group of worker honeybees in the test cages was not simply an aversive response to high concentrations of any odoriferous substance. The fact that the bees usually exhibited only weak responses (a category reserved for observed responses that are marginal) or did not respond at all, indicates that the presence of a strongly odoriferous compound is, in itself, insufficient to create an alarm response. There was, however, considerable fanning by the bees in the presence of these three compounds. Both tCNM and βI had been tested by Woodrow et al. (1965), who evaluated a large number of chemicals for attractiveness and repellency to bees for possible application with pesticides. tCNM was moderately repellent, and the bees did fan their wings during exposure.

From the group of sting-derived compounds tested, only the alcohols of

TABLE 5. CHI-SQUARE ANALYSIS OF INTENSITY OF RESPONSE TO 12 COMPOUNDS BY CAGED WORKER HONEYBEES FROM 2 DIFFERENT COLONIES

Compound	Colony	Intensity: No response	Weak	Medium	Strong	Very strong	χ^2	*df*
Isopentyl acetate	A	0	2	21	13	0	4.99	3
	B	1	7	20	8	0		
2-Heptanol	A	0	3	26	7	0	8.21*[a]	2
	B	0	13	17	6	0		
1-Octanol	A	1	7	16	3	0	8.86*	3
	B	5	17	14	0	0		
1-Acetoxy-2-octene	A	5	22	9	0	0	1.77	2
	B	9	21	6	0	0		
1-Acetoxy-2-nonene	A	0	10	17	9	0	9.82**	2
	B	0	22	12	2	0		
2-Heptyl acetate	A	3	11	18	4	0	4.08	3
	B	7	15	12	2	0		
1-Butanol	A	1	10	20	5	0	5.33	3
	B	5	14	15	2	0		
2-Nonyl acetate	A	15	24	28	5	0	10.97*	3
	B	19	38	14	1	0		
Methyl benzoate	A	19	15	2	0	0	3.86	2
	B	27	8	1	0	0		
Phenol	A	21	11	4	0	0	0.33	2
	B	20	13	3	0	0		
trans-cinnamaldehyde	A	22	14	0	0	0	4.40	2
	B	18	14	4	0	0		
β-Ionone	A	30	6	0	0	0	0.11	1
	B	31	5	0	0	0		
						summed χ^2	62.73	28
Total	A	117	135	161	46	0		
	B	142	187	118	21	0	26.68**	3
						heterogeneity χ^2	36.05	25

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

C_9 or less, or their acetates, were effective in eliciting an alarm response. The levels of response were not significantly different in speed, but did vary in intensity. All were significantly faster and stronger in their elicited response than were DA1 or phenol. These two compounds are apparently ineffective as alarm pheromones, since they produced no response more than half the time, and the responses that did occur were weak.

The only compound in the acetate-alcohol series of alarm pheromones that did not show an alarm function was DA1. As with its corresponding acetate, which was evaluated with the first group of compounds (Collins and Blum, 1982), in more than half the tests the bees did not respond at all. Including the compounds which had been previously tested, all the acetates and their alcoholic moieties from C_4 (*n*-butyl) to C_9 (*n*-nonyl), plus 1AO, 1AN, and BZA, can be considered as functional alarm pheromones. BZA1 was not effective in eliciting a response. Among the alarm pheromone group, the speeds of the response were not significantly different, but the intensity did differ significantly for some. However, at this juncture no general statement can be made about the relationship of chemical structure and its effect on the intensity of the alarm response.

These assays were based solely on responses with caged young bees using individual chemicals in amounts far exceeding those present in the stings of bees. Guard bees at the hive entrance under more normal conditions might have different response thresholds for the individual compounds. Also, interactions betweeen chemicals presented simultaneously may occur, but were not examined in the present study.

Acknowledgments—We are grateful to Dr. T.H. Jones for synthesizing some of the compounds utilized for this study. We thank Drs. R.W. Howard and H.M. Fales for aid in the structural studies of some of the sting-derived compounds. Thanks to Drs. R. Boch, N. Gary, and J. Schmidt for constructive criticism of the manuscript.

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FIELD RESPONSE OF THE DUTCH ELM DISEASE VECTORS, *Scolytus Multistriatus* (MARSHAM) AND *S. scolytus* (F.) (COLEOPTERA: SCOLYTIDAE) TO 4-METHYL-3-HEPTANOL BAITS CONTAINING α-, β-, γ-, OR δ-MULTISTRIATIN

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Abstract—The field responses of English populations of the Dutch elm disease vectors, *Scolytus multistriatus* and *S. scolytus* to baits containing 4-methyl-3-heptanol, a host synergist [(−)-α-cubebene or (−)-limonene] and (±)-α-, (+)-β-, (−)-β-, (±)-γ-, or (±)-δ-multistriatin were examined. (±)-α-Multistriatin, released at 5–10 μg/day, enhanced the response of *S. multistriatus* to baits containing 4-methyl-3-heptanol and either of the host synergists but had no effect on the capture of *S. scolytus*. The release of larger amounts (57 or 365 μg/day) of (±)-α-multistriatin interrupted the response of both species to the 4-methyl-3-heptanol baits. It appears that α-multistriatin has multiple functions as a behavior-modifying substance for the two beetles. The (+)-β-, (−)-β-, (±)-γ-, and (±)-δ-multistriatins were inactive when released at 5–10 μg/day. The results of these field experiments suggest that one bait can be formulated to capture both species.

Key Words—*Scolytus multistriatus*, *S. scolytus*, Coleoptera, Scolytidae, elm bark beetle, multistriatin stereoisomers, Dutch elm disease, aggregation pheromone, field responses, attractant baits.

INTRODUCTION

The European elm bark beetles, *Scolytus multistriatus* (Marsham), and *S. scolytus* (F.) are two important vectors of *Ceratocystis ulmi* (Buism.) C.

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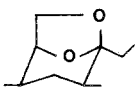
Moreau, the causal agent of Dutch elm disease. When these scolytids bore into, or feed on elm, complex mixtures of beetle- and host-derived volatile substances are produced, one of which is the bicyclic ketal, multistriatin.

(−)-α-Multistriatin (I) was identified (Pierce et al., 1975) as one of the components of the virgin female-produced aggregation attractant of North American populations of *S. multistriatus*. The other components were found



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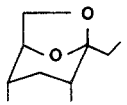

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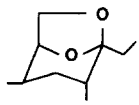

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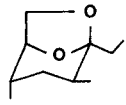

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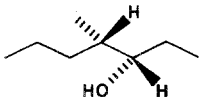

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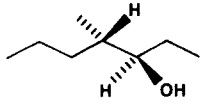

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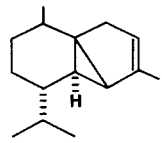

VII

to be (−)-threo-4-methyl-3-heptanol (V) and the elm sesquiterpene, (−)-α-cubebene (VII). Small amounts of the β-isomer (II) of multistriatin were also identified among the volatiles produced by infestations of female beetles. Of the four synthetic racemic stereoisomers, α-(I), β-(II), γ-(III), and δ-(IV), of multistriatin, only the α- (I), in admixture with 4-methyl-3-heptanol and α-cubebene, was found to be attractive to *S. multistriatus* in both laboratory and field tests; the other isomers were inactive (Lanier et al., 1977). Thus multilure, a mixture containing all four stereoisomers of multistriatin together with racemic 4-methyl-3-heptanol and distilled cubeb oil (containing 70% α-cubebene), is used to trap American populations of *S. multistriatus* (Lanier et al., 1976; Peacock et al., 1981).

European populations of *S. multistriatus* were also attracted to baits containing the mixed isomers of multistriatin (Vité et al., 1976), but Gerken et al. (1978) reported that Upper Rhine Valley populations of *S. multistriatus* were attracted to baits containing (−)-δ-multistriatin (IV), racemic 4-methyl-3-heptanol, and distilled cubeb oil. Addition of (−)-α-multistriatin (I) strongly reduced the attraction, and when (−)-δ-multistriatin was replaced by the (−)-α- isomer, the mixture was inactive. However, in further work with these same beetle populations it was found (Klimetzek et al., 1981) that mixtures of racemic α-multistriatin, racemic 4-methyl-3-heptanol, and distilled cubeb oil were attractive, but (−)-δ-multistriatin was inactive. In addition, Blight et al. (1980a) had previously demonstrated that English

populations of *S. multistriatus* were attracted to baits containing (−)-α-multistriatin, the (+)-α-isomer being inactive.

Female *S. scolytus*, like female *S. multistriatus*, also produce α-multistriatin (Blight et al., 1978b), together with unquantifiable trace amounts of β-multistriatin (Blight, Wadhams, and Wenham, unpublished). There is also evidence (Blight et al., 1979a) that trace amounts of α-multistriatin are produced by male *S. scolytus*, but this substance does not appear to be a constituent of the male-produced *S. scolytus* aggregation pheromone (Borden and King, 1977). The principal components of the latter are (−)-threo- (V) and (−)-erythro-4-methyl-3-heptanol (VI) (Blight et al., 1979b).

In a field trial in England, Blight et al. (1978a) found that the addition of a mixture of the four stereoisomers of multistriatin to 4-methyl-3-heptanol and cubeb oil interrupted (sensu Wood, 1977, page 372) the attraction of *S. scolytus* to the latter two substances. It appeared that this effect was mediated by α-multistriatin, but the involvement of the other multistriatin isomers could not be ruled out.

The field trials described below were performed to gain further information on the role of multistriatin in the behavior of both *Scolytus* species in Europe and to assist in the design of a single chemical bait which would effectively trap both beetles.

METHODS AND MATERIALS

Chemicals. The chemicals used in baits were: (1) Redistilled commercial 4-methyl-3-heptanol, 99.9% pure, consisting of (±)-threo- and (±)-erythro-4-methyl-3-heptanol in a 1.3:1 ratio (Aldrich Chemical Co., Gillingham, England); (2) (−)-limonene, >99.2% pure (Fluka A.G., Buchs, Switzerland); (3) (−)-α-cubebene, >99.0% pure, obtained from distilled cubeb oil (70% α-cubebene; Albany International, Chemicals Division, Columbus, Ohio) by preparative high-pressure liquid chromatography (HPLC); (4) (±)-α-multistriatin, >99.0% pure, synthesized using the method of Elliott and Fried (1976) and purified by preparative gas chromatography (GC). It contained no other multistriatin stereoisomers. (5) (±)-γ-multistriatin, >99.0% pure, and (6) (±)-δ-multistriatin, >99.0% pure. Both (5) and (6) were separated from a mixture of multistriatin isomers (Albany International) by preparative GC, and each purified isomer contained no other multistriatin isomers (7) (+)-β-multistriatin, 88.8% pure, containing 6.5% of (+)-δ-multistriatin and 4.7% of an unknown impurity; α- and γ-multistriatin were absent; (8) (−)-β-multistriatin, 86.5% pure, containing 8.5% of (−)-δ-multistriatin and 5% of an unknown impurity; α- and γ-multistriatin were absent. Both (7) and (8) were obtained by preparative HPLC of enantiomerically pure mixtures of the δ- and β-multistriatin stereoisomers (δ:β = 88:12–77:23) (Ottridge and

Blight, unpublished). (A glass vial containing a sample of (+)-β-multistriatin, 94% pure, in pentane, was exposed to light at room temperature (18–23° C) for four months, and periodic GC analysis showed that the β-multistriatin did not isomerize during this time). (9) Commercial multilure, consisting of 4-methyl-3-heptanol, multistriatin (mixed isomers containing 34% α-isomer), and distilled cubeb oil (containing 70% α-cubebene) and formulated in hollow fiber dispensers (Albany International, Controlled Release Division, Needham Heights, Massachusetts).

Field Experiments—Duration and Location. The field experiments, apart from the trial of β-multistriatin (see Results, trial B), were performed between May and September, 1980, on arable farmland located in a stream valley at Hollacombe, near Crediton, Devon, England. Scattered, discrete groups of English elm, *Ulmus procera* Salisbury, occurred throughout the area. Approximately 10% of the trees were healthy, 10–15% were infected with *C. ulmi*, and the remaining 75–80% were dead and unsuitable for beetle colonization. At one site (see Results, trial D) there were significant numbers of Wych elm, *U. glabra* Hudson, most of which were healthy.

The experiment with β-multistriatin was carried out during August and September, 1981, on mixed farmland at Aller Barton, northwest of Crediton. The site was a south-facing, gentle slope, which had originally been surrounded by hedgerow *U. procera*. At the time of the trial 5% of the elm was healthy, 20% was diseased and infested with *S. scolytus* and *S. multistriatus*, and the remaining 75% was dead and unsuitable for beetle colonization.

Trap Design. The fin traps described previously (Blight et al., 1980b) were used in the 1980 experiments. In order to facilitate the experimental design (see below), removable, disposable trap covers were constructed from white, flexible PVC, 140 μm thick. They were folded and stapled along one edge so as to match the profile of the trap. Heavy duty paper clips were placed at the top and bottom of each angle and along the outside edges of the traps to hold the covers in place.

Fully disposable fin traps constructed from corrugated white PVC, 4 mm thick, were used in the 1981 β-multistriatin experiment. They were of the same basic design as those used in 1980 except that each vane was 500 mm long $\times$ 250 mm wide, rather than 610 mm $\times$ 304 mm.

Both the covers and the corrugated PVC traps were coated with a sticky medium consisting of Hyvis 10 (B.P. Chemicals Ltd., London, England) and Epolene N-10 (Kodak-Eastman Chemicals, Hemel Hempstead, England) (93:7).

Dispensing and Release Rates of Baits. The individual components of each bait were released from separate vials. 4-Methyl-3-heptanol, limonene, and most multistriatin samples were released by diffusion through closed polythene vials (2.5 ml capacity, Fisons Scientific Apparatus, Loughborough,

Leics., England). The highest release of (±)-α-multistriatin, 365 μg/day, (see Results, trial C) was obtained from a glass vial-within-a-vial dispenser which consisted of a capped 36 × 11-mm glass vial (2 ml capacity) containing the multistriatin, inside a 60 × 22-mm glass vial (15 ml capacity). Each of these vials had a cap pierced by one 0.3-mm hole. α-Cubebene was released from open glass vials (36 × 11-mm, neck 6 mm diam.).

All release rates were determined in the laboratory at 20° C, in an airflow of 2.5 km/hr. Those from polythene vials were determined by the method used previously (Blight et al., 1980b). Release rates from glass vials were estimated by measuring the loss in weight after exposure for varying periods of time.

Experimental Design. A randomized block design, with periodic re-randomization within the blocks, was used for each trial. Each treatment occurred once in each block. The blocks were selected so that they were as homogeneous as possible with regard to trap exposure and proximity to infested elms. Because of active farming on all sites, the traps were placed, 30 m apart, at the edges of fields along fence lines and hedgerows. Trap height above ground varied, but was generally 2–2.25 m.

Each block was rerandomized after a beetle flight had occurred. In the 1980 experiments random numbers were used to assign treatments to trap positions and some treatments occurred more than once in the same trap position; in 1981 a rerandomization method was used to ensure that this did not occur. At the termination of a replication (i.e., rerandomization), the trap covers plus catch (1980 experiments) or the traps (1981 β-multistriatin experiment) were removed and placed in plastic bags for transportation to the laboratory. New covers (1980) or traps (1981) were then put in place. The duration of each replication varied from 2 to 21 days but most lasted 4–6 days. Each block was rerandomized between one and four times. The total number of replications per trial varied between 13 and 25.

Used covers and traps were stored at 2° C until the beetles were picked off and placed in light petroleum, bp 100–120° C, to remove the sticky material. The *S. scolytus* were both sexed and counted, while only the total numbers of *S. multistriatus* were recorded.

Catch data were subjected to the transformation $z = \log_e(y + 1.0)$ where z and y were the transformed and untransformed counts, respectively. Analyses of variance were performed on the transformed male, female, and total *S. scolytus* catch data and on the transformed total catch data of *S. multistriatus*. Where appropriate, multiple comparisons were made using the Newman-Keul's test.

In each trial, the sex ratios of captured *S. scolytus* were compared by performing analyses of variance of the differences between the transformed numbers of males and females trapped by individual treatments (DITT analysis). It should be noted that the *S. scolytus* sex ratio in the field varies

during the season, since from any given infestation males emerge slightly ahead of females (Blight, Fielding, King, Wadhams, and Wenham, unpublished).

RESULTS

Trial A: *Field Response of* S. multistriatus *and* S. scolytus *to Baits Releasing Small Quantities of Racemic* α-, γ-, or δ-*Multistriatin.* In this trial, the effect on the capture of *S. multistriatus* and *S. scolytus* of releasing 10 μg/day of (±)-α-, (±)-γ- or (±)-δ-multistriatin in combination with 4-methyl-3-heptanol (119 μg/day) and (−)-α-cubebene (319 μg/day) was tested. The mean numbers and transformed mean numbers of both species caught per trap are shown in Table 1. β-Multistriatin was unavailable for inclusion in this trial, but was tested the following year (see trial B, below).

Both *S. multistriatus* and *S. scolytus* were attracted to the mixture of 4-methyl-3-heptanol and α-cubebene (treatment 2 was significantly more attractive than treatment 1 at $P = 0.05$). The addition of either (±)-γ- (treatment 4) or (±)-δ-multistriatin (treatment 5) to this mixture had no significant effect on the capture of either species. However, there was a difference between the species in their response to the bait containing (±)-α-multistriatin (treatment 3). *S. multistriatus* was more strongly attracted to this treatment than to treatment 2, while the numbers of *S. scolytus* captured by treatments 2 and 3 were not significantly different (at $P = 0.05$).

The sex ratios of *S. scolytus* caught by treatments 2, 3, 4, and 5 differed significantly (DITT analysis $P < 0.001$) from the sex ratio of beetles caught by the blank. However, the latter caught only 1.7% of the total number of *S. scolytus* captured in the experiment.

Trial B: *Field Response of* S. multistriatus *and* S. scolytus *to Baits Releasing Small Quantities of* (+)-β-, (−)-β-, *or* (±)-α-*Multistriatin.* The effect on the capture of the *Scolytus* spp. of releasing (+)-β- (5 μg/day) or (−)-β-multistriatin (5 μg/day) in combination with 4-methyl-3-heptanol (119 μg/day) and (−)-α-cubebene (319 μg/day) was studied in this trial. A bait comprising (±)-α-multistriatin (10 μg/day), 4-methyl-3-heptanol, and (−)-α-cubebene (treatment 5) was included in the trial as a standard. The mean numbers and transformed mean numbers of beetles caught per trap are shown in Table 2.

Both species responded to the bait comprising 4-methyl-3-heptanol and (−)-α-cubebene (treatment 2), but the addition of either (+)-β- (treatment 3) or (−)-β-multistriatin (treatment 4) to this mixture made no significant difference to the number of beetles caught. The bait incorporating (±)-α-multistriatin (treatment 5) caught significantly more *S. multistriatus* than were caught by the other baits (treatments 2, 3, 4), but the numbers of *S. scolytus* captured were not increased.

TABLE 1. FIELD RESPONSE OF *S. multistriatus* AND *S. scolytus* TO BAITS RELEASING 10 μg/DAY OF RACEMIC α-, γ-, OR δ-MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-α-CUBEBENE (TRIAL A)

Treatment	Release rate (μg/day)	*S. multistriatus* Mean No. caught per trap (range)	*S. multistriatus* Transformed mean No. caught per trap[a]	*S. scolytus* Mean No. caught per trap (range)	*S. scolytus* Transformed mean No. caught per trap[a]	Sex ratio (♂:♀)
1. Blank		5.6 (0–22)	1.64a	0.8 (0–5)	0.45a	1:1.1
2. 4-Methyl-3-heptanol, (−)-α-cubebene	119 319	48.3 (4–369)	3.12b	13.1 (1–86)	2.21b	1:1.8
3. (±)-α-Multistriatin, 4-methyl-3-heptanol, (−)-α-cubebene	10 119 319	122.6 (8–424)	4.31c	12.7 (0–39)	2.20b	1:1.8
4. (±)-γ-Multistriatin, 4-methyl-3-heptanol, (−)-α-cubebene	10 119 319	40.5 (2–154)	3.22b	13.4 (1–68)	2.26b	1:1.8
5. (±)-δ-multistriatin, 4-methyl-3-heptanol, (−)-α-cubebene	10 119 319	26.6 (1–123)	2.75b	9.3 (0–42)	2.03b	1:2.1

[a]The transformation used was $z = \log_e (y + 1.0)$, where z = transformed catch, y = No. of beetles caught. The total number of replicates of each treatment was 25. The release ratio (in treatments 3, 4, and 5) was multistriatin–α-cubebene–4-methyl-3-heptanol = 0.08:2.68:1. ANOVA indicated a highly significant difference ($P < 0.001$) between the blank and treatment traps (for both species). Treatments within a column followed by the same suffix letter are not significantly different from one another (Newman-Keuls test, $P = 0.05$). The sex ratios of *S. scolytus* beetles caught by treatments 2, 3, 4, and 5 differed significantly ($P < 0.001$) from the sex ratio of beetles caught by the blank. (ANOVA–DITT analysis, see Methods and Materials).

TABLE 2. FIELD RESPONSE OF *S. multistriatus* AND *S. scolytus* TO BAITS RELEASING SMALL QUANTITIES OF (+)-β-MULTISTRIATIN, (−)-β-MULTISTRIATIN, OR (±)-α-MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-α-CUBEBENE (TRIAL B)

Treatment	Release rate (μg/day)	*S. multistriatus*		*S. scolytus*		
		Mean No. caught per trap (range)	Transformed mean No. caught per trap[a]	Mean No. caught per trap (range)	Transformed mean No. caught per trap[a]	Sex ratio (♂:♀)
1. Blank		9.52 (0–93)	1.30a	1.28 (0–13)	0.45a	1:0.60
2. 4-Methyl-3-heptanol,	119	59.24 (0–197)	3.48b	13.40 (0–66)	2.19b	1:0.68
(−)-α-cubebene	319					
3. (+)-β-Multistriatin,	5	40.08 (0–131)	3.18b	25.04 (0–284)	2.26b	1:0.83
4-methyl-3-heptanol,	119					
(−)-α-cubebene	319					
4. (−)-β-Multistriatin,	5	54.60 (0–207)	3.36b	13.32 (1–77)	2.29b	1:1.04
4-methyl-3-heptanol,	119					
(−)-α-cubebene	319					
5. (±)-α-multistriatin,	10	175.20 (7–569)	4.67c	12.72 (1–39)	2.34b	1:0.88
4-methyl-3-heptanol,	119					
(−)-α-cubebene	319					

[a]The transformation used was $z = \log_e (y + 1.0)$, where z = transformed catch, y = No. of beetles caught. The total number of replicates of each treatment was 25. The release ratio for β-multistriatin–α-cubebene–4-methyl-3-heptanol was 0.04:2.68:1 in treatments 3 and 4. For (±)-α-multistriatin–α-cubebene–4-methyl-3-heptanol the release ratio was 0.08:2.68:1 (treatment 5). ANOVA indicated a highly significant difference ($P < 0.001$) between the blank and treatment traps for both species. There was no other significant effect for *S. scolytus*, but for *S. multistriatus* treatment 5 was significantly different from treatments 3 and 4 ($P < 0.01$). Treatments within a column followed by the same suffix letter are not significantly different from one another (Newman-Keuls test, $P = 0.05$). The DITT analysis (see Methods and Materials) indicated that the sex ratios of *S. scolytus* caught by treatments 3 and 4 were significantly different from one another (at $P < 0.05$) but there were no significant differences by the Newman-Keuls test at $P = 0.05$.

TABLE 3. FIELD RESPONSE OF *S. multistriatus* TO BAITS RELEASING DIFFERENT AMOUNTS OF RACEMIC α-MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-LIMONENE (TRIAL C).

Treatment	Release rate (μg/day)	Release ratio multistriatin–limonene–4-methyl-3-heptanol	*S. multistriatus*	
			Mean No. caught per trap (range)	Transformed mean No. caught per trap[a]
1. Blank			1.9 (0–12)	0.74
2. 4-Methyl-3-heptanol,	119		4.3 (0–32)	1.07
(−)-limonene	998			
3. 4-Methyl-3-heptanol,	119	0.04:8.4:1	9.3 (0–31)	1.86
(−)-limonene,	998			
(±)-α-multistriatin	5			
4. 4-Methyl-3-heptanol,	119	0.48:8.4:1	11.7 (0–97)	1.59
(−)-limonene,	998			
(±)-α-multistriatin	57			
5. 4-Methyl-3-heptanol,	119	3.07:8.4:1	3.5 (0–13)	1.20
(−)-limonene,	998			
(±)-α-multistriatin	365			

[a]The transformation used was $z = \log_e(y + 1.0)$, where z = transformed catch, y = number of beetles caught. The total number of replicates of each treatment was 13. The analysis of variance of this data is shown in Table 5.

Differences in the sex ratios of *S. scolytus* caught by the treatments incorporating (+)-β- (treatment 3) or (−)-β-multistriatin (treatment 4) were significant ($P < 0.05$) according to the DITT analysis but were not significant by the Newman-Keul's test.

Trial C: Field Response of S. multistriatus *and* S. scolytus *to Baits Releasing Different Amounts of Racemic α-Multistriatin.* This trial tested the effect on the capture of *S. multistriatus* and *S. scolytus* of the incorporation of different concentrations of (±)-α-multistriatin into baits comprising 4-methyl-3-heptanol (119 μg/day) and (−)-limonene (998 μg/day). [The latter substance was used as an alternative to (−)-α-cubebene (Blight et al., 1980b).] Untransformed and transformed catch data for *S. multistriatus* and *S. scolytus* are shown in Tables 3 and 4, and the analyses of variance of the data are shown in Tables 5, 6, and 7.

(±)-α-Multistriatin affected the capture of both species of beetles but in different ways. Analysis of variance (Table 5) indicated a significant multistriatin effect ($P < 0.05$) vs. *S. multistriatus*. Two of the baits incorporating α-multistriatin (treatments 3 and 4) captured larger numbers of *S. multistriatus* than the 4-methyl-3-heptanol/limonene bait. Moreover, the bait (treatment 3) which released the lowest amount of α-multistriatin (5 μg/day,

TABLE 4. FIELD RESPONSE OF *S. scolytus* TO BAITS RELEASING DIFFERENT AMOUNTS OF RACEMIC α-MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-LIMONENE (TRIAL C)

Treatment	Release rate (μg/day)	Release ratio multistriation–limonene–4-methyl-3-heptanol	*S. Scolytus*				
			Mean No. males caught per trap (range)	Transformed mean No. males caught per trap[a]	Mean No. females caught per trap (range)	Transformed mean No. females caught per trap[a]	Sex ratio (♂:♀)
1. Blank			3.4 (0–12)	1.06	4.5 (0–23)	1.21	1:1.3
2. 4-Methyl-3-heptanol,	119		10.1 (0–36)	1.80	10.3 (2–31)	2.17	1:1.0
(−)-limonene	998						
3. 4-Methyl-3-heptanol	119	0.04:8.4:1	8.3 (1–22)	1.95	10.9 (1–28)	2.21	1:1.3
(−)-limonene,	998						
(±)-α-multistriatin	5						
4. 4-Methyl-3-heptanol,	119	0.48:8.4:1	8.1 (1–51)	1.76	6.9 (1–28)	1.74	1:0.9
(−)-limonene,	998						
(±)-α-multistriatin	57						
5. 4-Methyl-3-heptanol,	119	3.07:8.4:1	6.4 (0–21)	1.56	4.9 (0–16)	1.39	1:0.8
(−)-limonene,	998						
(±)-α-multistriatin	365						

[a]The transformation used was $z = \log_e(y + 1.0)$, where z = transformed catch, y = number of beetles caught. The total number of replicates of each treatment was 15. The analyses of variance of these data are shown in Tables 6 and 7. The DITT analysis (see Methods and Materials) indicated that (±)-α-multistriatin had a significant linear effect ($P < 0.05$) on the sex ratio of beetles caught.

TABLE 5. ANALYSIS OF VARIANCE: RESPONSE OF *S. multistriatus* TO BAITS RELEASING DIFFERENT AMOUNTS OF RACEMIC α-MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-LIMONENE (TRIAL C) [VARIANT = $\log_e$ (CATCH + 1.0)]

Source of variation	Degrees of freedom	Mean square	Variance ratio	Critical value of the *F* statistic
Sites (i.e., blocks)	4	1.382	2.08	
Rerandomization (replication)	8	3.013	4.53	
Treatments (broken down into)	4	2.507	3.77[a]	3.73
4-Methyl-3-heptanol-limonene	1	4.956	7.45[a]	7.2
Multistriatin				
Linear component	1	0.718	1.08	
Quadratic component	1	0.474	0.71	
Component which fails to fit quadratic	1	3.879	5.83[b]	4.04
Residual	48	0.666		
Total	52	0.807		
Grand total	64			

[a]Significant at $P = 0.01$.
[b]Significant at $P = 0.05$.

α-multistriatin to 4-methyl-3-heptanol release ratio, α-M–H = 0.04:1) was the most attractive. When the α-multistriatin release rate increased from 5 to 365 μg/day (with the α-M–H release ratio increasing from 0.04:1 to 3.07:1), there was an approximate linear decrease (Figure 1) in the number of beetles captured.

In contrast to the situation with *S. multistriatus*, the addition of (±)-α-multistriatin to the mixture of 4-methyl-3-heptanol and (−)-limonene did not result in the capture of significantly more *S. scolytus*. There was an inverse linear relationship (Figure 1) between the number of beetles captured and the release rate of α-multistriatin. The bait (treatment 5) which incorporated the highest release of α-multistriatin (365 μg/day, release ratio α-M–H = 3.07:1) captured appreciably fewer *S. scolytus* of both sexes than the 4-methyl-3-heptanol/limonene bait (treatment 2). However, the analyses of variance (Tables 6 and 7) indicated that α-multistriatin had a significant (linear) effect ($P < 0.01$) on the capture of female *S. scolytus* only (Figure 1). In addition, the DITT analysis revealed that (±)-α-multistriatin had a significant linear effect ($P < 0.05$) on the sex ratio of beetles caught (Table 4).

Trial D: Comparison of Field Response of S. multistriatus *and* S. scolytus *to 4-Methyl-3-heptanol and Multilure Baits.* In this trial the efficacy of a bait releasing 789 μg/day of 4-methyl-3-heptanol was compared with that of a commercial multilure bait with nominal release rates of 100 μg/day of

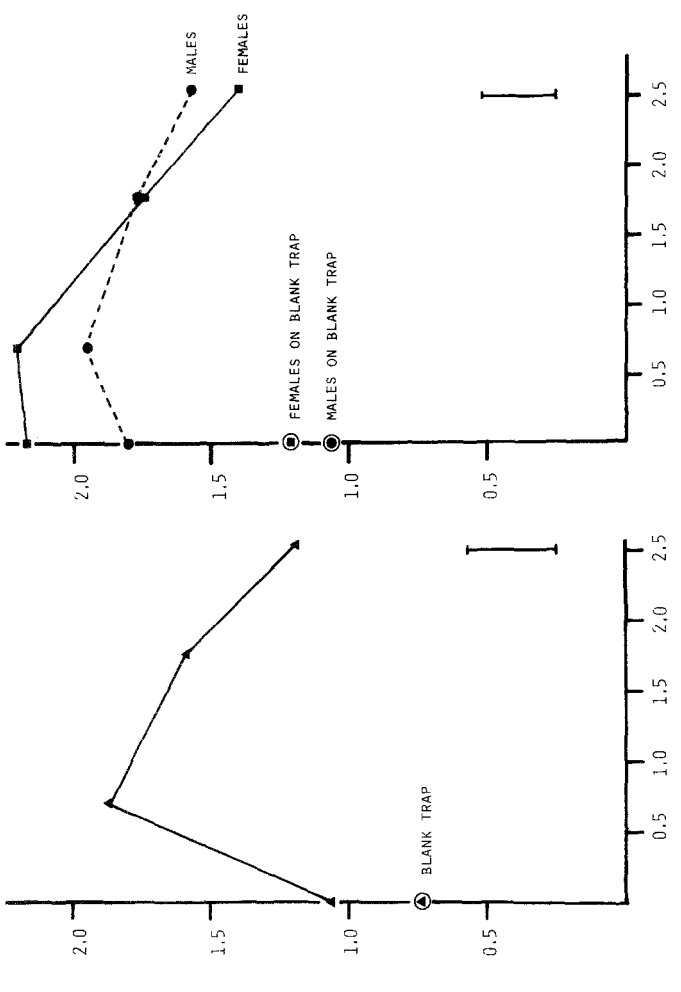

FIG. 1. Field responses of *S. multistriatus* and *S. scolytus* to baits releasing different amounts of (±)-α-multistriatin in combination with 4-methyl-3-heptanol (119 μg/day) and (−)-limonene (998 μg/day). The bar on each graph indicates the standard error of the differences between two treatment means.

TABLE 6. ANALYSIS OF VARIANCE RESPONSE OF MALE *S. scolytus* TO BAITS RELEASING DIFFERENT AMOUNTS OF RACEMIC α-MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-LIMONENE (TRIAL C) [VARIANT = $\log_e$ (CATCH + 1.0)]

Source of variation	Degrees of freedom	Mean square	Variance ratio	Critical value of the *F* statistic
Sites (i.e., blocks)	4	2.722	4.98	
Rerandomization (replication)	10	2.566	4.70	
Treatments (broken down into)	4	1.789	3.28[b]	2.55
4-Methyl-3-heptanol-limonene	1	6.014	11.01[a]	7.1
Multistriatin				
Linear component	1	0.920	1.69	
Quadratic component	1	0.039	0.07	
Component which fails to fit quadratic	1	0.182	0.33	
Residual	56	0.546		
Total	60	0.629		
Grand total	74			

[a]Significant at $P = 0.01$.
[b]Significant at $P = 0.05$.

TABLE 7. ANALYSIS OF VARIANCE: RESPONSE OF FEMALE *S. scolytus* TO BAITS RELEASING DIFFERENT AMOUNTS OF RACEMIC α-MULTISTRIATIN IN COMBINATION WITH 4-METHYL-3-HEPTANOL AND (−)-LIMONENE (TRIAL C) [VARIANT = $\log_e$ (CATCH + 1.0)]

Source of variation	Degrees of freedom	Mean square	Variance ratio	Critical value of the *F* statistic
Sites (i.e., blocks)	4	1.477	2.75	
Rerandomization (replication)	10	1.594	2.97	
Treatments (broken down into)	4	3.010	5.60[a]	3.7
4-Methyl-3-heptanol-limonene	1	5.367	9.99[a]	7.1
Multistriatin				
Linear component	1	5.533	10.30[a]	7.1
Quadratic component	1	1.084	2.02	
Component which fails to fit quadratic	1	0.055	0.10	
Residual	56	0.537		
Total	60	0.702		
Grand total	74			

[a]Significant at $P = 0.01$.

multistriatin isomers (containing 34% α-isomer), 400 μg/day of 4-methyl-3-heptanol, and 800 μg/day of distilled cubeb oil (containing 70% α-cubebene). Untransformed and transformed catch data for *S. multistriatus* and *S. scolytus* are shown in Table 8.

Both baits (treatments 2 and 3) attracted significantly more beetles of both species than the blank. However, the data clearly indicated that the commercial multilure bait was more effective than the 4-methyl-3-heptanol bait for trapping *S. multistriatus* (treatment 2 was significantly different from treatment 3 at $P < 0.01$), whereas the converse was true for *S. scolytus* (treatment 2 was significantly different from treatment 3 at $P < 0.05$).

The sex ratios of *S. scolytus* beetles caught by treatments 2 and 3 differed significantly ($P < 0.01$) from the sex ratio of beetles caught by the blank, but only 10% of the total *S. scolytus* captured in this experiment were on blank traps.

DISCUSSION

The results of the field trials reported in this paper have confirmed and amplified previous knowledge (Blight et al., 1980a) of the response of flying English *S. multistriatus* to multistriatin. In three separate experiments (Tables 1, 2, and 3) baits which released small amounts (5–10 μg/day) of (±)-α-multistriatin in combination with 4-methyl-3-heptanol and a host synergist were more attractive than baits which contained only the latter two substances, while the admixture of (+)-β-, (−)-β-, (±)-γ- or (±)-δ-multistriatin to 4-methyl-3-heptanol and α-cubebene had no effect on the numbers of beetles trapped. Thus, all the recent studies on English (this paper; Blight et al., 1980a), German (Klimetzek et al., 1981), and Swiss populations (M.E. Angst, personal communication) of *S. multistriatus* have disproved the suggestion (Gerken et al., 1978) that European populations respond to baits containing δ-, rather than α-multistriatin. In addition, these studies suggest that European *S. multistriatus* resemble American populations of this species. In support of the latter proposition, we have identified α-multistriatin from English female *S. multistriatus* (M.M. Blight and M.J. Wenham, unpublished).

In contrast to the effect on the catch of *S. multistriatus*, the addition of small amounts of α-multistriatin to the 4-methyl-3-heptanol baits did not result in the capture of more *S. scolytus*. Klimetzek et al. (1981) have also observed that α-multistriatin had no effect on the numbers of *S. scolytus* captured, but in this work the release rate of α-multistriatin was not determined. However, Swiss populations of *S. scolytus* were more highly attracted to a low release rate (approx. 8 μg/day) of α-multistriatin in combination with 4-methyl-3-heptanol and α-cubebene, than to 4-methyl-3-

TABLE 8. COMPARISON OF FIELD RESPONSE OF *S. multistriatus* AND *S. scolytus* TO 4-METHYL-3-HEPTANOL AND MULTILURE BAITS (TRIAL D)

Treatment	Release rate (μg/day)	*S. multistriatus*		*S. scolytus*		
		Mean No. caught per trap (range)	Transformed mean No. caught per trap[a]	Mean No. caught per trap (range)	Transformed mean No. caught per trap[a]	Sex ratio (♂:♀)
1. Blank	—	17.9 (1–166)	2.30a	3.8 (0–17)	1.15a	1:1.5
2. 4-Methyl-3-heptanol	789	39.8 (3–129)	3.29b	21.7 (5–83)	2.85b	1:1.9
3. Multilure:		142.1 (39–557)	4.74c	12.4 (1–38)	2.31c	1:2.0
Multistriatin (mixed isomers, ~34% α)	100					
4-Methyl-3-heptanol	400					
Cubeb oil (70% α-cubebene)	800					

[a]The transformation used was $z = \log_e (y + 1.0)$, where z = transformed catch, y = number of beetles caught. The total number of replicates of each treatment was 24. Treatments within a column followed by different suffix letters are significantly different from one another (Newman-Keuls test, $P = 0.05$ for *S. scolytus*, and $P = 0.01$ for *S. multistriatus*). The sex ratios of *S. scolytus* beetles caught by treatments 2 and 3 differed significantly ($P < 0.01$) from the sex ratio of beetles caught by the blank.

heptanol and α-cubebene alone (M.E. Angst, personal communication). It is not clear whether the difference between these observations and our own resulted from differences in behavior between English and Swiss *S. scolytus* populations. It could have been caused by differences in experimental design.

High release rates of α-multistriatin interrupted the response of both *S. multistriatus* and *S. scolytus* to traps baited with 4-methyl-3-heptanol and α-cubebene. The results of trial C confirmed that the decrease in the number of *S. scolytus* trapped by baits containing the mixed isomers of multistriatin (Blight et al., 1978a) was mediated by the α-isomer. Cuthbert and Peacock (1978) also observed a reduction in trap catch of American *S. multistriatus* when the multistriatin (mixed isomers)–4-methyl-3-heptanol release ratio in multilure baits increased from 1:1 to 4:1. This represented an increase in the release rate of (±)-α-multistriatin from 17 μg/day to 68 μg/day (with the release rate of 4-methyl-3-heptanol held constant). It is clear, therefore, that the release rate of α-multistriatin has a critical effect on the activity of the substance in the field. The release ratio of 4-methyl-3-heptanol to α-multistriatin is probably also important, but further work is needed to establish this.

Although trace amounts of β-multistriatin are produced by females of both species, both (−)-β- and (+)-β-multistriatin were inactive in the field experiment described above. The indication from the DITT analysis (see Table 2) that (−)-β-multistriatin may affect the sex ratio of responding *S. scolytus* must be treated with caution since this result was not confirmed by Newman-Keul's multiple-range test and was contradicted by the analysis of variance. The inactivity of β-multistriatin cannot be explained by the hypothesis (Gerken et al., 1978) that β-multistriatin isomerizes immediately to the (inactive) unnatural δ-isomer (see Methods and Materials). However, the possibility that β-multistriatin may affect short-range beetle behavior cannot be ruled out, and this is being investigated.

From present knowledge it appears that α-multistriatin has multiple functions in the behavior of the two *Scolytus* species, and in this respect it resembles several other scolytid pheromonal substances (Rudinsky, 1973a,b; Rudinsky et al., 1974; Bedard et al., 1980). In addition to functioning as a component of the aggregation pheromone of *S. multistriatus*, α-multistriatin may play a role in interspecific attraction between the two *Scolytus* species. *S. multistriatus* was attracted to billets artificially infested with both sexes of *S. scolytus* (Blight et al., 1980b). Under these conditions both 4-methyl-3-heptanol and α-multistriatin are simultaneously produced by male and female beetles (Blight, Wadhams, and Wenham, unpublished). Thus, *S. scolytus*, which normally emerges earlier in the season, colonizes the trunk and larger branches of stressed elm, and produces a bouquet of chemicals similar to multilure. The latter attracts the sympatric *S. multistriatus* to the smaller-diameter branches.

The release of large amounts of α-multistriatin may be part of a spacing mechanism for both species, by which beetle attack is shifted to less heavily colonized areas of the same tree, or to other trees. The known production of large quantities of α-multistriatin, and little or no 4-methyl-3-heptanol, by mated female *S. multistriatus* (Gore et al., 1977), and the results of the field experiment with *S. scolytus*-infested billets (Blight et al., 1980b) support this hypothesis. In the latter experiment only small numbers of *S. scolytus* were attracted to billets containing a high proportion of galleries bored by females.

The field trial results reported in this paper have important practical implications. Trial D indicated that multilure baits do not attract *S. scolytus* effectively, and the results of trial C suggest that multilure may not be the optimum formulation for attracting *S. multistriatus*, since it releases 17–68 μg/day of ($\pm$)-α-multistriatin (see footnote 2, page 372, in Cuthbert and Peacock, 1978). However, it is clear that one bait can be designed to attract both species. A small quantity of the mixed isomers of multistriatin, formulated with 4-methyl-3-heptanol and a host synergist will attract *S. multistriatus* effectively but will not interrupt and response of *S. scolytus* to the latter substances.

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INITIAL FIELD TRIALS WITH THE SYNTHETIC SEX PHEROMONE OF THE PROCESSIONARY MOTH *Thaumetopoea pityocampa*[1] (DENIS AND SCHIFF.)

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Abstract—Synthetic (*Z*)-13-hexadecen-11-ynyl acetate has been shown to be highly active in catching *Thaumetopoea pityocampa* (Denis and Schiff.) males in field trials carried out in different parts of Spain. A variety of formulations containing antioxidants or solid paraffin were tested. Formulations containing 3 and 10% of *E* isomer showed a decrease of activity compared with those prepared with pure *Z* isomer. Dodecyl acetate, also found in the virgin female gland, did not show any synergistic effect when tested in a 9:1 mixture with the synthetic pheromone. The product exhibited a remarkable persistence of activity under the field conditions even in the absence of stabilizer.

Key Words—Sex attractant, sex pheromone, processionary moth, *Thaumetopoea pityocampa*, Lepidoptera, Notodontidae, (*Z*)-13-hexadecen-11-ynyl acetate, field test.

INTRODUCTION

The processionary moth, *Thaumetopoea pityocampa* (Denis and Schiff.) (Lepidoptera, Notodontidae) is a major defoliator pine pest in southern Europe and North Africa. During the day, the larvae leave the nest and march in procession to feed on the young tender branches of the pine. They burrow

[1]Lepidoptera: Notodontidae.

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ca. 20–30 cm deep into the ground around the tree in late February to pupate through June. Some adults may emerge in July–October according to the climate, geographic distribution, and altitude, and the remaining pupae may enter into diapause for a second generation (Demolin, 1969).

The estimated level of damage caused in Spain by the processionary moth in 1977–1978 amounted to 10 million dollars. Defoliation threatens 21% of the total pine grove area, according to a recent study by the Servicio Nacional de Plagas and Instituto para la Conservación de la Naturaleza (ICONA).

Recently, we have described (Guerrero et al., 1981) the identification of (*Z*)-13-hexadecen-11-ynyl acetate (Structure I) as a potential sex pheromone of the processionary moth, isolated from the virgin female gland. Two different syntheses of this compound have been published (Camps et al., 1981a,b; Michelot et al., 1981).

STRUCTURE I

A response of 2.2 mV in the EAG was elicited by 1 μg of compound I, whereas the same amount of natural pheromone gave a 2.8-mV signal. A 1-μg sample of the *E* isomer of I elicited a response of only 0.6 mV.

METHODS AND MATERIALS

Chemicals. Syntheses of I and its corresponding E isomer (>99% stereochemical purity by GC analysis using a 2-m × 1/8-in. glass column of OV-101 3% on Chromosorb (Chr) W and 2-m × 1/8-in. glass column of FFAP 5% on Chr. W) were carried out from (*Z*)-3-hexen-1-yn or (*E*)-3-hexen-1-yn and 10-tetrahydropyranyloxy-1-bromodecane as previously described (Camps et al., 1981a,b).

Field Trapping. Field trials were performed in Spain from July to September 1981. Traps most extensively used were ICONA cups (7 cm upper diameter × 6 cm lower diameter, 15 cm high), made of paraffined cardboard. They were provided with a removable cap and impregnated internally with a Tanglefoot-type glue. Other traps used were the commercially available Inra (Société Léglise, Talence Cedex), Pherocon 1CP and 1CPY (Zoecon Corp., Palo Alto, California) and Southampton Delta (University of Southampton) traps. Traps were hung on trees at a height of 1.5–2 m above ground and

spaced 4–5 trees apart (10–15 m). They were set out in statistically randomized blocks and rerandomized or rotated at least three times in each trial. Cylinders of dental cotton, rubber septa (red, 5 × 9 mm, A.H. Thomas Co. Philadelphia, Pennsylvania), and polyethylene vials (Kartell Co.) were used as dispensers. When cylinders of dental cotton were employed, they were replaced every 3–4 days, and the captures were generally recorded every day.

Trap catches were subjected to a square root transformation followed by analysis of variance, and the data were analyzed statistically for significance according to Duncan's new multiple-range test.

RESULTS AND DISCUSSION

The first field trials were carried out in infested pine groves in Mora de Rubielos (Teruel). Several formulations of I plus the corresponding *E* isomer or dodecyl acetate were tested, and the amount of lure varied from 0.05 μg to 1 mg (0.05 μg was the GLC estimated amount of natural pheromone contained

TABLE 1. CAPTURE OF *Thaumetopoea pityocampa* MALES USING ICONA TRAPS AND CYLINDERS OF DENTAL COTTON AS DISPENSERS (MORA DE RUBIELOS, TERUEL, JULY 12–31, 1981

Attractant composition	Amount of lure (μg)	Males captured[a]
I	0.05	2a
	0.5	7ab
	5	33abc
	1000	157e
I + 3% isomer E	0.05	1a
	0.5	10ab
	1000	68cd
I + 10% isomer E	0.05	13ab
	0.5	5ab
	1000	89cd
Isomer E	0.05	0a
	0.5	24abc
	5	15ab
	1000	45bcd
I + 12:Ac (9:1)	0.05	29abc
	0.5	10ab
	5	19ab
	1000	82cd

[a] Treatments followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test. Four replicates per treatment.

in the crude extract of 10 female abdominal tips cut 3–10 hr after adult emergence). Four replicates for each treatment were carried out in ICONA baited-traps using cylinders of dental cotton as dispensers. As shown in Table 1, the best results were obtained by using 1-mg doses of isomerically pure I which were significantly better ($P < 0.05$) than those containing blends of both isomers. On the other hand, all formulations with lower doses gave inconclusive results. The slight activity of the isomerically pure *E* isomer (>99%) could be attributed to a partial isomerization to the *Z* isomer by sunlight.

Traps baited with a 9:1 blend of I and dodecyl acetate (also found in the virgin female gland) did not show a clear synergistic effect compared with those baited with compound I alone.

The relative efficiency of several kinds of traps was determined in two separate experiments. The first trial was carried out in Mora de Rubielos (Teruel) using rubber septa and polyethylene vials as dispensers. The traps used were ICONA cups, Inra and Pherocon 1 CP traps. With ICONA traps, six replicates per treatment were performed and only three replicates per treatment for the remaining traps (Table 2).

Inra and Pherocon 1CP traps were significantly superior ($P < 0.05$) to ICONA cups but, apparently, the influence of both types of dispenser tested was not relevant. During the experiment, placement of a 500-W light trap allowed us to follow the flight curve of the moth. As shown in Figure 1, the

TABLE 2. INFLUENCE OF TRAP DESIGN AND TYPE OF DISPENSER ON CATCHES OF *Thaumetopoea pityocampa* MALES USING (*Z*)-13-HEXADECE-11-YNYL ACETATE (I) AS LURE (MORA DE RUBIELOS, TERUEL, JULY 12–31, 1981)

Trap	Dispenser	Lure dose (μg)	Total catches	Mean catch/trap/day[a]
ICONA	Septum	50	6[b]	0.044a
		250	91[b]	0.669ab
		1000	169[b]	1.243bcd
	Vial	50	53[b]	0.390ab
		250	134[b]	0.985bc
		1000	198[b]	1.456cd
Inra	Septum	1000	216[c]	3.60ef
	Vial	1000	248[c]	4.133f
Pherocon 1CP	Septum	1000	238[c]	3.967f
	Vial	1000	214[c]	3.567ef

[a] Means followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.
[b] Six replicates per treatment.
[c] Three replicates per treatment.

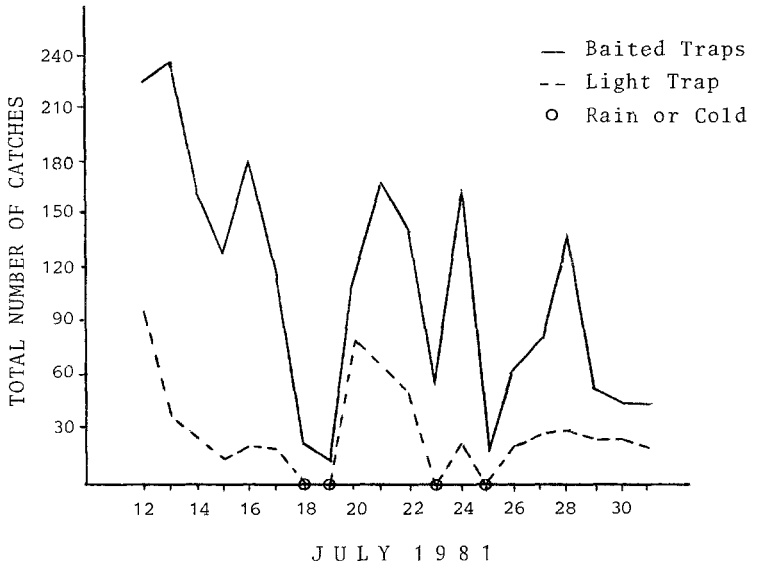

FIG. 1. Total number of catches of *Thaumetopoea pityocampa* males in traps baited with (*Z*)-13-hexadecen-11-ynyl acetate (I) and light trap (Mora de Rubielos, Teruel, July 12–31, 1981).

total number of catches in pheromone traps and in the light trap per day exhibited a good correlation, although the profile of both curves suggested that the field test was initiated after the maximum emergence. Unfavorable weather conditions on the 18th, 19th, 23rd, and 25th may account for the low level of catches on these dates. It is note worthy that there were some catches in the lure-baited traps after two months in the field, whereas after this time there were no captures in the light trap.

The second field experiment to test the efficiency of the traps was carried out in pine groves located in La Marina (Alicante). The traps were distributed at random in seven parcels spaced 150 m apart. In each parcel the traps were aligned and spaced 50 m apart. Every day the traps were statistically rerandomized, and the results are summarized in Table 3. Again, the maximum number of males was caught using the Inra traps and polyethylene vials as dispensers. Somewhat lower level of catches resulted using the Pherocon 1CP traps and vials as dispensers. Clearly inferior results were given by the Pherocon Delta, Pherocon 1CPY, Southampton Delta, and ICONA traps regardless of the type of dispenser used.

To test the possible improvement of efficiency of the lure by the use of antioxidants, several mixtures of isomerically pure compound I (1 mg) with a variety of antioxidants in a 1 : 1, 1 : 2, and 1 : 4 ratio were placed on rubber septa

TABLE 3. INFLUENCE OF TRAP DESIGN AND DISPENSER USING 1 MG OF COMPOUND I PER TRAP (LA MARINA, ALICANTE, SEPTEMBER 15–OCTOBER 3, 1981)

Trap	Dispenser	Total catches	Mean catch/trap/day[a]
Southampton Delta	Septum	119[b]	4.72bcd
	Vial	116[b]	3.22ab
Pherocon Delta	Septum	253[c]	4.72bcd
	Vial	186[b]	5.17bcd
Pherocon 1CPY	Septum	129[c]	2.39a
	Vial	116[c]	2.17a
Pherocon 1CP	Septum	472[d]	4.39abcd
	Vial	517[e]	5.78cd
Inra	Septum	294[c]	5.44bcd
	Vial	226[b]	6.28cd
ICONA	Septum	469[f]	3.72abc

[a] Means followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.
[b] Two replicates per treatment.
[c] Three replicates per treatment.
[d] Six replicates per treatment.
[e] Five replicates per treatment.
[f] Seven replicates per treatment.

and exposed to sunlight and atmospheric conditions (Shani et al., 1980). The antioxidants used were BHB (*n*-butyl-4-hydroxybenzoate), α-T (α-tocopherol), TBH (*t*-butylhydroquinone), PG (*n*-propyl gallate), BHA (*t*-butylhydroxyanisole), MHB (methyl *p*-hydroxybenzoate), DT (*N,N*-diethyl *m*-toluamide), BHT (*t*-butylhydroxytoluene), and TG (α-thioglycerine). After 2, 5, 9, 15, and 22 days under atmospheric conditions, the septa were extracted with 2 ml of nanograde hexane and concentrated to a volume of 100 μl. Gas chromatographic analysis (OV-101 3% on Chr. W glass column 2 m × 1/8 in. and FFAP 5% on Chr. W glass column 2 m × 1/8 in.) showed compound I and its *E* isomer as the only volatile compounds. After completion of the experiment, the formulation with α-T contained an average of 16.9% of the *E* isomer, that with PG 18.4%, and the remaining ones over 20%. Under the same conditions, formulations with pure I revealed the presence of 22% of the *E* isomer. The amount of compound I recovered from blends with PG was on average 49% higher than that recovered from the standard formulation containing pure I alone. On the other hand, the recoveries from the formulations with α-T and BHA were only 9.2 and 8.2%, respectively, higher than that from the standard. The best formulations thus found were applied in the field along with the natural pheromone (50 and 500 female equivalent). The test was performed in Sta. María del Tiétar (Avila) using 1 mg of

compound I on ICONA traps and septa as dispensers. The results, summarized in Table 4, were in close agreement with those expected from the laboratory assay.

The formulation of I + PG in a 1:1 ratio proved to be the most efficient (118 males caught), followed by I + BHB in a 1:1 ratio (103 males caught). Eventually, in some cases the higher ratio of antioxidant (1:4) appears to be detrimental to the biological activity. The excellent capture of formulation I + solid paraffin in a 1:5 ratio (104 males) should be noted. The low level of efficiency of 50 and 500 FE can be attributed to the lower concentration of the natural pheromone compared with the synthetic material.

Finally, a field experiment to check the attractiveness of compound I at several distances was planned. Three different lines of 10 ICONA baited traps with septa as dispensers were used. Minimum distance between lines was 200 m. The lines were placed at 25, 50, and 75 m from the border of the pine grove, and the traps were revised every other day. When at least 50% of the traps of

TABLE 4. EFFECT OF SEVERAL FORMULATIONS ON NUMBER OF CATCHES OF *Thaumetopoea pityocampa* MALES USING 1 MG OF I ON SEPTA IN ICONA TRAPS (STA. MARIA DEL TIETAR, AVILA, AUGUST 7–29, 1981)

Attractant composition	Antioxidant	Attractant Antioxidant	Total catches[b]	Mean catch/trap/day[a]
Natural pheromone				
50 FE			0	0a
500 FE			17	0.075a
Compound I			68	0.302b
I + solid paraffin (1:5)			104	0.462cd
I	BHB	1:1	103	0.457cd
I	BHB	1:4	90	0.40bcd
I	α-T	1:1	78	0.342bc
I	α-T	1:4	79	0.351bc
I	TBH	1:1	85	0.378bcd
I	TBH	1:4	96	0.426bcd
I	PG	1:1	118	0.524d
I	PG	1:4	92	0.409bcd
I	BHA	1:1	96	0.427bcd
I	BHA	1:4	64	0.284b
I	Uvinul[c]	1:1	94	0.418bcd
I	Uvinul	1:4	95	0.422bcd

[a] Means followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.
[b] Ten replicates per treatment.
[c] Rhone-Poulenc Ind., Vitry.

TABLE 5. ATTRACTIVENESS OF ICONA TRAPS BAITED WITH 1 MG OF I ON *Thaumetopoea pityocampa* MALES AT SEVERAL DISTANCES FROM PINE GROVE (LA MARINA, ALICANTE, SEPTEMBER 16–24, 1981)

	Total number of catches					
Line	25 m	50 m	75 m	125 m	150 m	175 m
A	109[a]	→	→	67[b]–50[c]–26[d]		
B		75[a]–32[b]	→	→	56[c]–31[d]	
C			125[a]–46[b]–40[c]	→	→	6[d]

[a] Revised on September 18.
[b] Revised on September 20 (bad weather).
[c] Revised on September 22.
[d] Revised on September 24.

the farthest line caught males, the closest line to the grove was moved away in order to be the farthest. The experiment was considered completed when less than 50% of the traps of the farthest line caught males. The results are summarized in Table 5.

As is shown, only six males were caught in three baited-traps after moving line C to 175 m away from the pine grove. This was therefore the maximum distance found at which compound I was still active. Farther displacements of lines A, B, or C did not catch insects in 50% of the traps. Independent field trials in Israel have shown the synthetic pheromone I to be active at a distance of 1400 m (A. Shani and J. Halperin, personal communication, 1981).

The present preliminary results reported here show that (*Z*)-13-hexadecen-11-ynyl acetate (I) can be very valuable for monitoring *Thaumetopoea pityocampa*. However, extensive field tests should be carried out to define different parameters, such as trap design, formulation, etc., to optimize these results.

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Spodoptera Exigua[1]: Capture of Feral Males in Traps Baited with Blends of Pheromone Components[2]

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Abstract—Field tests were conducted with various blends of acetates and alcohols previously identified as components of the sex pheromone for the beet armyworm (BAW), *Spodoptera exigua* (Hübner). The compounds were formulated on rubber septa and placed in sticky traps positioned in fields populated with *Amaranthus* sp., a favored host of this species. Moth captures were highest in traps baited with septa containing a blend of 0.1 mg (*Z,E*)-9,12-tetradecadien-1-ol acetate (*Z*9,*E*12-14:Ac) and 0.01 mg (*Z*)-9-tetradecen-1-ol (*Z*9-14:OH). Additionally, we confirmed that all combinations of acetates tested alone captured significantly fewer males than blends containing a 10:1 ratio of *Z*9,*E*12-14:Ac and *Z*9-14:OH. A 10:1 formulation of *Z*9,*E*12-14:Ac and *Z*9-14:OH in hollow fibers was also attractive to feral BAW males.

Key Words—*Spodoptera exigua*, Lepidoptera, Noctuidae, pheromone, rubber septa, (*Z,E*)-9,12-tetradecen-1-ol acetate, (*Z*)-9-tetradecen-1-ol, beet armyworm, trapping sticky trap.

INTRODUCTION

Recently we reported the identification of a pheromone blend from beet armyworm (BAW), *Spodoptera exigua* (Hübner), females (Tumlinson et al., 1981). Eleven compounds were identified from the active fractions obtained

[1]Lepidoptera: Noctuidae.
[2]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.
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from hexane washes of virgin females during the period of maximum calling activity: tetradecan-1-ol (14:OH), (*Z*)-9-tetradecen-1-ol (*Z*9–14:OH), (*E*)-9-tetradecen-1-ol (*E*9–14:OH), (*Z,E*)-9,-12-tetradecadien-1-ol (*Z*9,*E*12–14:OH), (*Z,Z*)-9,12-tetradecadien-1-ol (*Z*9,*Z*12–14:OH), the acetates of these alcohols, and (*Z*)-7-tetradecen-1-ol acetate (*Z*7–14:Ac). Not all of these compounds appeared necessary to elicit responses by males in the laboratory assay. However, it was necessary throughout the fractionation to combine the alcohol fractions with the acetate fractions to obtain activity. Subsequent field trapping tests comparing equivalent amounts of crude natural extracts (20 FE) and pure synthetic compounds on filter paper in sticky traps revealed that a blend of 25 ng *Z*9,*E*12–14:Ac and 20 ng *Z*9–14:OH was equivalent to virgin females in capturing males. Also the two-component blend was equal to or better than any other mixture, including the 11-component blend (Tumlinson et al., 1981).

In contrast, Persoons et al. (1981) reported that trap captures of BAW males in sticky traps baited with polyethylene caps containing a blend of *Z*9,*E*12–14:Ac (0.6 mg) and *Z*9–14:Ac (0.3 mg) were equivalent to those baited with virgin BAW females. They also reported *Z*9–14:OH not to be an essential component of the BAW pheromone.

Recently Rogers and Underhill (1981) reported significant captures of BAW moths in sticky traps baited with rubber septa containing 0.1 mg *Z*9,*E*12–14:OH and 0.01 mg *Z*9,*E*12–14:Ac (ca. 75 males/replicate). The lack of a BAW colony precluded these workers from determining the performance of these baits relative to a standard trap baited with virgin females.

Since different compounds are known to be released from rubber septa at different rates (Butler and McDonough, 1981) and alcohols are released more rapidly than acetates of equivalent chain length, we tested various ratios of components in addition to blends of different components. During the summer of 1981, we conducted field tests with various blends of synthetic compounds formulated on rubber septa for entrapment of BAW males. Results of these tests are reported here.

METHODS AND MATERIALS

The bait chemicals were dispensed from rubber septa (5 × 9 mm, No. 8753-D22, A.H. Thomas Co., Philadelphia, Pennsylvania) suspended just above the sticky surface of Pherocon® 1C traps. The traps were positioned ca. 1 m above the ground and spaced ca. 30 m apart in a line perpendicular to the prevailing wind in and around fields infested with pigweed, *Amaranthus* sp. (Amaranthaceae), a favored host of the BAW. Treatments were arranged in randomized complete blocks (2 or 3/experiment). Insects were counted and

the treatments were rerandomized every 1–3 days throughout the duration of each test; each collection was considered a replicate. When more than one block of treatments from the same or different experiments were run simultaneously, the blocks were located in widely separated areas to minimize chances for trap interference. The data were converted to $\sqrt{\bar{X} + 0.5}$ for statistical analysis (ANOV, Duncan's multiple-range test).

BAW females used as bait were reared in the laboratory on artificial diet (Burton, 1969). The females were 2 days old when placed in the trap, and they were replaced every 1–2 days. The test chemicals were obtained either from commercial sources or synthesized in our laboratory. The synthetic compounds were purified by HPLC and analyzed by capillary GC on SP-2340® and OV-101® and minimum purities were established as follows: *Z*9,*E*12–14:Ac, 98.5%; *Z*9–14:OH and *Z*9–14:Ac, 99.5%; all other chemicals tested were at least 98% pure.

RESULTS

Preliminary experiments were conducted during early spring 1981 using different dosages (0.3, 1.0, and 3.0 mg/septum) of the 5:4 blend of *Z*9,*E*12–14:Ac and *Z*9–14:OH previously reported as attractive to BAW males when dispensed from filter paper (Tumlinson et al., 1981). These tests were carried out during a period of low adult BAW populations as indicated by the relatively few numbers of males captured in female-baited traps ($\bar{X} = 3.4$). Nevertheless, results of these preliminary studies indicated that the 5:4 blend

TABLE 1. CAPTURE OF *Spodoptera exigua* MALES OVER A 7-WEEK PERIOD IN STICKY TRAPS BAITED WITH RUBBER SEPTA CONTAINING DIFFERENT DOSAGES OF (*Z*,*E*)-9,12-TETRADECADIEN-1-OL ACETATE: (*Z*)-9-TETRADECEN-1-OL (50:1) (ALACHUA COUNTY, FLORIDA, 1981)

Dosage (mg)		Mean (±SE) males captured in week[a]			
*Z*9,*E*12–14:AC/*Z*9–14:OH		1	3	5	7
3	0.06	2.5 ± 1.5d	29.9 ± 5.7a	18.9 ± 3.4ab	13.6 ± 3.9a
1	0.02	6.8 ± 1.6bc	33.2 ± 4.8a	16.3 ± 2.5bc	13.1 ± 3.7a
0.3	0.006	8.4 ± 2.0bc	29.8 ± 5.9a	16.2 ± 4.2bc	9.9 ± 2.1ab
0.1	0.002	15.6 ± 3.9a	16.0 ± 3.5b	18.1 ± 4.9bc	9.0 ± 2.6b
0.03	0.0006	10.8 ± 3.2ab	10.6 ± 5.2bc	7.0 ± 2.2c	2.4 ± 1.4c
0.01	0.0002	4.3 ± 1.2dc	4.6 ± 2.6c	0.7 ± 0.4d	1.6 ± 0.7c
3 females		7.5 ± 2.6bc	15.5 ± 3.2b	29.3 ± 6.1a	6.0 ± 2.1bc

[a] Means in each column followed by the same letter are homogeneous at the 5% level, DMRT. Ten replicates/treatment/week.

was relatively unattractive to BAW males when dispensed from rubber septa. In a subsequent test, a 4:1 blend of *Z*9,*E*12,–14:Ac (0.15 mg) and *Z*9–14:OH (0.04 mg) on rubber septa was somewhat more attractive to BAW males, suggesting that the ratio of acetate to alcohol in the mixture possibly could be increased.

A study (test 1) was then initiated to determine the response of BAW males to different dosages of a 50:1 blend of *Z*9,*E*12–14:Ac and *Z*9–14OH. The test was continued over a period of 7 weeks to determine the effects of aging on the attractant mixture.

Captures of BAW males with the different bait dosages relative to females are shown in Table 1. The highest trap captures during the first week were with the 0.1-mg dosage. The 3.0-mg treatment dosage captured only ca. 15% as many BAW males as were captured in the 0.1-mg treatment. However, no statistical difference was noted between these treatments at the end of the second week (data not shown in Table 1), thus indicating that at least initially the 3-mg dosage was too high for maximum captures of BAW males. The

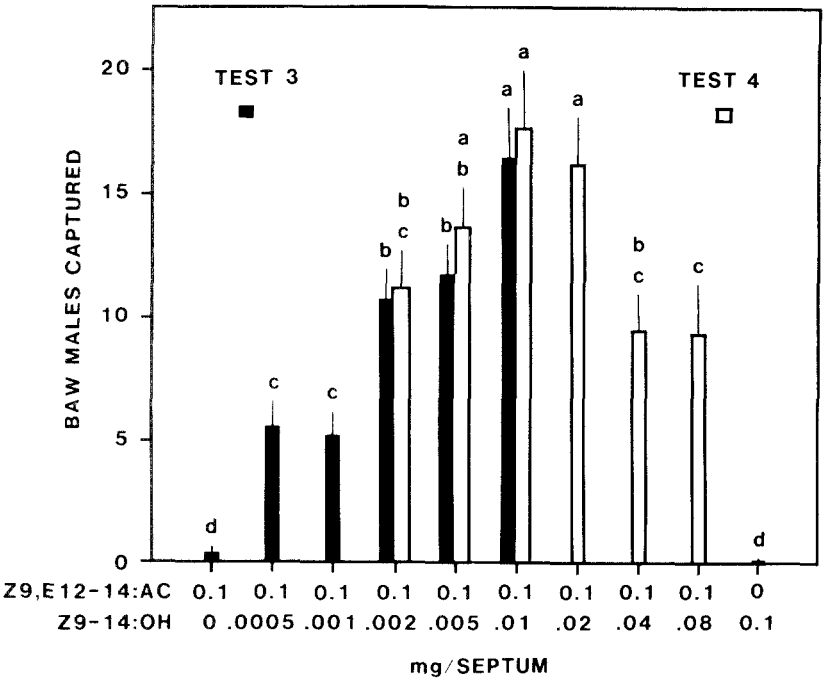

FIG. 1. Response of *Spodoptera exigua* males to sticky traps baited with rubber septa containing the sex pheromone. Narrow lines indicate standard errors of the means. Means in the same test followed by different letters differ significantly at the 5% level, DMRT.

0.1-mg dosage continued to entrap males in numbers comparable to females throughout the 7 weeks of the test. Survey traps baited with 0.1 mg of acetate and alcohol blend continued to attract significant numbers of BAW males even after 6 months in the field, although the numbers of insects captured were somewhat reduced compared to fresh septa.

The effects of isomeric purity of the alcohol component upon BAW captures was investigated by admixing *E*9–14:OH to the above mixture (0.1 mg *Z*9,*E*12–14:Ac and 0.002 mg *Z*9–14:OH) in quantities ranging from 5 to 50% of the *Z* isomer (test 2, 19 replicates). Mean catches (±SE) for the 0,5, 10, 25, and 50% levels of *E*9–14:OH were 8.4 ± 1.7, 10.6 ± 2.0, 9.2 ± 1.3, 7.2 ± 1.0, and 9.9 ± 1.8, respectively. Differences between means were not statistically different (5% level), indicating that the isomeric purity of the alcohol component was not critical to the capture of male BAW.

In another series of experiments (tests 3 and 4), various quantities of *Z*9–14:OH were admixed with 0.1 mg *Z*9,*E*12–14:Ac on rubber septa to establish the optimum ratio for maximum attractiveness to BAW males. The addition of 5–20% *Z*9–14:OH to *Z*9,*E*12–14:Ac gave the highest captures of BAW males (Figure 1). Traps baited with only *Z*9,*E*12–14:Ac or *Z*9–14:OH were virtually ineffective.

Another series of experiments (tests 5 and 6) were conducted to determine if other chemicals previously identified from hexane washes of calling BAW females (Tumlinson et al., 1981) were essential to the capture of BAW males in sticky traps. Ten of the 11 previously identified were admixed in various combinations in the approximate ratio in which they were found in the crude pheromone extract; *E*9–14:OH was not tested here because an earlier study (test 2) had shown that this compound was not essential to the capture of BAW males. Results in Table 2 show that *Z*9–14:OH was essential to entrapment of BAW but that the other compounds tested did not enhance trap captures. On the contrary, one of the components, *Z*9,*E*12–14:OH, appeared to have a suppressive effect on trap captures.

The BAW sex attractant pheromone proposed by Persoons et al. (1981)—a mixture of *Z*9,*E*12–14:Ac (0.6 mg) and *Z*9–14:AC (0.3 mg)—was formulated on rubber septa and field tested for capture of BAW males (tests 7 and 8). In these tests (Table 3), traps baited with our blend of 0.1 mg *Z*9,*E*12–14:Ac and 0.01 mg *Z*9–14:OH were superior in catches to traps baited with either virgin females or the Persoons' mixture in capturing BAW males. Female-baited traps were also more effective in capturing BAW males than traps baited with the acetate blend. These data agree closely with previous results (Table 2) which showed a significant increase in trap captures of BAW males when 14-carbon acetates were blended with *Z*9–14:OH.

Significantly more male BAW were captured in traps baited with females (Table 4, tests 9 and 10) or our most effective bait mixture (test 11, 0.1 mg

TABLE 2. CAPTURE OF *Spodoptera exigua* IN STICKY TRAPS BAITED WITH RUBBER SEPTA CONTAINING SYNTHETIC PHEROMONE COMPONENTS IDENTIFIED FROM HEXANE WASHES OF CALLING FEMALES (ALACHUA COUNTY, FLORIDA, 1981)

(*Z*,*E*)-9,12-Tetradecadien-1-ol acetate (0.1 mg) admixed with (mg)[a]									Mean no. males captured (±S.E.)[b]	
A	B	C	D	E	F	G	H	I	Week 1 (18)	Week 2 (18)
Test 5										
0.01									18.0 ± 2.8ab	25.6 ± 3.6a
0.01	0.04	0.0005	0.05	0.002	0.001	0.005	0.01	0.001	6.0 ± 1.1d	12.2 ± 3.1b
0.01	0.04								9.5 ± 1.8c	16.9 ± 4.3b
0.01		0.0005							15.2 ± 2.6b	21.1 ± 3.5a
0.01			0.05						20.5 ± 3.3a	22.6 ± 3.2a
Test 6										
0.01									19.3 ± 2.7ab	20.8 ± 4.3a
0.01		0.0005	0.05	0.002		0.005		0.001	18.3 ± 3.3b	25.9 ± 3.4a
0.01				0.002					23.8 ± 3.2a	25.8 ± 2.7a
	0.04								2.4 ± 0.6c	1.7 ± 0.3b
	0.04		0.05						2.9 ± 0.6c	1.5 ± 0.3b
		0.0005	0.05	0.002		0.005		0.001	0.9 ± 0.4c	0.8 ± 0.5b

[a] A = *Z*9-14:OH; B = *Z*9,*E*12-14:OH; C = *Z*7-14:OH; D = *Z*9-14:Ac; E = *Z*9,*Z*12-14:Ac; F = *Z*9,*Z*12-14:OH; G = 14:Ac; H = 14:OH; I = *E*9-14:Ac.

[b] Means in same test week followed by different letters differ significantly at the 5% level, DMRT. Numbers in parentheses are replicates per test per week.

TABLE 3. CAPTURE OF *Spodoptera exigua* MALES IN STICKY TRAPS BAITED WITH RUBBER SEPTA CONTAINING (*Z,E*)-9,12-TETRADECADIEN-1-OL ACETATE AND (*Z*)-9-TETRADECEN-1-OL ACETATE OR (*Z*)-9-TETRADECEN-1-OL (ALACHUA COUNTY, FLORIDA, 1981)

Dosage (mg)			Mean No. males captured (±SE)[a]
*Z*9,*E*12-14:Ac	*Z*9-14:Ac	*Z*9-14:OH	
Test 7 (6)			
0.1	0	0.01	37.7 ± 3.9a
0.6	0.3	0	0.5 ± 0.2b
	3 females		21.5 ± 3.2c
Test 8 (13)			
0.1	0	0.01	21.5 ± 4.8a
0.6	0.3	0	0.7 ± 0.3b
	3 females		6.3 ± 1.5c
	Control		0 ± b

[a] Means in the same test followed by different letters differ significantly at the 5% level, DMRT. Numbers in parentheses are replicates per treatment.

*Z*9,*E*12-14:Ac and 0.01 mg *Z*9-14:OH) than any of the traps baited with various dosages of *Z*9,*E*12-14:OH and *Z*9,*E*12-14:Ac including the three blends previously reported as being attractive to the BAW by Rogers and Underhill (1981). Furthermore, when 3 mg of *Z*9,*E*12-14:OH were admixed with a blend of *Z*9,*E*12-14:Ac (0.3 mg) and *Z*9-14:OH (0.006 mg), captures of male BAW were reduced ca. 85% relative to the mixture without *Z*9,*E*12-14:OH.

In test 12, Conrel® black hollow fibers filled with a hexane solution containing a 10:1 mixture of *Z*9,*E*12-14:Ac and *Z*9-14:OH were evaluated as an alternative dispensing system for the BAW sex pheromone. Sticky traps were baited with either 1, 5, or 10 fibers; a rubber septum containing 0.1 mg *Z*9,*E*12-14:Ac and 0.01 mg *Z*9-14:OH served as the standard. Mean captures/trap (±SE) for the treatments (9 replicates over 14 days) were: 1 fiber, 21.0 ± 4.1*; 5 fibers, 31.7 ± 4.5**; 10 fibers, 28.7 ± 3.6**; and rubber septum 36.0 ± 3.8** (means followed by the same symbol are homogeneous at the 5% level, DMRT). In another trial (test 13) a trap baited with 30 fibers captured a mean (±SE) of 26.8 ± 4.8 males compared to 20.8 ± 4.8 males captured in a control trap baited with 3 BAW females (5 replicates; difference between means not statistically different at the 5% level, Student's *t* test). The 30-fiber packet was aged in the field for 9 days prior to initiation of the 5-day test period. These results indicate that hollow fibers are an effective dispensing system for the BAW pheromone with an effective life of at least 2 weeks in the field.

TABLE 4. RESPONSE OF *Spodoptera exigua* MALES TO RUBBER SEPTA CONTAINING DIFFERENT DOSAGES OF (*Z,E*)-9,12-TETRADECADIEN-1-OL ACETATE ADMIXED WITH (*Z,E*)-9,12-TETRADECADIEN-1-OL AND/OR (*Z*)-9-TETRADECEN-1-OL (ALACHUA COUNTY, FLORIDA, 1981)

Chemical and dosage (mg)			Mean No. males captured (± SE)[a]
*Z*9,*E*12-14:Ac	*Z*9,*E*12-14:OH	*Z*9-14:OH	
Test 9			
0.01	0.1	0	1.6 ± 0.5c
0.02	0.02	0	5.7 ± 1.7b
0.002	0.02	0	0.1 ± 0.1c
	3 Females		21.9 ± 3.8a
Test 10			
3	90	0	1.1 ± 0.4cdef
3	30	0	3.1 ± 1.1cde
3	3	0	3.6 ± 1.5cd
0.3	9	0	0.8 ± 0.4def
0.3	3	0	1.9 ± 0.5cdef
0.3	0.3	0	3.8 ± 1.1c
0.03	0.9	0	0.2 ± 0.2f
0.03	0.3	0	0.3 ± 0.2f
0.03	0.03	0	0.6 ± 0.2ef
0.3	0	0.006	16.8 ± 3.5b
0.3	3	0.006	2.5 ± 1.2cdef
	3 Females		26.8 ± 7.9a
Test 11			
0.1	0.1	0	2.8 ± 1.2b
0.1	0.04	0	2.7 ± 1.3b
0.1	0.01	0	0.7 ± 0.3c
0.1	0.004	0	0.9 ± 0.3c
0.1	0.001	0	0.9 ± 0.6c
0.1	0	0.01	15.4 ± 3.3a

[a] Means in the same test followed by different letters differ significantly at the 5% level, DMRT. Fifteen replicates per treatment in each test.

DISCUSSION

The data presented here clearly demonstrate the attractant qualities of the sex pheromone previously identified for the BAW, a mixture of *Z*9,*E*12-14:AC and *Z*9-14:OH (Tumlinson et al., 1981). The optimum dosage on rubber septa for trapping BAW males was ca. 0.1 mg of the acetate to 0.01 mg of the alcohol. This bait combination gave consistently high trap catches compared to standard traps baited with virgin females. None of the other nine pheromone components identified from whole body washes enhanced trap

captures when admixed with the attractant blend. Research is underway to identify the role that each of the 11 compounds play in the sexual behavior of the BAW. It is possible that some of the compounds identified play no particular role in conspecific sexual communication but rather serve to maintain species integrity among the *Spodoptera* complex, several of which have one or more pheromone components in common (Campion, 1975).

The 10:1 attractant blend that proved so effective for BAW in the present study was considerably different from the 5:4 blend of *Z*9,*E*12–14:Ac and *Z*9–14:OH used in the original pheromone identification study. The difference in ratios may be related to the choice of dispensers used in the earlier investigation (filter paper and polyethylene vials) and the present study (rubber septa). Alcohols and acetates are known to be released at different rates from rubber septa (Butler and McDonough, 1981); hence an adjustment in the ratio of *Z*9,*E*12–14:Ac to *Z*9–14:OH in the pheromone blend was necessary to achieve the high level of male response recorded. The 10:1 ratio of acetate to alcohol also appears to be a useful combination for the hollow fiber dispenser system.

We did not confirm the attractant activity of the BAW pheromone reported by Persoons et al. (1981), a blend of *Z*9,*E*12–14:Ac (0.6 mg) and *Z*9–14:Ac (0.3 mg), using the rubber septum dispenser (they used polyethylene caps). Persoons et al. (1981) also claimed that *Z*9–14:OH is not essential to attraction of BAW males in the field, although they did note a significant level of response by BAW males in electroantennogram studies with this compound. We were unable to capture significant numbers of males without the addition of *Z*9–14:OH, thus indicating that this compound is indeed essential to attraction of BAW in Florida. It is possible that we are dealing with geographic races in the BAW similar to those demonstrated for the European corn borer, *Ostrinia nubilalis* (Hübner) (Klun and cooperators, 1975). However, this appears unlikely because the pheromone identified by Persoons et al. (1981) was made from BAW introduced into the Netherlands from Florida in 1976. On the other hand, their field tests were carried out in California. Cooperative tests on the two pheromone blends have been arranged and the outcome of these trials will be reported at a later date.

The low catches associated with the *Z*9,*E*12–14:OH and *Z*9,*E*12–14:Ac combination reported as attractive to BAW males by Rogers and Underhill (1981) was not surprising inasmuch as they had no comparative data with standard female-baited traps. During their work with *Z*9–*E*12–14:OH, the sex pheromone of the sunflower moth, *Homoeosoma electellum* (Hulst), it was discovered that mixtures of this alcohol and the corresponding acetate analog captured BAW males. The senior author (C.E.R.) has advised us that the tests were carried out during a period of high adult BAW populations. Under such conditions, it is not uncommon to record relatively high catches of nontarget

insects in traps baited with incomplete pheromone mixtures. For example, we recently captured large numbers of male granulate cutworm moths, *Feltia subterranea* (F.) (unpublished data), in pheromone traps baited with (*Z*)-9-dodecen-1-ol acetate, the sex attractant pheromone for the fall armyworm, *S. frugiperda* (J.E. Smith). It is possible that the granulate cutworm has *Z*9–12: Ac as a component of its sex pheromone, although this has not been verified. In recent years reports of interspecific sex attraction to pheromone traps baited with females have become more frequent. The chemical basis for such interspecific attraction is usually unknown, although the species involved frequently have one or more pheromone components in common. In several cases, the cross sex attraction appeared to have resulted from having fortuitously positioned the attractant source in an area heavily populated by a favored host during a period of peak emergence by the species captured.

Sex attractant pheromones are becoming increasingly used as tools for monitoring seasonal population fluctuations and timing insecticide applications for insect pests in a wide variety of cropping situations. The results reported here demonstrate that the BAW sex pheromone identified by Tumlinson et al. (1981) can now be used to monitor adult populations of this pest. However, further research is needed to relate pheromone trap catches to larval populations and plant damage levels before control strategies utilizing the BAW pheromone can be devised.

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DEPENDENCE OF NECROPHORIC RESPONSE TO OLEIC ACID ON SOCIAL CONTEXT IN THE ANT, *Pogonomyrmex badius*

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Abstract—The response of the southern harvester ant, *Pogonomyrmex badius*, to oleic acid was found to depend on social context. Social context was specified as the number of ants engaging in each of five categories of behavior. When a large percentage of the colony is doing midden work or nest maintenance, papers treated with oleic acid are taken to the midden, as previously reported. However, when a large percentage of the colony is foraging or convening, treated papers are taken into the nest as if they were food items.

Key Words—*Pogonomyrmex badius*, Hymenoptera, Formicidae, oleic acid, social context, midden, pheromone, harvester ant, necrophoric.

INTRODUCTION

This study reports upon the response of the southern harvester ant, *Pogonomyrmex badius*, to oleic acid. Wilson et al. (1958) reported that objects treated with oleic acid were "invariably" carried to the colony's refuse pile, or midden. Experiments by Haskins and Haskins (1974) suggested that oleic acid produces this necrophoric effect in the ant *Myrmecia vindex* also. Blum (1970) concluded from similar experiments that oleic acid evokes necrophoric behavior in the ant *Solenopsis saevissima*. The idea that oleic acid is the primary releaser of the necrophoric response in *P. badius* occurs elsewhere in the literature (Wilson, 1963; Howard and Tschinkel, 1976; Howard et al., 1982).

It is well known that social factors can affect the response of an animal to a chemical cue. For example, honeybee "queen substance" (9-oxodec-*trans*-

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enoic acid) attracts a retinue of workers inside the nest, but attracts only drones during mating flights outside the nest (Gary, 1970). Also, readiness to mate is a significant factor in the response of male moths to sex attractant pheromones (Jacobson, 1972).

In an effort to continue the work of Wilson et al. (1958), I examined the effect of social context on the response of *P. badius* colonies to oleic acid. By "social context," I mean the social activities of the colony at a given time. I found that colonies responded differently to oleic acid in different social contexts, carrying it to the midden only in certain situations.

METHODS AND MATERIALS

Experiments were performed in the laboratory using four colonies of *P. badius*, each containing a queen, and ranging in size from about 350 to 650 workers. The experimental procedure followed that of Wilson et al. (1958). In each trial ten pieces of filter paper, each 2 × 2 mm, were placed within 10 cm of the main nest entrance of the colony. Of the ten pieces, five were treated with 1 μl each of oleic acid (Sigma Chemical Co., 99%), while five were left untreated as controls. The treated and untreated papers were placed in the terrarium in two separate piles about 3 mm apart. In each trial, the colony was observed for one hour, and the time of removal from the original site and the destination of any removed papers were recorded. Twenty-two trials were made.

After the first six trials, all of the colony social activities observed thus far were classified into five groups (Table 1): (1) midden work, (2) feeding and drinking, (3) nest maintenance, (4) patrolling, and (5) convening. In the subsequent 16 trials, the activities of the ants were recorded at the beginning of the trial and at 5-min intervals throughout the hour. Only ants on the terrarium surface could be observed; the activities of the ants inside the nest were not considered. Colony activities were recorded by noting the number of ants engaging in each of the five categories of behavior. The five numbers together operationally specify "social context."

RESULTS

The control papers were left in place significantly longer than those treated with oleic acid (Wilcoxon's signed-ranks test, $P > 0.005$). The mean time for removal of control papers was 37.9 min, and for treated papers, 15.3 min. The controls were invariably taken to the midden. Only 45% of all treated papers offered to the colonies were taken to the midden. Forty-nine percent were taken into the nest, while 6% were left in place during the hour trial.

TABLE 1. CLASSIFICATION OF COLONY ACTIVITIES

Midden work	Feeding	Nest maintenance	Patrolling	Convening
Repiling midden	Taking hold of food bits	Carrying sand out of nest	Patrolling edges	Standing together under lamp
Carrying objects to midden	In water tube	Relocating sand on terrarium surface	Walking around quickly	Grooming each other
Carrying dead ants	Piling sand on food		Pawing at sand and inspecting it with antennae	Standing together wiping off antennae
	Taking food into nest			

The data were analyzed using multivariate analysis of variance, with the percentages of ants in all five activity types as observation variables. The data were classified into two groups according to the destination (midden or nest) of treated papers. The mean activity vector when treated papers were taken to the midden was found to differ highly significantly ($P > 0.001$) from the mean activity vector when treated papers were taken into the nest. Except in the case of patrolling, the percentages of ants in each activity when treated papers were

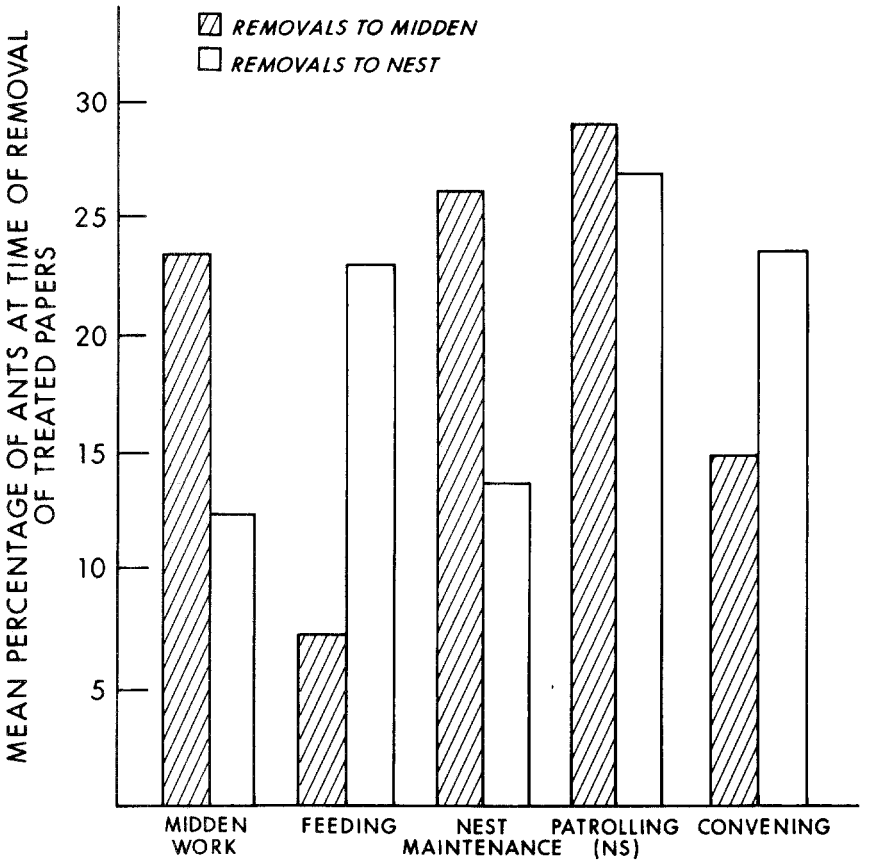

FIG. 1. The shaded bars show the distribution of ants among the five activities when treated papers were taken to the midden. The unshaded bars show the distribution of ants among the five activities when treated papers were taken to the nest. The ordinate displays the mean percentages of all ants on the terrarium surface engaged in a given activity. The percentages are averaged both over a 15-min time interval including the time of removal, and over all observations of treated papers transported to the same destination. The mean percentages represented by the shaded bars sum to approximately 100%, and similarly for the unshaded bars.

taken to the midden differed significantly ($P > 0.05$) from the percentages when treated papers were taken into the nest.

These results are shown in Figure 1. When a large percentage (>>15%) of the ants were doing midden work or nest maintenance, treated objects were usually taken to the midden. When a large percentage (>>15%) of the ants were feeding or convening, treated objects were usually taken into the nest. The percentages of ants patrolling were about the same regardless of the destination of the treated papers.

I attempted to duplicate the finding (Wilson et al., 1958) that live workers treated with oleic acid were taken directly to the midden. In 15 trials, a worker was chilled, then treated with a drop of pure oleic acid on the abdomen and replaced into the terrarium still thoroughly chilled.[1] In two of these trials, the treated worker was carried to the midden. In the remaining 13 trials, the treated ant revived, showed mild alarm behavior, was antennated by several nestmates, and then was left alone to groom herself or to go back into the nest. During the two trials in which treated workers were carried to the midden (as well as during six of the remaining 13 trials), more ants were doing midden and nest maintenance work than were feeding or convening.

DISCUSSION

As stated, colony response to oleic acid depends on social context. However, long-term patterns of social activity are different from colony to colony. As a result, some colonies took treated papers more often to the midden, while others took them more often to the nest. For example, in one colony a tunnel collapse in the terrarium caused the ants to devote much of the next six weeks to rebuilding and other nest maintenance activities. Most treated papers offered to this colony went to the midden. It is possible that Wilson et al. (1958) studied a colony in which midden or nest maintenance work was the most common activity. If so, this may account for their observation that treated objects were always taken to the midden.

The main result of this study may be explained as follows. *P. badius* colonies respond to oleic acid by quickly relocating it to destinations that are appropriate to their current activities. It is assumed that if the percentage of the colony's outside work force doing a certain activity is large, the likelihood that an ant encountering a treated paper will be doing that activity is similarly high. Thus, if most of the ants on the mound surface are engaged in midden work, an object treated with oleic acid will probably be discovered by a

[1]This procedure was used at the suggestion of Dr. E.O. Wilson, in order to duplicate as closely as possible the procedure used in Wilson et al. (1958). In nine previous trials in which workers were not chilled when treated, no treated workers were taken to the midden.

midden worker. The worker will carry the object off to the midden as if it were refuse. On the other hand, if a large percentage of the ants outside the nest are feeding or are part of a group being recruited to a food source, a forager will probably discover the treated object and carry it into the nest as a food item.

There is some evidence that the distinction between food and refuse is not as sharp as previously thought. I have observed *P. badius* taking fresh food to the midden, and have also observed both dead ants and old and seemingly undesirable bits of food being brought into the nest. Finally, oleic acid has been shown to be attractive as food to other species of ants (Marshall et al., 1979).

The reactions to oleic acid of ants doing nest maintenance work require further explanation. In general, ants engaged in nest maintenance activities were relocating nest materials such as sand to some destination outside the nest. When they encountered treated papers, they took them to the appropriate outside-of-nest location, which in this situation is the midden.

Finally, when a large percentage of the ants are convening, treated papers are taken into the nest. Convening ants mill around slowly, grooming each other and themselves. There is no obvious explanation for their reactions to oleic acid.

Division of labor in *P. badius* is not yet understood. Several alternative mechanisms might account for the behavior observed in this study. First, if temporal polyethism exists in *P. badius*, a change in an individual's response to oleic acid could accompany its change in task. Alternatively, both particular tasks and response to oleic acid may vary according to size differences among *P. badius* workers (Wilson, 1978). I am currently investigating polyethism in *P. badius*, using labeled individuals. However, in the present study, observations of colony behavior are used to arrive at a prediction about colony response to oleic acid.

The response of *P. badius* to oleic acid depends on colony activities at the time treated objects are encountered. It is clear that it can be misleading to disregard social context as a factor in research on chemical communication.

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FIELD TRAPPING OF DIAMONDBACK MOTH *Plutella xylostella* USING AN IMPROVED FOUR-COMPONENT SEX ATTRACTANT BLEND[1,2]

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Abstract—In addition to three known sex lure components [(*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecenal, and (*Z*)-11-hexadecenol], (*Z*)-9-tetradecenyl acetate was field-proven as a trace coattractant for male *Plutella xylostella*, with an optimal content below 0.01% in blends. This potent four-component lure for diamondback males also attracted *Crymodes devastator* males, and in this respect is not different in its attractancy from virgin diamondback females. Replacement of (*Z*)-9-tetradecenyl acetate in the four component lure with (*Z*)-9-tetradecen-1-ol, at the level of 10% of the total lure mixture, did not alter its attractancy for diamondback males, but it did inhibit attraction of *Crymodes devastator*. The status of biologically active components as possible sex pheromones or parapheromones is discussed.

Key Words—Diamondback moth, *Plutella xylostella*, *Crymodes devastator*, Lepidoptera, Yponomeutidae, Noctuidae, sex attractant, (*Z*)-11-hexadecenyl acetate, (*Z*)-11-hexadecen-1-ol, (*Z*)-11-hexadecenal, (*Z*)-9-tetradecenyl acetate, (*Z*)-9-tetradecen-1-ol.

INTRODUCTION

The diamondback moth, *Plutella xylostella* (L.), is a worldwide pest. In Canada it has caused economic damage to cruciferous vegetable crops in Ontario, Quebec, and British Columbia, and to rapeseed crops (*Brassica*

[1]Lepidoptera: Yponomeutidae.
[2]NRCC No. 20570.

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napus L. and *B. compestris* L.) in the prairie provinces (Putnam, 1975; Beirne, 1971). Chow et al. (1974, 1977) showed that diamondback males respond sexually to partially purified female abdominal tip extract in a laboratory bioassay, and the extract also attracted males to field traps. Tamaki et al. (1977) identified (*Z*)-11-hexadecenal (*Z* 11–16: Ald) and (*Z*)-11-hexadecenyl acetate (*Z* 11–16: Ac) in extracts of female abdominal tips. Chisholm et al. (1979) reported an electroantennogram (EAG) study and optimum conditions for field trapping of diamondback males using blends of *Z* 11–16: Ald and *Z* 11–16: Ac. They also reported that (*Z*)-9-tetradecenyl acetate (*Z* 9–14: Ac) inhibited attraction of males to chemically baited field traps. Koshihara and Yamada (1980) noted that the attractiveness of a mixture of *Z* 11–16: Ald and *Z* 11–16: Ac to diamondback males was synergized by (*Z*)-11-hexadecen-1-ol (*Z* 11–16: OH).

We report here a species-specific four-component attractant for male diamondback moths. We also discuss the status of biologically active components as possible pheromones or parapheromones.

METHODS AND MATERIALS

The chemical compounds were either synthesized in this laboratory or purchased from Chemical Samples Company, Columbus, Ohio, and purified by procedures previously described (Steck et al., 1980). Purity of *Z* 11–16: OH, *Z* 11–16: Ac, and *Z* 11–16: Ald was better than 99.7%; *Z* 9–14: OH, *Z* 11–14: OH, *Z* 9–14: Ac, and other chemicals used was $> 97\%$, and the *Z* 9–14: OH was free of *Z* 9–14: Ac at the detection limit of 0.005%.

Pherocon 1-CP traps (Zoecon Corp., Palo Alto, California) were baited with lures prepared by adding appropriate concentrations of chemical in hexane solution to red rubber spectra (Arthur H. Thomas 8753-D22). Trapping was carried out in rapeseed plots at an experimental farm near Saskatoon, Canada. The traps, attached to wood stakes, were randomized and set 12 m apart in a straight line along the edge and near the canopy of the blooming rapeseed plants. Sticky trap bottoms were changed daily and recorded catches were summed for each trap position. Replicated data were transformed ($\sqrt{x + 1}$) and then analyzed for rank order of treatment effect using Duncan's new multiple-range test.

RESULTS

A basic bait composed of *Z* 11–16: Ald (70 μg) + *Z* 11–16: Ac (30 μg) attracted diamondback male moths to field traps, and the addition of *Z* 9–14: Ac (50 μg) to the bait reduced captures (Chisholm et al., 1979).

Subsequently, many structural analogs of the basic bait components were tested as possible coattractants. Summed duplicate trap captures were used to measure the effect of adding 1% and 10% of each analog to the basic bait. Some preliminary field trials (1979–1980) suggested Z 11–16:OH and Z 11–14:OH at both levels of addition increased male captures. In additional replicated field tests, adding 1% Z 11–16:OH to the basic bait gave significantly more captures than either 0.1% or 10%, in accord with Koshihara and Yamada (1980). When Z 11–14:OH was retested at the 0.1, 1.0, and 10% levels in the basic bait, the 1% addition captured the most moths. It is known that some compounds present in greater than optimal ratios in lures frequently inhibit male attraction (Roelofs, 1978), but at lower concentrations many have been found to function as trace coattractants (Steck et al., 1980). However, Z 9–14:Ac, a known inhibitor at high ratios in the basic bait, was not observed to function as a trace coattractant when retested as a third component (0.5% and 0.05% levels) in the basic bait.

In 1980 preliminary field tests of fourth components were carried out near the end of the flight period. The fourth component was added to the basic bait combined with 1% of either Z 11–14:OH or Z 11–16:OH. At 1% and 10% levels Z 9–14:OH increased male diamondback captures, while Z 9–14:Ac decreased captures when combined with the basic bait and Z 11–16:OH. When Z 9–14:OH or Z 9–14:Ac was tested with the basic bait + Z 11–14:OH, a less pronounced but similar moth capture trend was apparent. Z 11–14:OH as a fourth component with the basic bait + Z 11–16:OH had no effect.

TABLE 1. EFFECT OF ADDITION OF Z 11–14:OH OR Z 11–16:OH AND DIFFERENT AMOUNTS OF Z 9–14:AC ON CAPTURES OF *P. xylostella* MALE MOTHS WHEN ADDED TO BASIC BAIT: Z 11–16:ALD(70μg) + Z 11–16:Ac(30μg)

Bait (μg)	Total males captured[a]
Basic	60f
Basic + Z 11–14:OH(1)	282b
Basic + Z 11–14:OH(1) + Z 9–14:Ac(0.1)	152cde
Basic + Z 11–14:OH(1) + Z 9–14:Ac(0.02)	234bc
Basic + Z 11–14:OH(1) + Z 9–14:Ac(0.01)	172cde
Basic + Z 11–16:OH(1)	145cde
Basic + Z 11–16:OH(1) + Z 9–14:Ac(0.1)	146cde
Basic + Z 11–16:OH(1) + Z 9–14:Ac(0.02)	200bcd
Basic + Z 11–16:OH(1) + Z 9–14:Ac(0.01)	421a
Basic + Z 9–14:Ac(0.02)	69f

[a] July 6, 1981. 3× replicated: numbers followed by the same letter are not significantly different ($P = 0.05$).

The results obtained in the 1981 field trials corroborated and extended these preliminary findings (Table 1). Addition of either Z11-14:OH or Z11-16:OH at 1% of the basic lure increased significantly the number of diamondback males captured. Although the three-component lure containing Z11-14:OH attracted more males than did the one with Z11-16:OH, this effect was reversed with the addition of trace amounts of Z9-14:Ac. The four-component bait composed of Z11-16:Ald (70 μg) + Z11-16:Ac (30 μg) + Z11-16:OH (1 μg) + Z9-14:Ac (0.01 μg) captured the greatest number of diamondback males. Another interesting result was that the addition of the same level of Z9-14:Ac to the basic two-component lure resulted in no potentiation of moth captures, a result which clearly establishes a hierarchical order of lure components; Z11-16:OH must be present in the basic bait in order for Z9-14:Ac to elicit a positive response from male diamondback moths.

Table 2 shows that increasing the ratio of Z9-14:Ac in a bait composed of Z11-16:Ald (70 μg) + Z11-16:Ac (30 μg) + Z11-16:OH (1 μg) inhibits capture of male diamondback moths. While the total capture of diamondback males with the basic bait + Z11-16:OH (1 μg) + Z9-14:Ac (0.1 μg) is higher in Table 2 than male capture by the same bait in Table 1, the capture rate by the basic bait alone in Table 2 is also proportionally higher.

Table 3 compares the three-component lure with Z9-14:Ac (0.01 μg) added, to the same lure with increasing amounts of Z9-14:OH. The relative inhibitory effect of Z9-14:OH on *P. xylostella* and *Crymodes devastator* (Brace), a sympatric noctuid species attracted by diamondback female moths (Chisholm *et al*, 1979), is evident. Addition of 1% Z9-14:OH did not reduce captures of either species significantly, but at 10% *C. devastator* was excluded, although the number of diamondback males captured was not significantly altered. At 100% addition the diamondback capture was reduced significantly.

TABLE 2. EFFECT OF ADDING Z11-16:OH AND VARIOUS AMOUNTS OF Z9-14:AC TO BASIC BAIT: Z11-16:ALD(70 μg) + Z11-16:AC(30 μg)

Bait (μg)	Total males captured[a]
Basic	137b
Basic + Z11-16:OH(1) + Z9-14:Ac(0.1)	393a
Basic + Z11-16:OH(1) + Z9-14:Ac(1)	132b
Basic + Z11-16:OH(1) + Z9-14:Ac(10)	37c

[a]June 29-July 2, 1981. 3× replicated: numbers followed by the same letter are not significantly different ($P = 0.05$).

TABLE 3. RELATIVE CAPTURES OF *P. xylostella* AND *C. devastator* MALE MOTHS WHEN FOURTH COMPONENT IS ADDED TO *Z* 11-16:ALD(70 μg) + *Z* 11-16:AC(30 μg) + *Z* 11-16:OH(1 μg)

Component added (μg)	Total males captured[a]	
	P. xylostella	*C. devastator*
Z 9-14:Ac(0.01)	484a	38y
Z 9-14:OH(1)	438a	24y
Z 9-14:OH(10)	398a	1z
Z 9-14:OH(100)	125b	0z

[a] July 28–August 4, 1981. 3× replicated: numbers followed by the same letter are not significantly different ($P = 0.05$).

DISCUSSION

The hierarchical order of action of the trace bait components (Table 1) suggests that *Z* 11-16:OH and *Z* 9-14:Ac might be part of the diamondback moth's pheromone system. *Z* 11-14:OH is possibly a parapheromone, as *Z* 9-14:Ac is active in combination with *Z* 11-16:OH but not with *Z* 11-14:OH. Also, *Z* 9-14:Ac elicits a mating behavior response from diamondback males in laboratory bioassay (Chow et al., 1974), and its EAG antennal response with significant (Chisholm et al., 1979). Tamaki, et al. (1977) isolated *Z* 11-16:Ald and *Z* 11-16:Ac from female abdominal tip extracts; however, trace quantities of *Z* 11-16:OH and *Z* 9-14:Ac could have been present in amounts below their level of detection. Also the trapping ratio of diamondback to *C. devastator* by four-component synthetic blends containing either *Z* 9-14:Ac (0.01 μg) or *Z* 9-14:OH (1 μg) (Table 3) are very near the capture ratio for these two species by live diamondback female moths (Chisholm et al., 1979).

It is noteworthy (Table 3) that blends containing *Z* 9-14:Ac at the 0.01% level or *Z* 9-14:OH at the 1 or 10% levels are equally attractive to diamondback males, while *C. devastator* is excluded from traps by 10% *Z* 9-14:OH. The presence of 1% or more *Z* 9-14:Ac (Table 2) reduced diamondback captures significantly. A possible explanation is that a portion of the relatively larger amounts of *Z* 9-14:OH may be undergoing acetylation on the antennae of the diamondback males. It is known that (*Z*)-7-dodecenyl acetate (*Z* 7-12:Ac) bound to the sensillae of the antennae of *Trichoplusia ni* (Hübner) is degraded to the free alcohol (Rockstein, 1978). Therefore, the reverse reaction, biological acetylation, may be possible.

This report also shows that a synthetic bait—in this case composed of Z11-16:Ald (70 μg) + Z11-16:Ac (30 μg) + Z11-16:OH (1 μg) + Z9-14:OH (10 μg)—can be more specific than the natural pheromone. We recommend the foregoing blend as a bait for monitoring diamondback moth populations.

The hierarchical action in a multicomponent attractant has been recognized in at least one other insect species, *Euxoa ochrogaster* (Guenee) (Steck et al., 1982); it is also apparent for the same species in the data published by Struble (1981).

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INFLUENCE OF DITERPENE RESIN ACIDS ON FEEDING AND GROWTH OF LARCH SAWFLY, *Pristiphora erichsonii* (HARTIG)[1]

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Abstract—Diterpene resin acids significantly affect consumption rates, feeding efficiencies, and growth rates of the larch sawfly, *Pristiphora erichsonii* (Hartig) when topically applied to their natural food, tamarack *Larix laricina* (DuRoi) K. Koch. Abietic acid, neoabietic acid, dehydroabietic acid, and isopimaric acid significantly reduced consumption rates, feeding efficiencies, and growth rates. Sandaracopimaric acid reduced growth and efficiency but did not influence consumption rate. Two-way analysis of variance indicates a significant interaction between chemical and concentration for growth rate, feeding efficiency, and consumption rate. This interaction indicates that increasing chemical concentrations do not influence the larch sawfly in a uniform manner, supporting the concept of concentration-dependent biological activity of allelochemics.

Key Words—Larch sawfly, *Pristiphora erichsonii* (Hartig), Hymenoptera, Tenthredinidae, feeding behavior, nutritional indices, abietic acid, dehydroabietic acid, neoabietic acid, isopimaric acid, sandaracopimaric acid, resin acids.

INTRODUCTION

The importance of chemical deterrency in host–herbivore interactions is widely accepted (Beck, 1965; Waiss et al., 1977; Rosenthal and Janzen, 1979;

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Maxwell, 1977). Terpenoids are one of a wide variety of secondary plant chemicals known to influence host plant utilization by insects. Terpenoids are basically of three types: monoterpenes, sesquiterpenes, and diterpenes. The role of monoterpenes in insect attraction and host plant resistance have been discussed in several reviews (Hanover, 1975; Smith, 1972; Callaham, 1966). Sesquiterpenes also have been implicated as allelochemics (Mabry et al., 1977; Waiss et al., 1977). Gossypol is a widely recognized sesquiterpene that is an important resistance mechanism in cotton. Of the three terpene types, diterpenes have been investigated the least, and their role is poorly understood.

Research on the mechanisms by which diterpenes confer resistance in plants has followed two main lines of investigation: feeding inhibition and interference with metabolic processes essential to insect growth. Investigating the feeding behavior of certain *Neodiprion* sawfly larvae, All and Benjamin (1975a,b) discovered that the monophagous species *Neodiprion swainei* Midd. and *N. rugifrons* Midd. did not feed on juvenile foliage and were inhibited by antifeedants present in juvenile foliage of their host jack pine, *Pinus banksiana* Lamb. Ikeda et al. (1977) identified the important component as a diterpene resin acid 13-keto-8(14)-podocarpen-18-oic acid. Ohigashi et al. (1981) identified several diterpene resin acids as antifeedants for the larch sawfly *Pristiphora erichsonii*. Wagner and Benjamin (1981) implicated abietic acid as a growth retardant chemical for *P. erichsonii*. This paper reports on the growth-retardant and feeding-inhibitory characteristics of several highly pure resin acids.

METHODS AND MATERIALS

Diterpene resin acids used in this study were obtained from B.C. Research, Vancouver, Canada. Percent purity range was dehydroabietic acid, 99+; abietic, 90–95; neoabietic, 99+; isopimaric, 99+; sandaracopimaric, 85–90.

Methods for handling larch sawfly larvae and calculating nutritional indices have been described previously (Wagner and Benjamin, 1981). Nutritional indices utilized included: relative consumptive rate (RCR), relative growth rate (RGR), and efficiency of conversion of ingested food to body substance (ECI) (Waldbauer, 1968).

Resin acids were dissolved in methanol and applied to the natural host food, i.e., needle tufts, by pipetting. The solvent was allowed to evaporate and recently molted fifth instar larvae were placed individually on test foliage. Experiments were conducted at 22° C under continuous light for 72 hr. Controls consisted of foliage handled in the same manner except treated with only the solvent. Each experiment was replicated 15 times, and analysis of

data was conducted on ten randomly selected subsamples of larvae successfully feeding for the duration of the experiment. Because of the use of natural host food, precise percent resin acid could not be calculated. However, estimates indicate the 5 mg/ml concentration used in the experiments is roughly equivalent to one percent resin acid by weight, and extrapolation from this estimate gives rough percent resin acid figures of 4, 2, and 0.5 for the mg/ml concentrations 20, 10, and 2.5, respectively.

Data from each resin acid test were analyzed using a one-way analysis of variance with Duncan's New Multiple-Range Test (significance level 0.05). Two-way analysis of variance with Duncan's New Multiple Range Test (significance level 0.05) was used to test for interaction between chemicals and concentration.

RESULTS AND DISCUSSION

Abietic acid significantly reduced RCR, ECI, and RGR at 20 mg/ml (Table 1). This activity, however, did not follow a typical dosage–response curve (i.e., increasing concentrations increase the response) in that low levels (2.5 mg/ml) actually enhanced RCR. Growth was adversely affected at each concentration, although neither ECI or RGR exhibited a typical dosage–response curve.

Dehydroabietic acid at 20 mg/ml adversely affected growth, but this was primarily as a result of a significant reduction in efficiency rather than a reduction in consumption (Table 2). Significant effects on efficiency and growth were evident at the highest concentration. The only significant reduction in consumption occurred at the 10 mg/ml concentration.

Isopimaric acid was similar to dehydroabietic acid in that the only significant reduction in consumption occurred at the 10 mg/ml concentration

TABLE 1. INFLUENCE OF ABIETIC ACID ON CONSUMPTION, EFFICIENCY, AND GROWTH OF *Pristiphora erichsonii*

Concentration (mg/ml)	RCR[a]	ECI	RGR
20	2.10a	13.36ab	0.28a
10	2.24ab	15.22bc	0.32b
5	2.36ab	14.82bc	0.35b
2.5	2.94c	12.07a	0.35b
Control	2.54b	16.00c	0.40c

[a] Values followed by the same letter are not significantly different using Duncan's New Multiple-Range Test at the 0.05 level. Designations apply for all following tables.

TABLE 2. INFLUENCE OF DEHYDROABIETIC ACID ON CONSUMPTION, EFFICIENCY, AND GROWTH OF *Pristiphora erichsonii*

Concentration (mg/ml)	RCR	ECI	RGR
20	2.48a	10.79a	0.26a
10	2.10b	17.43c	0.36b
5	2.61a	15.12b	0.39b
Control	2.54a	16.00bc	0.40b

TABLE 3. INFLUENCE OF ISOPIMARIC ACID ON CONSUMPTION, EFFICIENCY, AND GROWTH OF *Pristiphora erichsonii*

Concentration (mg/ml)	RCR	ECI	RGR
20	2.30b	11.74a	0.26a
10	1.84a	17.23b	0.31b
5	2.90c	12.78a	0.36c
Control	2.54bc	16.00b	0.40d

TABLE 4. INFLUENCE OF SANDARACOPIMARIC ACID ON CONSUMPTION, EFFICIENCY, AND GROWTH OF *Pristiphora erichsonii*

Concentration (mg/ml)	RCR	ECI	RGR
20	2.54a	13.12a	0.33a
10	2.47a	13.28a	0.33a
5	2.80a	12.65a	0.35a
Control	2.54a	16.00b	0.40b

(Table 3). Efficiency was equally reduced at the 5 mg/ml and 20 mg/ml concentrations. Only growth followed a typical dosage–response curve.

Sandaracopimaric acid had no significant effect on consumption, and, although efficiency and growth were reduced at all concentrations, there was no dosage response (Table 4).

Neoabietic acid influence was similar to that of abietic acid in that low levels actually stimulated feeding, but at high concentrations the primary effect was a reduction in feeding efficiency (Table 5). Again, the typical dosage–response curve was not exhibited.

The resin acids tested significantly reduced larval growth, but with the one exception of isopimaric acid, the growth reductions did not exhibit a typical dosage response. The apparent reduction in larval growth suggests that the main mechanism of action is an interference with metabolic pathways. It has been suggested by Elliger et al. (1976) that diterpene resin acids may in some way interfere with steroid metabolism. Three of the resin acids tested (dehydroabietic acid, abietic acid, and isopimaric acid) have some feeding-inhibitory action in addition to reducing growth.

Two-way analysis of variance for RCR (Table 6), ECI (Table 7), and RGR (Table 8) indicated a significant interaction between resin acids tested and concentration. This interaction supports the previously stated conclusion that increasing concentrations do not influence larch sawfly consumption, efficiency, and growth in a uniform manner, and it supports the concept of concentration-dependent biological activity of allelochemics.

The potential usefulness of diterpenes in insect control is far-reaching. Diterpene resin acids are under strong genetic control, which allows for the selection of genotypes that have combinations and concentrations of these materials suited to specific protection needs. We might speculate that the use of digestibility-reducing compounds such as resin acids as a mechanism of plant resistance may be superior to the use of toxic compounds because of lower selection pressure for insect resistance. More research is needed to

TABLE 5. INFLUENCE OF NEOABIETIC ACID ON CONSUMPTION, EFFICIENCY, AND GROWTH OF *Pristiphora erichsonii*

Concentration (mg/ml)	RCR	ECI	RGR
20	2.59a	13.16a	0.34a
10	2.77a	15.29b	0.42b
5	3.17b	13.81a	0.43b
Control	2.54a	16.00b	0.40b

TABLE 6. TWO-WAY ANALYSIS OF VARIANCE FOR RELATIVE CONSUMPTIVE RATES (RCR) OF LARCH SAWFLY FED LARCH FOLIAGE TREATED WITH DITERPENE RESIN ACIDS

Concentration (mg/ml)	Abietic	Dehydroabietic	Isopimaric	Sandaracopimaric	Neoabietic	$\bar{X}$
20	2.10ab	2.48bcde	2.30bc	2.54cde	2.59cde	2.40
10	2.24abc	2.10ab	1.84a	2.47bcde	2.77def	2.28
5	2.36bcd	2.61cde	2.90ef	2.80ef	3.17f	2.77
$\bar{X}$	2.23	2.40	2.35	2.60	2.84	2.48

Analysis of variance

Source	*df*	SS	MS	*F*	Significance level
Treatments	14	16.779	1.199	6.823	0.01
Chemicals	4	7.007	1.752	9.973	0.01
Concentration	2	6.391	3.195	18.198	0.01
Chem × Conc.	8	3.380	0.422	2.406	0.05
Error	135	23.712	0.176		
Total	149	40.491			

TABLE 7. TWO-WAY ANALYSIS OF VARIANCE FOR EFFICIENCY OF CONVERSION OF INGESTED FOOD (ECI) OF LARCH SAWFLY FED LARCH FOLIAGE TREATED WITH DITERPENE RESIN ACIDS

Concentration (mg/ml)	Abietic	Dehydroabietic	Isopimaric	Sandaracopimaric	Neoabietic	$\bar{X}$
20	13.36bcd	10.79a	11.74ab	13.12bcd	13.16bcd	12.43
10	15.22de	17.43f	17.23ef	13.28bcd	15.29de	15.69
5	14.81cd	15.12d	12.79abc	12.65abc	13.81bcd	13.84
$\bar{X}$	14.47	14.45	13.92	13.02	14.09	13.99

Analysis of variance

Source	*df*	SS	MS	*F*	Significance level
Treatments	14	484.266	34.590	7.241	0.01
Chemicals	4	41.934	10.483	2.194	0.07
Concentration	2	266.803	133.402	27.926	0.01
Chem. × Conc.	8	175.529	21.941	4.593	0.01
Error	135	644.929	4.777		
Total	149	1129.195			

TABLE 8. TWO-WAY ANALYSIS OF VARIANCE FOR RELATIVE GROWTH RATE (RGR) OF LARCH SAWFLY FED LARCH FOLIAGE TREATED WITH DITERPENE RESIN ACIDS

Concentration (mg/ml)	Abietic	Dehydroabietic	Isopimaric	Sandaracopimaric	Neoabietic	$\bar{X}$
20	0.28ab	0.26a	0.26a	0.33c	0.34c	0.29
10	0.32c	0.37cd	0.31b	0.33c	0.42ef	0.35
5	0.35cd	0.39de	0.36cd	0.35cd	0.43f	0.38
$\bar{X}$	0.32	0.34	0.31	0.33	0.40	0.34

Analysis of variance

Source	*df*	SS	MS	*F*	Significance level
Treatments	14	0.364	0.026	13.40	0.01
Chemical	4	0.140	0.035	18.07	0.01
Concentration	2	0.179	0.089	44.67	0.01
Chem. × Conc.	8	0.045	0.006	2.83	0.01
Error	135	0.262	0.002		
Total	149	0.626			

assess the extent to which diterpene resin acids exist in economic plants and how they interact with important herbivores on those plants.

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SEX-SPECIFIC RESPONSES TO AGGREGATION PHEROMONE: Regulation of Colonization Density in the Bark Beetle *Ips paraconfusus*

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Abstract—About equal numbers of each sex of flying *Ips paraconfusus* Lanier (Coleoptera: Scolytidae) were caught on traps several meters downwind from a male-infested ponderosa pine log releasing pheromone while a significantly different ratio of over four times more females than males were caught at the pheromone source. Females oriented directly to higher concentrations of colonizing males in a felled tree while males tended to land on the host in adjacent uncolonized areas. The attraction response of walking males to a 1 : 1 : 1 mixture of the synthetic pheromone components ispenol–ipsdienol–*cis*–verbenol was reduced progressively at higher concentrations while female response continued to increase. These responses may function to regulate density of colonization and limit intraspecific competition.

Key Words—Coleoptera, Scolytidae, *Ips paraconfusus*, bark beetle, *Pinus ponderosa*, ipsenol, ipsdienol, *cis*-verbenol, pheromone, attractant, intraspecific competition.

INTRODUCTION

Most bark beetle species studied, including *Ips paraconfusus* Lanier (Coleoptera: Scolytidae), use pheromones to attract individuals of both sexes to a suitable host tree for breeding (Borden, 1977). Each species probably has evolved behavioral and physiological mechanisms which attenuate aggregation that would otherwise result in overcolonization and severe competition

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for food and space. In the bark beetle, *Dendroctonus brevicomis* (Renwick and Vité, 1970; Bedard et al., 1980a), *D. pseudotsugae* (Rudinsky et al., 1972, 1974), and *Trypodendron lineatum* (Nijholt, 1973) inhibitory pheromones may assist in regulating the density of colonization and/or termination of aggregation on host materials. The reduction or inhibition of attraction response in these beetles either affects both sexes or may be limited to one, such as *T. lineatum* in which effects on females have not been determined. One of the intraspecific inhibitors of attraction response in *D. brevicomis*, verbenone, also decreases response of its competitor, *I. paraconfusus*, to its conspecific pheromone (Byers and Wood, 1980). Attractive pheromone components of certain *Ips* species may, in addition, function to inhibit response of competing bark beetle species to their conspecific pheromone (Birch and Wood, 1975; Birch et al., 1980; Byers and Wood, 1981).

Male *I. paraconfusus* begin colonizing ponderosa pine, *Pinus ponderosa* Laws., by excavating a "nuptial chamber" under the bark cortex primarily in the phloem layer. Two attractive pheromone components, ipsenol and ipsdienol (Silverstein et al., 1966), are synthesized only in males from the host terpene, myrcene (Hughes, 1974; Byers et al., 1979; Hendry et al., 1980). A third pheromone component, *cis*-verbenol, is synthesized from another host compound, α-pinene, in both sexes (Renwick et al., 1976; Byers, 1981a). The three components together are attractive to both males and females in the field (Wood et al., 1968). *I. paraconfusus* males appear to regulate their attack density since Struble and Hall (1955) reported a maximum of 374 attacks/m^2 with the majority of samples ranging from 121 to 186/m^2. The density of females infesting pine is proportional to the density of males which allow entry of up to five females (usually three) into the nuptial chamber. Subsequently, others seeking entry are forcefully rejected (Barr, 1969). A recent study has indicated that the attraction of male *I. paraconfusus* to logs infested with males was inhibited by volatiles from an additional log infested with mated males and females (Byers, 1981b). This study implicated an olfactory mechanism that may regulate the attack density. The present study reports differences between the sexes in their upwind flight to a male-infested log and in their attraction to a concentration series of pheromone components. In addition, differences between the sexes in landing patterns on a felled tree under colonization reveal a mechanism of male inhibition by high levels of pheromone, which appears to regulate colonization density and intraspecific competition.

METHODS AND MATERIALS

Upwind Flight Response to Pheromone. The pattern of flying beetles of each sex orienting to pheromone released by 50 males boring in a ponderosa

pine log was determined by intercepting their flight with a grid of sticky-traps placed downwind from the infested log (September 20–28, 1975). The grid of traps (Figure 1) was located in a narrow valley in the Sierra National Forest (Miami Creek drainage at 1200 m elevation, Mariposa County, California). Observations of a wind-vane placed in various positions in the grid indicated a consistent wind direction perpendicular to the trap rows throughout the flight period (time of catch from about 0800 to 2000) probably due to solar heating at lower elevations forcing air upslope in a laminar flow.

The male-infested log was screened and prepared as described earlier (Byers and Wood, 1980). It was placed inside a tubular sticky-trap (19 cm diam × 30.5 cm high) made of 6.3-mm mesh screen coated with Stickem Special® and 1.2 m above the ground (Bedard and Browne, 1969). Each row of traps had a nylon line perpendicular to the wind direction which suspended 15 flat sticky-trap screens (each 30.5 × 61 cm high) coated with Stickem Special and spaced at 1.5 m intervals 1.2 m above ground. The sticky-traps were

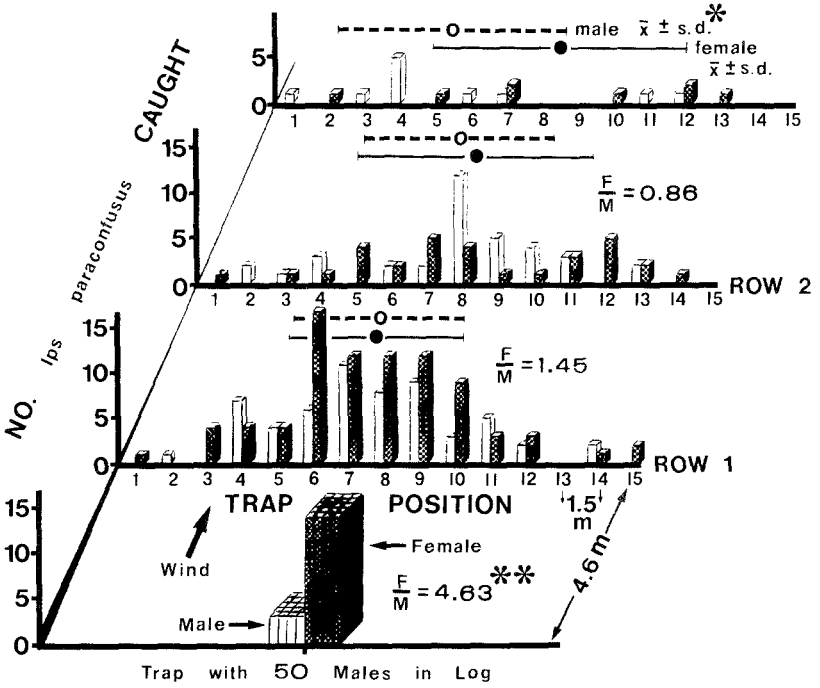

FIG. 1. Distribution of male and female *Ips paraconfusus* caught on sticky-traps spaced in a grid downwind from a log infested with 50 males in the Sierra National Forest, California (September 20–28, 1975). *Mean of distribution of catch ± standard deviation. **Female/male sex ratio was significantly different from sex ratios in either row 1 or 2 ($P < 0.001$, chi-square).

monitored for beetles two days prior to introducing the infested log and six days after its removal. The "weighted" mean distances of catch from the pheromone source for each row and sex were calculated from numbers caught on traps 3–13 (and their respective distances) in each row.

Walking Response to Synthetic Pheromone Components. Beetles used for tests in a laboratory olfactometer (Byers et al., 1979, modified from Browne et al., 1974) were collected from naturally infested pine logs obtained from the same area described above. Walking beetles of each sex were tested for their attraction response to a series of increasing concentrations of a 1 : 1 : 1 mixture of ipsenol–ipsdienol–*cis*–verbenol (racemic chemicals >97% pure, Chemical Samples Co.). Concentrations of each compound ranged in decimal steps from 10^{-11} to 10^{-6} g/μl diethyl ether.

Landing on Trunk of Felled Pine during Colonization. Numbers of male and female *I. paraconfusus* caught on sticky-traps along the trunk of a felled pine were analyzed to determine whether behavioral differences between the sexes existed under conditions of actual colonization. An apparently healthy ponderosa pine tree (0.36 m diam at 1.5 m height) was felled at 08:00 on October 1, 1978, and all branches were removed except those above the 16.3 m height (trap 1). Eighteen sticky-trap screens, 21 × 21 cm, were placed horizontally on the upper side of the felled tree at intervals of 0.76 m beginning 3.6 m from the "top" (0.1 m diam) and extending along the trunk to 3.3 m from the base. An aggregation of *I. paraconfusus* near the top of the tree was induced by baiting with a log that had been infested two days earlier with 50 males. The male-infested log was removed 24 hr later. The portion of the tree that was under attack, as indicated by boring dust piles, was noted each day.

The slopes of linear regression of male versus female catch on the 18 traps for successive two-day periods (October 1–8, 1978) were compared to corresponding 95% lower binomial confidence limits (LBCL) of sex ratios of catch (Byers and Wood, 1980) with a *t* test method (Mendenhall, 1967). The total male and female catch and the percentage of males in the catch per day during the mass aggregation period were summarized.

RESULTS

Upwind Flight Response to Pheromone. The distribution of catch of *I. paraconfusus* on the grid of traps appeared as a narrowing pattern focusing to the pheromone source (Figure 1). This pattern is to be expected if beetles are flying upwind toward the source of pheromone. The broad overlaps of the standard deviations of the sexes caught within each row of traps (Figure 1) indicated that both sexes initially directed their flight upwind toward the pheromone source. The increase in catch on trap rows closer to the source was probably a function of an increase in the proportion of the cross-sectional area

of the pheromone "odor plume" that was intercepted by trap area. About equal numbers of male and female beetles appeared to enter the grid area since catches of each sex on rows 3 and 2 were almost identical. However, proportionately fewer males than females were attracted upwind to the source, as the sex ratio caught there was significantly less than on row 1 (Figures 1 and 2), and row 1 was significantly less than rows 2 and 3 ($P < 0.05$). No beetles were caught after removing the infested log.

Walking Response to Synthetic Pheromone Components. Male and female response was similar at lower release rates (10^{-11}–10^{-9} g/μl) of ipsenol–*cis*-verbenol–ipsdienol. However, attraction of males, but not females, was inhibited at higher release rates (10^{-8}–10^{-6} g/μl, Figure 3). The actual release rate (g/min) was estimated to be about 2.2 times the g/μl concentration, which assumes that the compounds were released in proportion to the volume reduction of solvent (Byers and Wood, 1981). These results indicated that response of males increased with an increase in pheromone concentration to a threshold above which a reduction in attraction occurred. On the other hand,

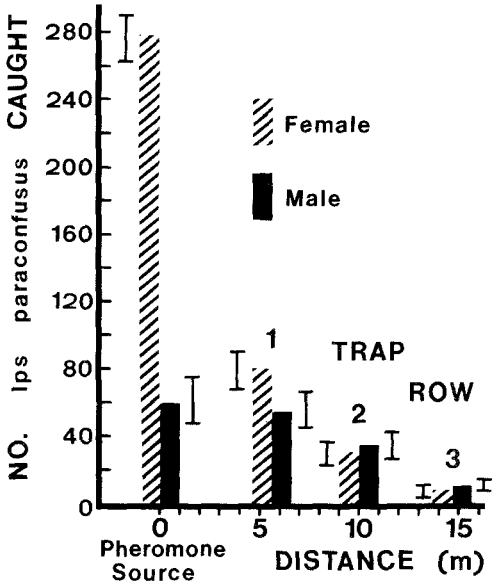

FIG. 2. Total number of male and female *Ips paraconfusus* caught at the log infested with 50 males and on sticky-traps (nos. 3–13) in rows of increasing distance downwind from the pheromone source, Sierra National Forest, California (September 20–28, 1975). The distance from the pheromone source for each row is based on the average trap distance weighted by the catch. Brackets represent 95% asymetric binomial confidence limits.

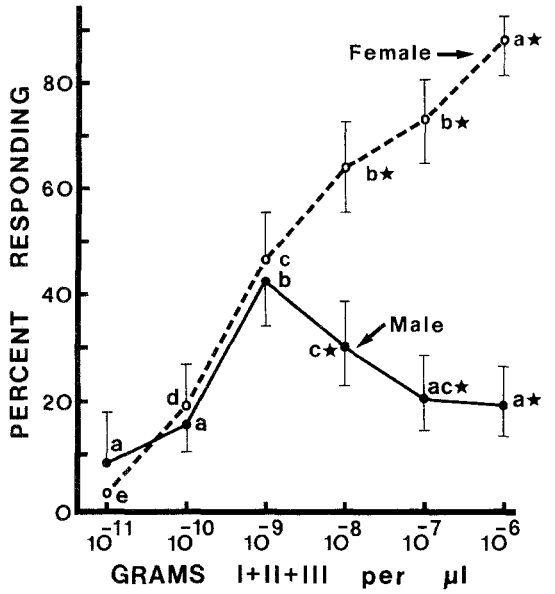

FIG. 3. Response of male and female *Ips paraconfusus* to a 1:1:1 mixture (separate weights) of the pheromone components ipsenol (I), *cis*-verbenol (II), and ipsdienol (III) in the laboratory olfactometer. Means compared within each sex between concentrations followed by the same letter were not significantly different at $\alpha = 0.05$, and means compared at the same concentration between sexes followed by star symbols were significantly different at $\alpha = 0.01$ (chi-square). Brackets represent 95% binomial confidence limits, $N = 90$–120.

the attraction of females continued to increase in proportion to the logarithm of the concentration of pheromone components.

Landing on Trunk of Felled Pine during Colonization. Traps progressively further from the origin of colonization at the "top" of the felled tree caught increasingly higher proportions of males on the first, second, and third days of aggregation (Figure 4). In order to observe these trends in sex ratio it was necessary to combine the catches of each three successive traps for a total of six groups. Numerous piles of boring dust were observed only at the "top" of the tree beginning on the second day. On the third day piles appeared further along the tree corresponding to the highest catches. By the fourth day, the entire surface that was covered with traps appeared to be relatively uniformly attacked (about 110–150 attacks/m^2) and no trends in sex ratio then were observed (Figure 4).

Males responded differently from females to areas of colonization corresponding to the 18 trap locations on all four two-day periods since the slopes of the regression lines of male against female catch were significantly

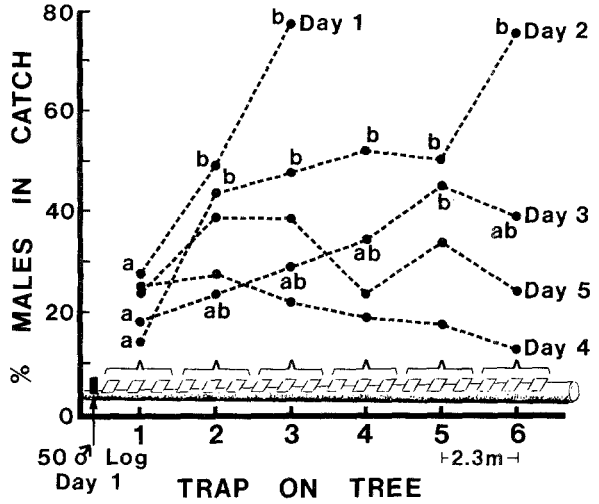

FIG. 4. Trends in the proportion of male *Ips paraconfusus* caught on sticky-traps distributed distally from the origin of colonization on the trunk of a felled ponderosa pine in the Sierra National Forest, California (October 1–5, 1978). An aggregation of *I. paraconfusus* near the top of the tree was induced by placing a 50-male-infested log there for one day only. Proportions within each day followed by the same letter were not significantly different at $\alpha = 0.05$ (chi-square).

TABLE 1. COMPARISON OF SEX RATIO OF TOTAL CATCH OF *Ips paraconfusus* AND LINEAR REGRESSION OF MALE AND FEMALE CATCH ON 18 STICKY-TRAPS ALONG TRUNK OF FELLED PONDEROSA PINE TREE, SIERRA NATIONAL FOREST, CALIFORNIA (OCTOBER 1–8, 1978)

Date	Sex ratio (♂ / ♀)	LBCL (95%)[a] sex ratio	Regression slope (x = ♀ vs. y = ♂)	Difference between LBCL and regression slope (P value)[b]
Oct. 1–2	0.660	0.511	0.107	<0.001
Oct. 3–4	0.346	0.274	0.093	0.034
Oct. 5–6	0.418	0.297	0.141	0.030
Oct. 7–8	0.300	0.185	−0.078	0.027

[a] Lower binomial confidence limit (95%) of the sex ratio of total catch.
[b] A P value less than 0.05 indicates the regression slope was significantly less than the LBCL of the sex ratio.

less than the corresponding LBCL (95%) sex ratios of catch on all traps (Table 1). Similarly, compared to the first and second days of attack, the proportion of males that landed on the fourth day significantly decreased (each $P < 0.001$, chi-square) when female aggregation was the most intense (Figure 5).

DISCUSSION

Previous reports indicated that males were less responsive to pheromone than females based on their attraction in the laboratory to naturally produced pheromone (Wood, 1962) or to gut extracts of fed males (Pitman et al., 1965). The attraction of walking males to pheromone has been described as an "area orientation" (higher number of turns per unit distance traveled) compared to the female's "straight-line orientation" (Wood and Bushing, 1963). It was further speculated that males would be attracted to host substrates under colonization but that after landing thigmotactic or gustatory stimulants might induce attacks on the bark before the source of pheromone, a male entrance tunnel, was reached. The present study indicates that these earlier observations were the result of an inhibition of male response to higher concentrations of ipsenol, *cis*-verbenol, and ipsdienol. Furthermore, my laboratory bioassays indicate that the differences in sex ratio of catch in the grid trap experiment

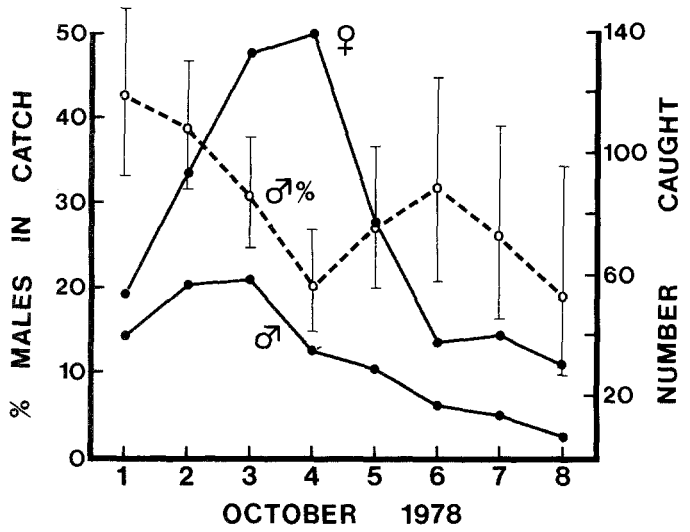

FIG. 5. Relationship between proportion of male *Ips paraconfusus* caught and the number of each sex caught daily on sticky-traps on the trunk of a felled ponderosa pine during colonization in the Sierra National Forest, California (October 1–8, 1978).

were probably due to inhibition of male response by higher concentrations of these pheromone components encountered when the male-infested log was approached. Colonizing males produce the highest amounts of ipsenol and ipsdienol during the first eight days of an attack (Byers, 1981b), so these levels of pheromone were available to inhibit the response of males. In the first experiment, females were not allowed to join males at the pheromone source so the possibility of release of inhibitors by females, or female-induced release of inhibitors by males, cannot account for the sex-specific differences in attraction.

Sex-specific responses were clearly shown in the experiment that monitored beetles landing on the felled ponderosa pine tree being colonized. The highest proportion of males in the trap catches occurred farthest from the origin of attack during the first three days. By the fourth day male attacks had occurred throughout the trunk, and there were no significant trends in sex ratio along the trunk, probably because pheromone was being released at all trap locations. The regression slope of male to female catch was significantly less than the 95% lower binomial confidence limit of the overall sex ratio of the catch, a fact which may be explained by inhibition of male response, while female response increased at those areas of high colonization density and pheromone concentration. As the colonization of the tree progressed during the week, the proportion of males in the catch significantly decreased, and it was lowest at the peak of the aggregation when pheromone release was presumably the highest (Byers, 1981b). The responding population would probably arise from several brood locations not necessarily of the same age that each contributed a sex ratio of approximately 1:1 over the period (Struble and Hall, 1955). Furthermore, based on the beetle's longevity in the laboratory, it appears that the absolute sex ratio of the population in nature would be composed of several days' emergence as well as reemerged parent adults (Struble and Hall, 1955). Thus, the significant change in the sex ratio of catch observed during the first four days of colonization is probably a result of differential response of the sexes to varying pheromone release rates rather than a significant change in the sex ratio of the available adults.

Many investigators of bark beetle biology have wondered about the discrepancy between the approximate male–female ratio of emerging beetles (1:1) to the sex ratio caught on traps with male-infested logs (1:3) or the sex ratio in nuptial chambers (1:3) (Struble and Hall, 1955; Wood, 1962; Gara, 1963; Cameron and Borden, 1967). One previous hypothesis suggested that *I. paraconfusus* males sustain higher levels of mortality than females because of greater exposure to predation and host tree resin during the initial attack (Gara, 1963). The higher male mortality might result in absolute numbers of males of about 1/3 those of females in the responding population. However, the 1:0.86 male–female sex ratio of beetles believed to be entering the grid

area, and the 1:1.35 male–female ratio landing during the first day on the felled tree, suggests that differences in mortality between the sexes because of such differences in predation and death during initial burrowing does not appear to significantly change the sex ratio of flying beetles from that at emergence.

In this study, males and females appeared to be equally responsive to low concentrations of pheromone, but many of the males deviated from the path taken by females when flying to infested hosts to land on areas adjacent to those with higher densities of males which are releasing higher amounts of pheromone. This behavioral mechanism would function to regulate density of colonization and intraspecific competition for food and space. The sexual difference in response was shown to occur even before beetles landed, and it is probable that the effect intensifies after landing. Once males are on the host, other factors may contribute to density regulation and prevent new attacks in fully infested substrates. If no new attacks occur, the colonized areas would become nonattractive since ipsenol and ipsdienol quantities in mated males decrease to negligible levels after 7–10 days (Byers, 1981b).

In contrast to *I. paraconfusus*, both sexes of the bark beetle *Scolytus multistriatus* have been shown to respond in about equal numbers to sources of natural attraction (Peacock et al., 1971). Tilden et al. (1979) reported that more males than females of the monogamous bark beetle, *D. brevicomis*, were trapped close to the source of synthetic pheromones than on surrounding unbaited traps further away. However, the sex ratio was different only between the source and two comparisons on outlying traps while six other comparisons at similar distances were not significantly different. In other studies, the sex ratio of *D. brevicomis* was found not to be significantly different from unity during attraction to a trap releasing synthetic pheromones (Vité and Pitman, 1969; Wood, 1972; Wood et al., 1976; Byers and Wood, 1980; Bedard et al., 1980b) or, more importantly, to traps containing naturally infested host logs (Byers and Wood, 1980). Neither sex of *D. brevicomis* appeared to be inhibited by increasing the concentrations of its synthetic pheromone components in the same laboratory walking bioassay (Byers and Wood, 1981) that demonstrated inhibition of male *I. paraconfusus* in the present study. The responses of the sexes of *D. brevicomis* to individual components of its pheromone (*exo*-brevicomin and frontalin) were shown to be different (Vité and Pitman, 1969), but this may be an artifact since these compounds are never released alone during mass colonization, except by the first female (Byers and Wood, 1980). However, certain ratios of these pheromone components might function to regulate "close-range" behavior after landing such as mating and avoidance of competition. Verbenone, produced by male *D. brevicomis* (Renwick, 1967), inhibits the response of both sexes to their attractants in the laboratory (Hughes and Pitman, 1971) or

field (Bedard et al., 1980a) and may function to regulate attack density (Byers and Wood, 1980) or terminate aggregation (Renwick and Vité, 1970). *D. frontalis* males also release verbenone which inhibits male response (females were not tested; Rudinsky, 1973), and synthetic verbenone released at "higher concentrations" inhibited both sexes (Vité and Renwick, 1970; Payne et al., 1978).

The attack density and termination of mass attack in *D. pseudotsugae* may be controlled in part by release of 3-methyl-2-cyclohexene-1-one (MCH) from females when a male arrives and stridulates at the entrance hole (Rudinsky and Michael, 1972). On the other hand, Pitman and Vité (1974) found that males contain considerably more MCH than females, and they believe males are primarily responsible for release of the inhibitor. The responses to pheromone in both sexes are inhibited (Rudinsky et al., 1974), but males appear to be more strongly affected (Rudinsky et al., 1972). The density of *D. pseudotsugae* attack on a tree under colonization has been reduced by MCH released at multiple sources, and the distance of nearest attack from MCH elution points increased with the concentration of MCH (Furniss et al., 1974). Surprisingly, at the highest concentrations of MCH release, the attack density increased (Hedden and Pitman, 1978). Nijholt (1973) reported that males of *Trypodendron lineatum* released an unidentified inhibitory pheromone after joining the female. Unfortunately, the possibility of changes in the sex ratio of the responding beetles after inhibitors were released was not investigated. However, a recent study has shown that female *T. lineatum* decrease their release of the attractive pheromone, lineatin, after the male arrives (Klimetzek et al., 1981). Klimetzek et al. found evidence of a male-induced inhibition of response which appeared to operate only at close range and may "involve a communication system other than olfaction" (possibly acoustic).

The theory that sex-specific differences in response to intraspecific semiochemicals can function to regulate density of colonization has not been suggested elsewhere for *Ips* species. However, Lanier et al. (1972) found sex-specific differences in *I. pini* in which females, but not males, from Idaho could discriminate between New York and Idaho male-infested logs, while both sexes of New York beetles could differentiate Idaho and New York pheromone. These differences appear to be the result of differential response of the geographical populations to enantiomers of ipsdienol (Lanier et al., 1980). Mustaparta et al. (1980) showed that both Idaho and New York females (males not tested) had two receptor cells each specialized for a specific enantiomer of ipsdienol. Since the receptor systems for ipsdenol of the two populations are virtually identical, the response differences appear to be governed by the central nervous system rather than the peripheral receptors (Lanier et al., 1980). Indicative of the present study, Lanier et al. (1980) found

in the laboratory that the responses of male *I. pini* from New York or Idaho to odor from male-infested logs were always less than those for the same type of female. Electrophysiological studies may reveal whether receptors on the antenna of a male *I. paraconfusus* (and *I. pini*) adapt at lower concentrations of pheromone than the receptors of females, or whether the sexual difference in response is a consequence of the central nervous system.

In all *Ips* species distributed throughout the northern hemisphere, the males initiate the attack, are polygamous, and in many species studied, the males produce various ratios and amounts of ipsenol, *cis*-verbenol, and ipsdienol (Vité et al., 1972). Therefore, olfactory mechanisms similar to that described here may operate in reducing intraspecific competition in other species of the genus *Ips*.

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ALLOMONAL SECRETIONS IN COCKROACHES

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Abstract—We have analyzed the allomonal secretions in 10 species of cockroaches belonging to seven genera and made a detailed reanalysis of these secretions in three other species previously studied. Forty-three products were identified (quinones, phenols, acids, ketones, aldehydes, lactones, hydrocarbons, alcohols and various products). In many cases, the function of these secretions is unknown. We were able to test in the laboratory and also in natural conditions the different products identified, and we were able to test the reactions of several cockroaches when attacked by natural predators (beetles, lizards, toads, and bats). In *Eurycotis decipiens*, the secretion of the sternal glands has a strong deterrent effect on the predators. In *Eublaberus distanti*, the secretion of the glands associated with the second abdominal spiracle acts as an alarm pheromone. In several species of *Periplaneta*, the function of the sternal secretions is unknown. The components of these allomonal secretions are discussed.

Key Words—Cockroaches, Orthoptera, Blattidae, Blaberidae, allomones, defensive secretions, glands, deterrence, taxonomic position.

INTRODUCTION

The cockroaches have evolved a complex glandular exocrine system. In the head of the Blattaria, the exocrine glandular system exhibits an unsuspected complexity (Brossut, 1973). The distribution of the glands supports McKittrick's (1964) classification. Except for the mandibular glands of *Blaberus craniifer* and *Eublaberus distanti* (which secrete an aggregative pheromone, Brossut, 1970; Brossut et al., 1974), the function of the cephalic glands in unknown. The abdominal exocrine glandular system is also complex, consisting of tergal glands, sternal glands, and glands associated with the second abdominal spiracle. Generally the tergal glands are present only in adult males and their secretions act as aphrodisiacs (Brossut et al.,

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1975; Brossut and Roth, 1977). Several of the other glands, especially the sternal glands, have a defensive function (see the review of Roth and Alsop, 1978). In *Nauphoeta cinerea*, the sternal glands of the males produce a sexual pheromone: the "seducin" (Sreng, 1979). However, in many cases, the function of the secretions of these glands is unknown.

In this work we have studied the chemistry of the secretions emitted by the abdominal glands in 10 species belonging to seven genera and we have made a detailed reanalysis of the secretion in three other species previously studied (*Diploptera punctata*, Roth and Stay, 1958; *Eurycotis floridana* and *E. decipiens*, Dateo and Roth, 1967). The fine structure of five of these glands has been published elsewhere (Brossut and Sreng, 1980). In the laboratory, but also in natural conditions (the caves of Trinidad), we were able to test the different products identified, and the reactions of *E. decipiens*, *Periplaneta americana*, *P. brunnea*, and *E. distanti* when attacked by their natural predators (beetles, lizards, toads, and bats). The components of these allomonal secretions will be discussed.

METHODS AND MATERIALS

The list of the species used is as follows:

Blattidae: Polyzosterinae—*Eurycotis floridana*, *Eurycotis decipiens*; Blattinae—*Blatta orientalis*, *Periplaneta americana*, *Periplaneta brunnea*, *Periplaneta fuliginosa*.

Blaberidae: Diplopterinae—*Diploptera punctata*.

These species are reared in the laboratory (26° C humidity, photoperiod 12:12 light-dark).

The other species were collected in Trinidad in 1975 and 1977 and include:

Blaberinae—*Eublaberus distanti*, *Eublaberus posticus*, *Blaberus cranifer*, *Blaberus colosseus*, *Blaberus atropos*; Oxihaloinae—*Leucophaea maderae*.

Collection of Secretion. Different collection methods have been used according to the species studies. The secretion was collected directly from the gland opening with a capillary tube and stored at −20° C (*E. floridana*, 10 males, 7 females; *E. decipiens*, 3 males, 4 females; *E. distanti*, 15 males, 10 females).

Samples were also obtained by placing several insects in a flask (filled with pieces of filter paper) and subjecting them to a jet of carbon dioxide. This causes them to eject the secretion just before anesthesia sets in. The secretion was then collected from the filter paper by methylene chloride (*L. maderae*, 25 males, 25 females; *D. punctata*, 14 males, 17 females).

In another method the secretion was collected by dissecting the glands

which were dipped in methylene chloride or hexane (*D. punctata*, 15 males, 20 females; tergal and sternal glands of *B. orientalis*, 15 males, 15 females; and the three species of *Periplaneta* 20 males, 20 females).

Sometimes whole insects were dipped in methylene chloride (*E. distanti*, 600 males and females; *E. posticus*, *50 males and females; B. craniifer*, 100 males and females; *B. colosseus*, 35 males and females; *B. atropos*, 15 males, 17 females).

Chemical Analysis. Total extracts were chromatographed on small columns of silica gel (60–80 mesh, Merck) with pentane to elute the cuticular hydrocarbons. The other products, remaining on the column, are eluted by methylene chloride. Solvent extracts were treated with an 1% NaOH aqueous solution. The upper phase (solvent layer) was collected and analyzed, the lower phase (aqueous layer) was acidified up to pH 5 by HCl and then the acids were extracted with hexane. Silylations were done according to the classical methods; with BSA (*N,O*-bistrimethylsilyl acetamide) or with TSIM (*N*-trimethylsilylimidazole) or with HDMS (hexamethyldisilylazane) (Lavoué, 1976).

Extracts were chromatographed without further purification by gas chromatography on the following columns:

A 6-m × 2.5-mm stainless-steel column, coated with 15% Carbowax 20 M on Chromosorb 60–80 mesh; N_2 flow rate of 20 ml/min programed from 60° to 220° C at 3° C/min. Chromatographs were a Girdel 75 CSPT and a Girdel 3000.

A glass column coated with Carbowax 20 M (35 m × 0.7 mm, N_2 flow rate 5 ml/min programed from 40° to 110° C at 3° C/min). The chromatograph was a Girdel 3000.

Analysis of Sugars. After hydrolysis (methanol–HCl 0.5 M) and total evaporation, the *O*-methyl glycosides are dissolved in a mixture of methylene chloride–trifluoroacetic anhydride and heated at 150° C for 5 min and directly chromatographed on a stainless-steel column (3 m × 2.5 mm) coated with 5% OV-210 on gas-chrom Q, 100–120 mesh (N_2 flow rate 20 ml/min programed from 90° to 180° C at 4° C/min).

Analysis of Lactones. The extracts were trifluoroacetylated by a mixture of trifluoroacetic anhydride in methylene chloride (1 : 1), heated 1 hr at 100° C, and directly chromatographed on a stainless-steel column (3 m × 2.5 mm), coated with 3% OV-17 on gas-chrom Q, 100–120 mesh, programed from 120° to 200° C at 3° C/min (Lavoué, personal communication).

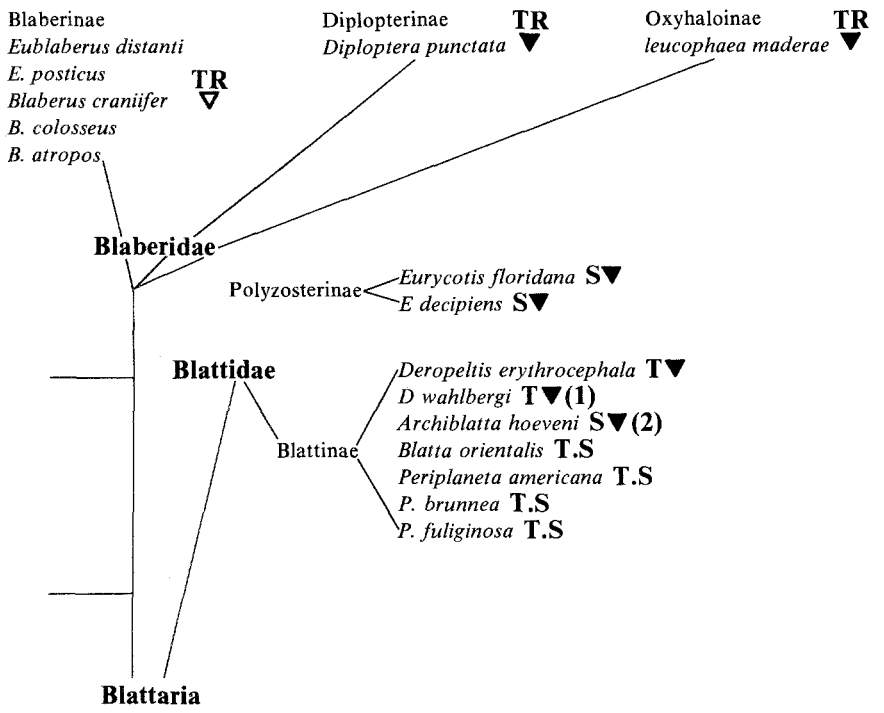
The identification of the different products was made by GC-MS (Girdel 3000-CH5 Mat Varian) using different type of columns. The carrier gas was helium (flow rate 20 ml/min). The mass spectra were compared with the spectra published by Stenhagen et al. (1969), Cornu and Massot (1975) and with spectra obtained from pure products purchased from Pfalz and Bauer and Sigma.

Observations and Bioassays. Observations were made in the caves of Trinidad (Lopinot, Guanapo, Calado) or in the laboratory on *E. decipiens*, *E. distanti*, *P. americana*, and *P. brunnea*. All observations were made under red light during the periods of activity of the insects.

The natural predators are the beetle *Morion* sp., the pedipalpa *Tarantula palmata*, the lizard *Anolis* sp., the toad *Bufo marinus*, and the bat *Phyllostomus hastatus*.

The activity of the different products identified in solution in ether was tested in the field and in the laboratory, especially on *E. distanti*. Papers impregnated with different products were placed in the groups (after total evaporation of the solvant), or wind puffs saturated with the products were spread on the group (with a vaporizer containing impregnated filter paper). In the laboratory 30 *E. distanti* were used (5 males, 10 females, 15 larvae) in each

TABLE 1. LOCALIZATION OF GLANDS SECRETING ALLOMONAL SECRETIONS ACCORDING TO PHYLOGENY OF BLATTARIA[a]



[a] S: sternal glands; T: tergal glands; TR: tracheal glands; ▽: secretion has no defensive value; ▼: secretion is defensive; (1): see the review of Roth and Alsop, 1978; (2): Maschwitz and Tho, 1978.

TABLE 2. QUANTITY OF SECRETION PER INSECT (AVERAGE) ACCORDING TO COLLECTION METHOD

Species	Average quantity of secretion collected from one insect (μl)	Average of the lower phase in the secretion (%)
Secretion collected with capillary tube		
Eurycotis floridana (10 ♂, 7 ♀)	30	15
Eurycotis decipiens (3 ♂, 4 ♀)	40	40
Eublaberus distanti (15 ♂, 10 ♀)	7	60
Insects subjected to carbon dioxide jet (estimation)		
Leucophaea maderae (25 ♂, 25 ♀)	5	60
Diploptera punctata (14 ♂, 17 ♀)	7	25
Secretion collected by dissecting the glands		
Diploptera punctata (25 ♂, 20 ♀)	10	25
Blatta orientalis (15 ♂, 15 ♀)		
Tergal glands	5	80
Sternal glands	3	60
Periplaneta americana (20 ♂, 20 ♀)		
Tergal glands	5	80
Sternal glands	6	60

experiment; they are placed in a wooden box measuring 1 × 1.5 m (without food). Generally after an exploration period of 10–20 min, they aggregate. Impregnated filter paper and wind puffs are then tested.

RESULTS

Origin of Secretions. The results are summarized in Table 1. For *E. posticus*, *B. craniifer*, *B. colosseus*, and *B. atropos*, only total extracts were analyzed. We were unable to localize the origin of these secretions, but in all likelihood they are secreted by the tracheal glands as in *E. distanti*, *L. maderae*, and *D. punctata*.

Quantities of Secretion. It was quite easy to measure the quantity of secretion when it was collected with capillary tubes. In the other cases, the quantity of secretion was measured after total evaporation of the solvent under a nitrogen flow with Hamilton syringes. In most cases, the secretion was a mixture of an upper phase (organic phase) and a lower phase (aqueous phase). In Table 2 the average quantity of secretion per insect is given according to the collection method. When the secretion was collected by total extraction, it was impossible to quantify the amount of secretion.

TABLE 3. PRODUCTS IDENTIFIED IN SECRETION OF GLANDS STUDIED[a]

Species	Main components	Minor components
BLATTIDAE		
Polyzosterinae		
Eurycotis floridana sternal glands	■ (*E*)-2-Hexenal (90%)	Methacrylic acid
	□ ■ Gluconic acid	Tiglic acid
	□ ■ Gluconolactone	Octanol
	□ ■ Glucose	□ Isovaleric acid
		□ Isobutyric acid
Eurycotis decipiens sternal glands	■ (*E*)-2-Hexenal (90%)	□ Hexanoic acid
	□ ■ Gluconic acid	□ Butyric acid
	□ ■ Gluconolactone	Methacrylic acid
	□ ■ Glucose	Tiglic acid
		Hexenol
Blattinae		
Blatta orientalis sternal glands	1,4-Benzoquinone (10%)	(*E*)-2-Hexenal
	p-Cresol (25%)	□ Gluconic acid
	2-Ethylphenol (15%)	□ Gluconolactone
	4-Ethylphenol (15%)	
Periplaneta americana, *Periplaneta brunnea* sternal glands	*p*-Cresol (40%)	(*E*)-2-Hexenal
	2-Ethylphenol (25%)	Undecene
	4-Ethylphenol (25%)	Cyclohexanedial
		□ Hexanoic acid
		□ ● γ-Dodecalactone
Periplaneta fuliginosa sternal glands	*p*-Cresol (40%)	□ γ-Hydroxybutyric acid
	2-Ethylphenol (30%)	□ ● α-Acetyl-γ-butyrolactone
	4-Ethylphenol (20%)	□ ● α-Acetyl-γ-methylbutyrolactone
Periplaneta americana tergal glands	□ ■ Proteins	■ 2-Pentanone
		■ 3-Pentanone
		■ 3-Octanone

		● 2,3,4-Methylcyclohexanone
Periplaneta brunnea, *Periplaneta fuliginosa*, *Blatta orientalis* tergal glands		2-Heptanone Undecene
Blaberidae		
Diplopterinae		
Diploptera punctata tracheal glands	■ 1,4-Benzoquinone (40%) ■ Toluquinone (35%) 2-Ethyl-*p*-benzoquinone	Methacrylic acid Tiglic acid
Blaberinae		
Eublaberus distanti tracheal glands *Eublaberus posticus*?	1,4-Benzoquinone (70%) 2,3-Dimethylbenzoquinone (15%) 2,5-Dimethylbenzoquinone (10%)	2-Ethylphenol 2-Decenal
Blaberus craniifer?	1,4-Benzoquinone (40%) *p*-Cresol (35%) Naphthol	Octanol
Blaberus colosseus? *Blaberus atropos*?	2,3-Dimethylbenzoquinone (15%) 2,5-Dimethylbenzoquinone (20%) *p*-Cresol (30%) Naphthol (25%)	
Oxyhaloinae		
Leucophaea maderae tracheal glands	● Anisaldehyde (60%) ● Anisole (25%) ● Anisic acid (10%)	● 2,2,4-Trimethylhexane

[a]The secretion of these glands is a mixture of organic phase (upper phase) and of an aqueous phase (lower phase). Compounds noted □ belong to the lower phase; ■, products have been previously identified (Roth et al., 1956; Roth and Stay, 1958; Dateo and Roth, 1967; Brossut et al., 1975). To our knowledge products marked ● have never been identified in insects.

In the cases of the two species of *Eurycotis* and *P. americana*, we were able to measure the composition of the secretion from one gland. In *E. decipiens* we have found one gland with 60 μl of upper phase (UP) without lower phase (LP) but also a case with 10 μl of UP and 30 μl of LP. In *E. floridana* we have found glands with 80 μl of UP and 2 μl of LP, but also an insect with 5 μl of UP and 60 μl of LP (similar results were obtained by Dateo and Roth, 1967, and Wallbank and Waterhouse, 1970, in several species of *Polyzosteria*).

In the sternal glands of *P. americana*, the UP represents about 40% of the whole secretion. Twenty analyses were made. In 14 cases the results were similar (cresol 40%, 2- and 4-ethyl phenol 25 and 25%, respectively), but in six cases we have been unable to detect any organic fraction; all the secretion is aqueous. We do not find the acids and lactone usually present in this fraction, but without further experiments, it is difficult to assert that this secretion consists only of water.

Composition of Secretion. Our results are summarized in Table 3. Other products have been identified in the exocrine secretions of several species that we did not study: 2-methylenebutanal (Waterhouse and Wallbank, 1967), 2-methylenepentanal, 2-octenal, 2-heptanal, 2-pentanal, hexanal, and (*E*)-2-octenal have been identified on Polyzosterinae species by Wallbank and Waterhouse (1970); terpineol by Meinwald et al. (in Roth and Alsop, 1978) in the secretion of the tergal glands of *Deropeltis wahlbergi*; and phenol by Maschwitz and Tho (1978) in the secretion of the sternal glands of *Archiblatta hoeveni*.

Field Observations and Experiments. In Trinidad, the caves provide a specialized habitat in communication with the floor of a tropical rain forest. The dark parts are characterized by permanent high humidity (95%), almost constant temperature (26–26.5° C), and a very high rate of input of organic matter in form of bat guano and waste food. The frugivorus bats (*Artibeus*), which live in great number in these caves, eat almost exclusively the seeds of a bush (*Pipper* sp., "candle stick"); they bring the fruit back to the caves, eat part of it, and drop the remainder. This waste food accumulates on the floor of the caves along with the guano.The terrestrial crabs (undetermined species) try to catch all the species of cockroaches but the latter can escape easily. The small gecko (*Anolis sp.*) lives on the walls and hunts with success *P. americana* and *P. brunnea*. *Tarantula palmata* lives also on the walls. Its strikes are very swift, and few cockroaches (larvae and adults of *P. americana* and *P. brunnea*) can escape. *Morion sp.*, the big carnivorous beetle, hunts the two species of *Periplaneta* very actively on the floor of the caves, but the cockroaches can escape easily. The main predators are the vertebrates: the big toad *Bufo marinus* and the "greater spearnosed bat" *Phyllostomus hastatus*.

E. decipiens is not a natural inhabitant of the caves and usually lives in the

forest. In two cases we have observed *E. decipiens* at the entrance of one cave attacked by *Anolis sp.* The gecko is strongly deterred by the secretion of the sternal glands; it runs away and hides in cracks of the wall. *E. distanti* are hunted by *B. marinus* when they eat fresh guano. The strikes elicit the disruption of the group and an escape behavior. The cockroaches which are caught are readily eaten. When living in small groups on the walls, *E. distanti* is hunted by the bat *P. hastatus*; the strikes elicit the disruption of the group and an escape behavior.

In these two cases, the escape behavior appears only when the prey is handled by the predator. The glands associated with the second abdominal spiracle secrete a mixture of 1,4-benzoquinone, 2,3- and 2,5-dimethylbenzoquinone and minutes amount of 2-decenal and 2 ethylphenol. When papers impregnated with this mixture were placed in the center of different groups, the insects slowly move away from the paper (17 positive responses on 20 experiments). Wind puffs saturated with these products elicit the disruption of the group and escape behavior: the insects quickly move away to about 60 cm (20 positive responses out of 25 experiments).

P. americana and *P. brunnea* are mainly hunted by *B. marinus* on the floor. Its strikes elicit a disruption of the group and an escape behavior. The sternal glands secrete a mixture of *p*-cresol, 2- and 4-ethylphenol. When papers impregnated with this mixture were placed inthe center of different groups, the insects slowly move away (8 positive responses out of 10 experiments). Wind puffs saturated with this mixture (10 experiments) but also wind puffs alone (10 experiments) have provoked a disruption of the group and an escape behavior.

Although *B. marinus* can jump very quickly, it moves slowly when hunting and the cockroaches do not react when it draws nearer. When the toad throws forward its tongue, its enough to elicit the escape behavior. Camhi et al. (1979) have determined that *p. americana* attacked by *B. marinus* begins its escape 17 msec after the toad's tongue appears.

Morion runs after the two species of *Periplaneta*. In five attacks that we have observed, the beetle killed two cockroaches. In three cases, the cockroaches were caught but struggled and could escape. In no cases did the beetle seem to be repelled by the secretion. All these attacks take place near groups of cockroaches without disturbing them.

It clearly appears that the secretion of the sternal glands, if emitted, has no defensive function and does not act like an alarm pheromone.

Laboratory Experiments on E. distanti. Results are summarized in Table 4. A mixture of the three quinones is necessary to elicit the disruption of the group and an escape behavior. Wind puffs alone provoke some disturbance in the group but do not elicit an escape behavior (seven experiments). These experiments strongly suggest the existence of an alarm pheromone emitted by

TABLE 4. ALARM PHEROMONE OF *E. distanti*: BIOLOGICAL ACTIVITY OF PRODUCTS IDENTIFIED IN SECRETION ACCORDING TO CONCENTRATION[a]

Exp No.	1,4-Benzoquinone (μl)	2,3-Dimethylbenzoquinone (μl)	2,5-Benzoquinone (μl)	2-Ethylphenol (μl)	2-Decenal (μl)	Activity
1	70	15	10	2	2	+++
2	70	15	10			+++
3	70	70	70			+++
4	70					++
5	100					++
6		15	10			++
7		70	70			++
8				2	2	−
9				50	50	−
10				100	100	+
11				200	200	+

[a] Wind puffs saturated with different products are sprayed on a group (5 males, 10 females, 15 larvae); 10 experiments in first case, 5 for the the 10 other cases. In the first experiment, the different products are in the same proportion as in the secretion. A mixture of the first three products is enough to elicit the escape behavior. A mixture of the products in columns 2 and 3 provokes strong disturbance but not an escape behavior. A mixture of the products in columns 4 and 5 provokes some disturbances in the group, but only in very high concentrations (very far from the natural concentrations). +++: disruption of the group and an escape behavior; ++: disruption of the group; +: some disturbance in the group but without disruption or escape behavior;—no effect.

the attacked insect only when it is handled by the predator. The secretion has no apparent defensive function.

DISCUSSION

Morphology. In a previous paper, we studied the fine structure of five of these glands (Brossut and Sreng, 1980): the sternal and tergal glands of *P. americana*, the sternal glands of *E. floridana*, and the glands associated with the second abdominal in *D. punctata* and *L. maderae*. Despite marked anatomical diversity of the glands, all the glandular cells are of type 3 (Noirot and Quennedey, 1974) and differ only in some details. For us, it is quite clear that in the other species studied here, the glandular units are of the same type.

In *L. maderae* and *E. floridana*, the numerous peroxysomes (microbodies) may play a part in the synthesis of the aldehydes [anisaldehyde, (*E*)-2-hexenal]. In *Eurycotis*, the abundant glycogen is perhaps a precursor of the gluconic acid and of the gluconolactone; but in many cases, it is difficult to find a relation between the probable mode of elaboration of the secretory material and the morphology of these glands.

Chemistry of secretions. Among the 43 products that we have identified, seven have been previously identified in the defensive secretions of cockroaches, six have never been previously identified in insects, and eight other products (not identified in the species studied here) have been identified in Polyzosterinae (see Table 3). Most of these products [(*E*)-2-hexenal, quinones, phenols, acids] are common in the defensive secretions of the insects (see the review of Weatherston and Percy, 1970, 1978; Blum and Hermann, 1978).

We must point out that the allomonal secretions are not responsible for the typical odor of each species of cockroaches. This suggests that there are additional compounds. The origin of these, or similar, products has been discussed in several reviews (Duffey, 1976; Araujo, 1978; Staddon, 1979) but remains in many cases highly speculative. Although for Staddon (1979) the significance of an aqueous phase in the scent glands of Heteroptera is not clear, its presence in the allomonal secretions of many cockroaches is necessary: many products (acids, lactones, proteins) are soluble only in water.

Variations in Secretion composition. As we have seen in *E. decipiens*, *E. floridana*, and *P. americana*, the composition and the quantity of the secretion change according to the insect. The same is true in many Polyzosterinae (Dateo and Roth, 1967; Wallbank and Waterhouse, 1970). This phenomenon seems to be related to the age of the insect and to the rhythm of the emission of the secretion. A cockroach which emits a large amount of secretion when captured lives 5–6 weeks, whereas undisturbed adults commonly live 9 months (Wallbank and Waterhouse, 1970).

Wheeler et al. (1979) found 99% of methacrylic acid and very low amounts of tiglic acid in freshly collected beetles (*Scaphinotus viduns*). After 3–6 weeks in captivity, the relative abundance had switched to 15% and 85%, respectively. It was suggested that diet might be the factor determining the change.

In *E. distanti*, the composition of the aggregative pheromone (secreted by the mandibular glands) varies according to the density of the population in the different caves (Brossut, 1978). In the same way, Tschinkel (1975) found that the variations of quinones and alkenes in a given species of *Eleodes* from one locality were as great as the variations found in the beetles from different localities.

Defensive Secretions. Many invertebrates are deterred by the quinones in the defensive secretions of *Diploptera punctata*. Ants, like *Pogonomyrmex*, avoid this insect. On the contrary, these cockroaches are eaten by *B. marinus* and by praying mantids (Roth and Stay, 1958). Eisner et al. (1959) observed that lizards which readily ate *Diploptera* learned how to discriminate against this blattid when allowed to choose a more palatable insect.

Quinones also act on the olfactory receptory sites by interacting with sulfur amino acids of the receptor proteins (Norris, 1976). Thus apart from the

irritating odor of quinones which can lead to immediate deterrence of predator, a closer contact with the liquid secretion instead of its vapors may result in a prolonged sensory handicap for the predator. The quinones can alter the dendritic membrane potential which in turn reduces the perception of the predator (concept of negative odors, Kittredge, 1974).

In the literature, we have many observations on the defensive behavior of the different species of Polyzosterinae, which mainly produce (*E*)-2-hexenal and 2-methylenebutanal. These secretions are strongly deterrent for invertebrates, especially ants. Ants affected by the material became disoriented and showed serious, although temporary incoordination (Blum, 1964; Waterhouse and Wallbank, 1967; Roth and Alsop, 1978).

(*E*)-2-hexenal is a very versatile product: it is a potent releaser of alarm pheromone in myrmicine ants (Blum and Crewe, 1970). In *Nezara viridula*, it can act as an alarm or an aggregative pheromone (Ishiwatari, 1974, 1976).

After the work of Levinson et al. (1974), it was concluded that a minimum chain length of C_6 and a terminal carbonyl group are essential functional characteristics of the natural alarm pheromone in bugs (2-hexenal, 2-octenal), but a *trans* double bond is not.

We have seen in Trinidad that the gecko *Anolis* is strongly repelled by the secretion of *E. decipiens*. On the contrary, Blum (1964) observed that *Anolis cristatellus* attacks *Pelmatosilpha coriacea* [which also secretes (*E*)-2-hexenal]; it eats the head first and then the abdomen (after emission of the secretion).

In the same way, Slobodchikoff (1978) shows that skunks can eat tenebrionids (with abundant quinoids defensive secretions). Skunks circumvent the effect of the secretions by manipulating the beetle and stimulating it to release the secretions into the substrate. This manipulative or rolling behavior appears to be innate.

Platyzosteria novaeseelandiae secretes a mixture of (*E*)-2-hexenal and 2-methylenebutanal. Benn et al. (1977) were able to test the reactions of two birds from New Zealand (*Gallirallus australis*, *Porphyrio melanotus*). Both birds devoured several cockroaches in succession, but with a developing reluctance, washing their beak after each insect has been consumed; then they became indifferent to the cockroaches.

The immediate deterrent value of a defensive secretion is only an obvious facet of the prey–predator interaction. The long-range effects of arthropod allomones are neglected phenomena and may be found to be more important than previously considered.

Alarm Pheromone. Eublaberus distani is devoid of defensive secretions, but the secretions of the glands of the tracheal trunk associated with the second abdominal spiracle act as an alarm pheromone promoting an escape behavior of the members of the groups when a predator handles a congener.

This behavior assumes an effective protection for all the members of the group. This is the first time that the existence of an alarm pheromone has been demonstrated in cockroaches. *E. posticus* secretes the same products, but we were unable to test their function. These secretions are a mixture of quinones. As far as we know, quinones have never been previously involved in an alarm communication; the function of the secretion is unknown.

Takahashi and Kitamura (1972) state that the secretion of the sternal glands of *P. americana* is repellent to the nymphs. For Brousse-Gaury (1975), this secretion acts as trail pheromones. *Tarantula palmata* lies in waiting for *P. americana* or *P. brunnea*, and few can escape. (The secretion of the sternal glands is a mixture of *p*-cresol and ethylphenol in very low amounts.) On the contrary, the defensive secretions of tenebrionids, like *Eleodes* (quinones) are strongly deterrent against *Tarantula aphonopelma* (Minch, 1978).

Kairomones. During their active period, both prey and predator live in total darkness. Although *Tarantula palmata* can react at the least vibration of the substrate, for the other predators, the recognition of prey is mainly mediated by olfaction. *P. hastatus* cannot use echolocation to hunt cockroaches on the walls; *B. marinus* is quite blind.

Deleporte has shown (1976) that more than 13% of the oothecae deposited by *P. americana* are parasitized by *Evania appendigaster* (Hymenoptera). The recognition of the oothecae is certainly olfactory. In all these cases the typical odor of the different cockroaches acts as a kairomone.

Defense against Microorganisms. Most arguments for the roles of insect allomones only focus on the external predators, and the biological activity is often depicted in the terms of the major components [(*E*)-2-hexenal, quinones, etc.]; *p*-cresol, naphthol, and quinones are common in several species of Blaberidae and Blattidae and can act against microorganisms (fungi, bacteria, etc.). We find many other examples in the literature (see the review of Duffey, 1975) but no real experiments have been made.

Possible Functions of "Inactive" Products. The mandibular glands of *E. distanti* secrete an aggregative pheromone, which is a mixture of four products, but contains 14 other products (Brossut, 1979). In *E. floridana*, the (*E*)-2-hexenal, the more active product, represents 90% of the secretion, but we find eight other products. In the secretion of the sternal glands of *P. americana* (which is behaviorally inactive) we find six products. We must emphasize that in the cockroaches the exocrine glandular system is very complex (Brossut, 1973; Brossut and Roth, 1977) and that in many cases we find multicomponent secretions.

Why do some insects evolve such chemically complex secretions? Although a predator may find an insect distasteful initially, it may become accustomed to the secretion. A secretion containing a variety of chemicals can have the potential to play upon a greater number of physiological suscep-

tibilities of the predator. These products can play a part in some micro-behaviors. We know that in several moths the different products of the sexual pheromones of the female can elicit successive behaviors: long-range attraction, short-range attraction, landing, walking, mating (Cardé et al., 1975). They can also act as organic carriers, surfactants, penetrators or evaporation retardants. We know nothing about these fundamental issues, and this underlines the fact that much work using a multi-disciplinary approach remains to be done.

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CHEMICAL DEFENSE OF A ROVE BEETLE (*Creophilus maxillosus*)[1]

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Abstract—The abdominal defensive glands of *C. maxillosus* secrete a mixture (70 μg/beetle) of isoamyl alcohol (I), isoamyl acetate (II), iridodial (III), actinidine (IV), dihydronepetalactone (VE), and (*E*)-8-oxocitronellyl acetate (X). When disturbed, the beetle everts the glands and revolves the abdomen so as to wipe the glands against the offending agent. Fecal fluid is commonly emitted at the same time and may become added to the glandular material. Ants (*Formica exsectoides*) are effectively fended off by the beetle and were shown in bioassays (*Monomorium destructor*) to be repelled by the four major components of the secretion (II, III, X, VE); the principal component (VE) was the most active. Some anatomical features of the glands are described.

Key Words—Coleoptera, Staphylinidae, *Creophilus maxillosus*, defensive secretion, ant repellent, bioassay, isoamyl acetate, isoamyl alcohol, iridodial, actinidine, dihydronepetalactone, (*E*)-8-oxocitronellyl acetate.

INTRODUCTION

The beetles of the family Staphylinidae, comprising the rove beetles, share with many other insects the possession of defensive glands. Although upwards of 25,000 species of staphylinids have been described, less than two dozen have been studied chemically (Weatherston and Percy, 1978). Even from this limited sample, it is clear that these beetles produce defensive substances of

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considerable diversity. Compounds identified to date include hydrocarbons, terpenes, quinones, aldehydes, and alkaloids, as well as the complex nonglandular product pederin (review in Weatherston and Percy, 1978).

We have recently collected large numbers of *Creophilus maxillosus*, a staphylinid not previously studied chemically, and here report on: (1) the structure and operation of its abdominal defensive glands, (2) the composition of the glands' secretion, and (3) the repellency of the secretory components to ants.

METHODS AND MATERIALS

Biology. C. maxillosus is one of the largest North American staphylinids (body length up to nearly 2 cm). It is commonly found in carrion. We collected several thousand specimens of both sexes at baits (dead fish and chickens; beef remains) at the Archbold Biological Station, Lake Placid, Florida, and in the environs of Ithaca, New York. In the laboratory they were caged in small groups and maintained for weeks on pieces of freshly killed cockroaches.

Beetles with everted glands were prepared for examination with the scanning electron microscope by holding the animals live in forceps so as to cause them to evert the glands, dropping them immediately in refrigerated (−195° C) liquid freon, and transferring them while still frozen to the stage of a freeze-drier for desiccation. They were thus preserved in the precise stance in which they died upon immersion in the freon.

Cuticular preparations of the glands were made by immersing freshly killed beetles overnight in cold 10% aqueous KOH, so as to dissolve away all cellular parts, and then mounting the excised and washed (H_2O) glands in glycerin (for light microscopy), or critical-point drying them after dehydration in alcohol (for scanning electron microscopy).

Since the beetles commonly defecate when everting their glands, it was not possible to obtain pure secretion by wiping the everted glands with filter paper. Instead, secretion was obtained by extraction of freshly excised glands. For this purpose, 1760 *Creophilus* were killed by freezing and then individually dissected to remove the glands. These were extracted with CH_2Cl_2 or CH_3OH (ca 5 μl per set of glands; one week at −10° C); male and female glands were kept separate. A "milking" was also obtained from several hundred live beetles of both sexes by holding their abdominal tips in glass vials and pinching their abdomens with forceps until their glands everted. The fluid that dribbled into the vials was inevitably contaminated with some rectal effluent.

The tests with ants (*Monomorium destructor*) were done on the trails of a natural colony that foraged in our laboratory at the Archbold Biological Station. Daily baitings with dilute honey and dead insects maintained ongoing ant activity at the experimental site.

Chemistry. Gas chromatographic analyses were carried out using a Varian 2100 instrument equipped with a 15-ft × 1/4-in 3% SE-30 column (temperature programed from 50° C to 250° C at 8° /min), or a Hewlett-Packard 5720A instrument equipped with a 8-ft × 1/4-in. 5% Carbowax 20 M column (operated isothermally at 200° C). Both instruments had a flame ionization detector and used N_2 as a carrier gas. GC-MS data were obtained using a Finnigan 3300 instrument with a 5-ft × 1/8-in. 3% OV-101 column. Helium was the carrier gas used to record electron impact mass spectra, and methane was used to obtain chemical ionization mass spectra. Ultraviolet spectra were obtained using a Beckman DB-G spectrophotometer. Infrared spectra were determined in $CDCl_3$ on a Perkin-Elmer 299B grating spectrophotometer. [^{1}H]NMR spectra were recorded on either a Varian EM-390 (90 MHz) spectrometer or a Varian CFT-20 (80 MHz) Fourier transform spectrometer. Solvents used were either $CDCl_3$ or C_6D_6. All chemical shifts are reported in parts per million (δ) downfield from internal tetramethylsilane.

GC-MS Analysis of Secretion Extracts. The extracts were analyzed using a 5-ft × 1/8-in. 3% OV-101 column, temperature programed from 20° C to 250° C at a rate of 8° /min. A typical chromatogram is shown in Figure 1. Peaks A through F gave the following mass spectra:

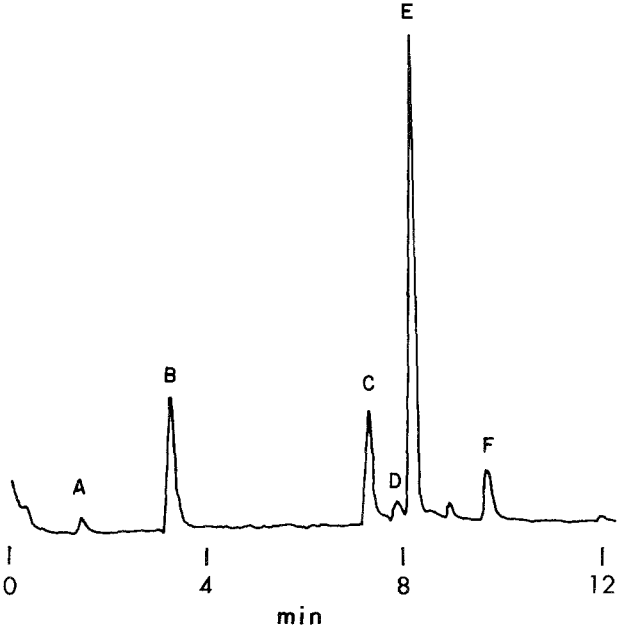


FIG. 1. Gas chromatogram (3% OV-101) of *C. maxillosus* secretion extract (methanol). Column conditions and details of lettering in text.

A: EI-MS *m/e*(rel. int.): 70(71), 55(100), 43(50), 42(65), 41(58).

B: EI-MS *m/e*(rel. int.): 87(10), 70(57), 55(45), 43(100). CI-MS *m/e* (rel. int.): 131(0.5) (M + 1).

C: EI-MS *m/e*(rel. int.): 168(2), 153(2), 150(8), 135 (43), 111(64), 109(58), 95(31), 93(48), 81(100), 71(52), 67(70), 58(81), 55(67), 43(47), 41(67). CI-MS *m/e*(rel. int.): 169(1.3) (M + 1).

D: EI-MS *m/e*(rel. int.): 147(51), 146(30), 132(100), 117(47). CI-MS *m/e* (rel. int.): 148(100) (M + 1), 176(25) (M + 29), 188(6) (M + 41).

E: EI-MS *m/e*(rel. int.): 168(4), 153(8), 138(17), 113(80), 110(70), 95(66), 81(100), 69(37), 67(54). CI-MS *m/e*(rel. int.): 169(40) (M + 1), 197(2) (M + 29), 209(3) (M + 41).

F: EI-MS *m/e*(rel. int.): 212(1), 170(4), 152(11), 97(33), 95(48), 81(34), 69(26), 67(35), 55(38), 43(100). CI-MS *m/e* (rel. int.): 213(8) (M + 1), 241(3) (M + 29), 253(2) (M + 41).

Isolation of Components B, C, E, and F. These four components were isolated as colorless liquids in <2-mg amounts each by preparative gas chromatography of defensive gland extract using the Varian 2100 GC with a 15-ft × 1/4-in. 3% SE-30 column temperature programed from 50° C to 250° C at a rate of 8° / min. The compounds were collected in glass capillaries cooled to −78° C, using a collector-detector ratio of 10:1. The following spectral data were recorded for these isolated compounds:

B: [^{1}H]NMR (80 MHz, $CDCl_3$): δ 4.09. 2H, t, J = 6.7 Hz; 2.04, 3H, s; 1.6–1.4, 3H, m; 0.92, 6H, d, J = 5.8 Hz.

C: [^{1}H]NMR (80 MHz, $CDCl_3$): δ 9.65–9.60, m; 1.12, d, J = 6.8 Hz; 1.08, d, J = 6.3 Hz. [^{1}H]NMR (80 MHz, C_6D_6): δ 9.48, d, J = 2.2 Hz; 9.38, d, J = 1.6 Hz; 0.92, d, J = 6.3 Hz; 0.83, d, J = 7.0 Hz. IR ($CDCl_3$): 2960, 2720, 1710 cm^{-1}.

E: [^{1}H]NMR (80 MHz, $CDCl_3$): δ 4.38, 1H, dd, J = 6, 11 Hz; 3.84, 1H, dd, J = 7, 11 Hz; 1.19, 3H, d, J = 5.8 Hz; 1.00, 3H, d, J = 6.3 Hz. [^{1}H]NMR (80 MHz, C_6D_6): δ 3.95, 1H, dd, J = 6, 11 Hz; 3.45, 1H, dd, J = 7, 11 Hz; 1.39, 3H, d, J = 6.3 Hz; 0.62, 3H, d, J = 6.0 Hz. IR ($CDCl_3$): 2990, 1740 cm^{-1}.

F: [^{1}H]NMR (80 MHz, $CDCl_3$): δ 9.40, 1H, 3, s; 6.48, 1H, br t, J = 6 Hz; 4.12, 2H, t, J = 6 Hz; 2.50–2.25, 2H, m; 2.04, 3H, s; 1.75, 3H, br s; 0.97, 3H, d, J = 5.6 Hz. [^{1}H]NMR (80 MHz) (C_6D_6): δ 9.34, 1H, s; 5.99, 1H, br t, J = 7 Hz; 4.17, 2H, t, J = 6.7 Hz; 1.87, 3H, s; 1.79, 3H, s; 0.83, 3H, d, J = 5.8 Hz. IR ($CDCl_3$): 2720, 1735, 1687, 1655 cm^{-1}. UV (Et_2O): λ_{max} = 232 nm.

Preparation of Dihydronepetalactones VC, VD, and VF from Puleganolide (VII). A slurry of 1.68 g (15.0 mmol) of potassium *tert*-butoxide in 15 ml of DMF freshly distilled from BaO was heated to 120° C under N_2.

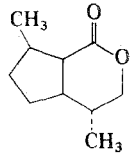
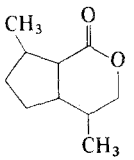
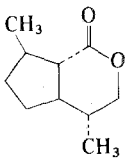
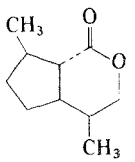
cis-Puleganolide (VII) (Wolinsky and Eustace, 1972) (2.32 g, 13.8 mmol) in 10 ml of DMF was added rapidly by syringe. The temperature was raised to 145° C and the solution was stirred at this temperature for 4.5 hr, then cooled and poured onto ice. The water–DMF was extracted with Et_2O, and the extract was dried over $MgSO_4$ and concentrated to remove 170 mg of the starting lactone. The basic aqueous solution was then acidified with dilute HCl and extracted with Et_2O. The combined extracts were washed with water and with saturated NaCl, dried over $MgSO_4$, and concentrated to yield 2.19 g of a light brown oil. A [^{1}H]NMR spectrum of this crude product showed a broad resonance (exchangeable with D_2O) centered at 6.5 ppm, broad singlets at 4.8 and 1.75 ppm, and a doublet at 1.1 ppm. These data suggest the presence of a significant amount of VIII accompanying IX in the reaction product.

The mixture of acids from the above was dissolved in 10 ml of THF and added slowly by syringe to a solution of 1.60 g (13.1 mmol) of crystalline 9-BBN in 40 ml of THF at 20° C. This solution was stirred for 1 hr, and then an additional 1.6 g of 9-BBN in 40 ml of THF was added. The resulting solution was stirred at 20° C for 12 hr. Afterwards the flask was cooled to 0° C and 2.0 g of KOH in 20 ml of H_2O was added, followed by the slow addition of 25 ml of H_2O_2 (50%). The resulting solution was then warmed to room temperature and stirred for an additional 13 hr. At this time, the solution was diluted with 200 ml of H_2O and extracted with Et_2O. The aqueous phase was acidified with dilute HCl and extracted with Et_2O. The extract was washed with water, saturated NaCl, dried over $MgSO_4$, and concentrated to yield 1.51 g of a light yellow oil. The [^{1}H]NMR spectrum of the crude product showed a broad resonance at 7.5 ppm (D_2O exchangeable) and a multiplet centered at 3.8 ppm. Its IR spectrum showed a broad absorption at 3500–3300 cm^{-1} and a carbonyl absorption at 1710 cm^{-1}, in accord with expectations for the desired hydroxy acids.

The lactonization of a portion of this acid mixture was carried out as described by Wolinsky and Eustace (1972). The mixture of hydroxy acids (450 mg) was heated under N_2 at 170° C for 1 hr. The resulting dark brown oil was taken up in 50 ml of Et_2O, washed with saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated to yield 380 mg of a brown oil. GC analysis on 5% Carbowax 20 M at 200° C showed three major peaks in a ratio of 1 : 1.25 : 1.5. Analytical samples of these three components were isolated by preparative GC and gave the spectral data summarized in Table 1 (peak 1 = VF, peak 2 = VC, peak 3 = VD).

In an independent experiment, 320 mg of the crude hydroxy acid mixture was dissolved in 10 ml of benzene and 50 mg of *p*-toluenesulfonic acid was added. The solution was heated to 80° C for 12 hr. At this point, examination of the reaction mixture using a 5% Carbowax 20 M GC column at 200° C showed two peaks in a 1:1 ratio that corresponded to peaks 2 and 3 in the high-temperature lactonization procedure.

TABLE 1. SPECTRAL DATA OF DIHYDRONEPETALACTONES V(C–F)

Lactone	[^{1}H]NMR ($CDCl_3$)	EI-MS *m/e* (rel. int.)	IR ($CDCl_3$)
 VC	δ = 4.21 1H dd J = 11.0, 3.7 Hz δ = 3.85 1H dd J = 11.0, 9.7 Hz δ = 2.98 1H dd J = 9.5, 8.4 Hz δ = 0.96 3H d J = 7.1 Hz δ = 0.95 3H d J = 5.9 Hz	168(1.1), 153(2.3), 113 (100), 95 (26.6), 81 (22.8), 69(12.6), 67 (26.9)	1715 cm^{-1}
 VD	δ = 4.08–3.97 2H m δ = 3.12 1H dd J = 10.0, 8.8 Hz δ = 0.99 3H d J = 7.1 Hz δ = 0.92 3H d J = 6.8 Hz	168(3.1), 153(1.4), 139 (5.7), 126(19.7), 113 (100), 95(28.9), 81 (35.5), 69(19.8), 67 (41.4)	1715 cm^{-1}
 VE	δ = 4.39 1H dd J = 11.5, 5.9 Hz δ = 3.82 1H dd J = 11.5, 7.8 Hz δ = 1.19 3H d J = 5.9 Hz δ = 0.99 3H d J = 6.2 Hz	168(0.9), 153(3.0), 138 (6.3), 113(59.2), 110 (42.5), 95(54.4), 81 (100), 69(41.5), 67 (59.4)	1740 cm^{-1}
 VF	δ = 4.38 1H dd J = 11.6, 6.6 Hz δ = 3.76 1H dd J = 11.6, 8.0 Hz δ = 1.16 3H d J = 6.0 Hz δ = 0.92 3H d J = 6.7 Hz	168(1.3), 153(2.1), 138 (7.1), 113(45.4), 110 (43.2), 95(52.0), 81 (100), 69(42.2), 67 (58)	1740 cm^{-1}

Epimerization of VC and VD to VE and VF: Samples of VC (8.3 mg) and VD (10.9 mg), isolated by preparative GC, were dissolved separately in 500 μl of xylene, and 1 mg of *p*-toluenesulfonic acid was added to each. The solutions were heated to reflux for 12 hr. At this point, examination by GC using a 5% Carbowax 20 M column at 200° C showed that lactone VD had given rise to a 3:1 mixture of VD and VF, and lactone VC had yielded a 5:1 mixture of VC and VE. Lactone VF proved to be identical ([^{1}H]NMR, GC-MS, IR) to peak 1 produced by the high-temperature lactonization method. Lactone VE gave spectral data summarized on Table 1. It was found to be indistinguishable from the *C. maxillosus* component E on the basis of the above data and by

coinjection of lactones VC, VD, VE, and VF with *C. maxillosus* component E on a Carbowax 20 M column.

(E)-8-Oxocitronellyl Acetate (X). Citronellyl acetate (2.07 g, 10.4 mmol) was dissolved in 30 ml of 95% ethanol, and 1.17 g of SeO_2 (10.5 mmol) was added in one portion. The slurry was heated to reflux for 12 hr, the solution was cooled, and the precipitate filtered. Analysis by GC using a 3% SE-30 column temperature programed from 100 to 200° C at a rate of 8°/min showed predominantly one new peak at a retention time slightly longer than that of the starting acetate. An analytical sample was obtained by preparative GC and gave the following data: MS *m/e*(rel. int.) 212(1), 170(11), 152(25), 126(28), 128(25), 109(30), 97(70), 95(100), 81(62), 69(38), 67(49). IR($CDCl_3$):

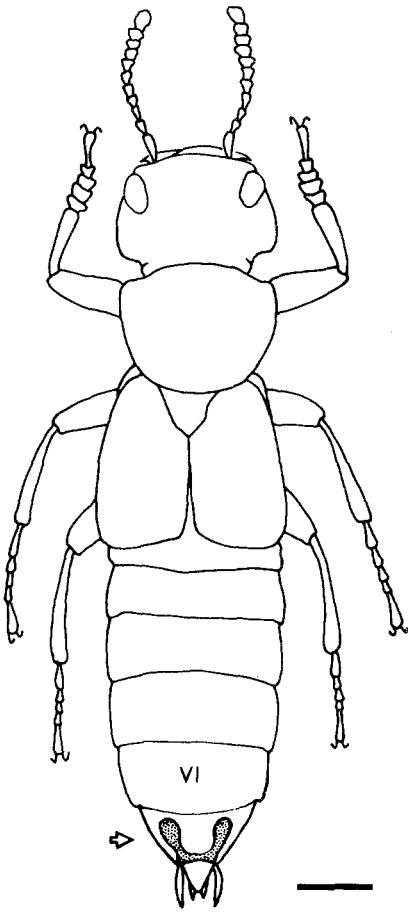


FIG. 2. Diagram of *Creophilus maxillosus*, showing the glands (arrow) in place. The VIth apparent (not actual) abdominal segment is numbered. Reference bar = 2 mm.

2720, 1736, 1687, 1655 cm^{-1}. UV (Et_2O):λ_{max} = 232 nm. [^{1}H]NMR (90 MHz, $CDCl_3$): δ 9.42, 1H, s; 6.48, 1H, tq, J = 7, 1 Hz; 4.12, 2H, t, J = 6 Hz; 2.5–2.2, 2H, m; 2.03, 3H, s; 1.75, 3H, s; 0.97, 3H, d, J = 5.5 Hz.

Quantitative Analysis. The amounts of compounds B, C, D, and F per individual were determined gas chromatographically using synthetic samples as standards and with the aid of a Spectra Physics Autolab Minigrator.

RESULTS

Structure and Operation of Glands. The beetle's glands (Figure 2) are two cuticular sacs that lie directly beneath the VIIth (apparent) abdominal tergite, and open in the intersegmental membrane between tergites VII and VIII. They are essentially involutions of this membrane. Casual observation showed the glands to be eversible and eversion to occur only in response to disturbance. Mild manipulation of the beetle rarely induced eversion, but pinching the body or an appendage with forceps almost invariably did. The eversion occurred promptly upon application of the pinch, lasted only for as long as the stimulus persisted, and as a rule involved no more than partial evagination of the glands.

Examination at higher magnifications of the abdominal tip of beetles being held and pinched with forceps showed defecation to be a common concomitant of gland eversion (Figure 3). Eversion usually occurred first, but defecation, heralded by protrusion of the anal tube, was often quick to follow. The grayish rectal fluid flowed over the abdominal tip, wetting the glandular "horns" and spreading over the cerci. Within the minute after emission, the effluent dried to a white semisolid paste, which clung persistently to the cercal and perianal hairs, unless the beetle wiped its rear against some object in the course of its abdominal wigglings.

Experimentation with tethered beetles showed that the animals make use of precisely directed abdominal motions when defending themselves. Five *Creophilus* of each sex were attached to metal rods cemented to their backs with wax, and subjected to "assault" by pinching legs, antennae, or some part of the body with forceps. Application of a pinch induced the anticipated gland eversion and (in many cases) rectal emission, but there was also an immediate flexing of the abdomen, executed in such fashion that the abdominal tip was brought into direct contact with the forceps. As a result, the latter were often visibly wetted by the beetle's defensive fluid. The abdominal tip is extraordinarily mobile. It can be flexed upward at a sharp angle, as well as forward beneath the body to almost within reach of the head (Figure 4). There is therefore no way that an enemy can seize *Creophilus* without incurring the risk of being contacted by the abdominal tip.

Dissection of freshly killed beetles under saline solution revealed some of

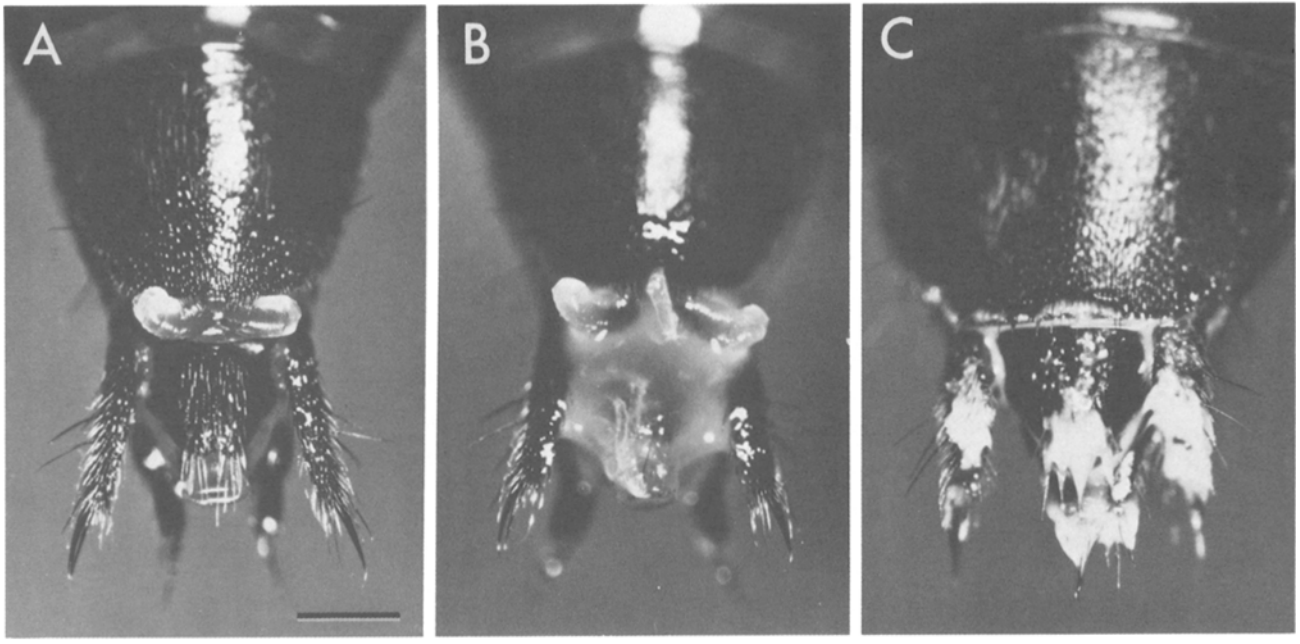


FIG. 3. Close-up view of abdominal tip of *C. maxillosus*, showing three consecutive stages in the defensive response. (A) glands are everted and anal tube is protruded; (B)rectal fluid has been discharged and has spread over abdominal tip and base of glands;(C)over 1 min later, the defensive fluid has dried to a white pasty remnant. Reference bar in A = 0.5 mm.

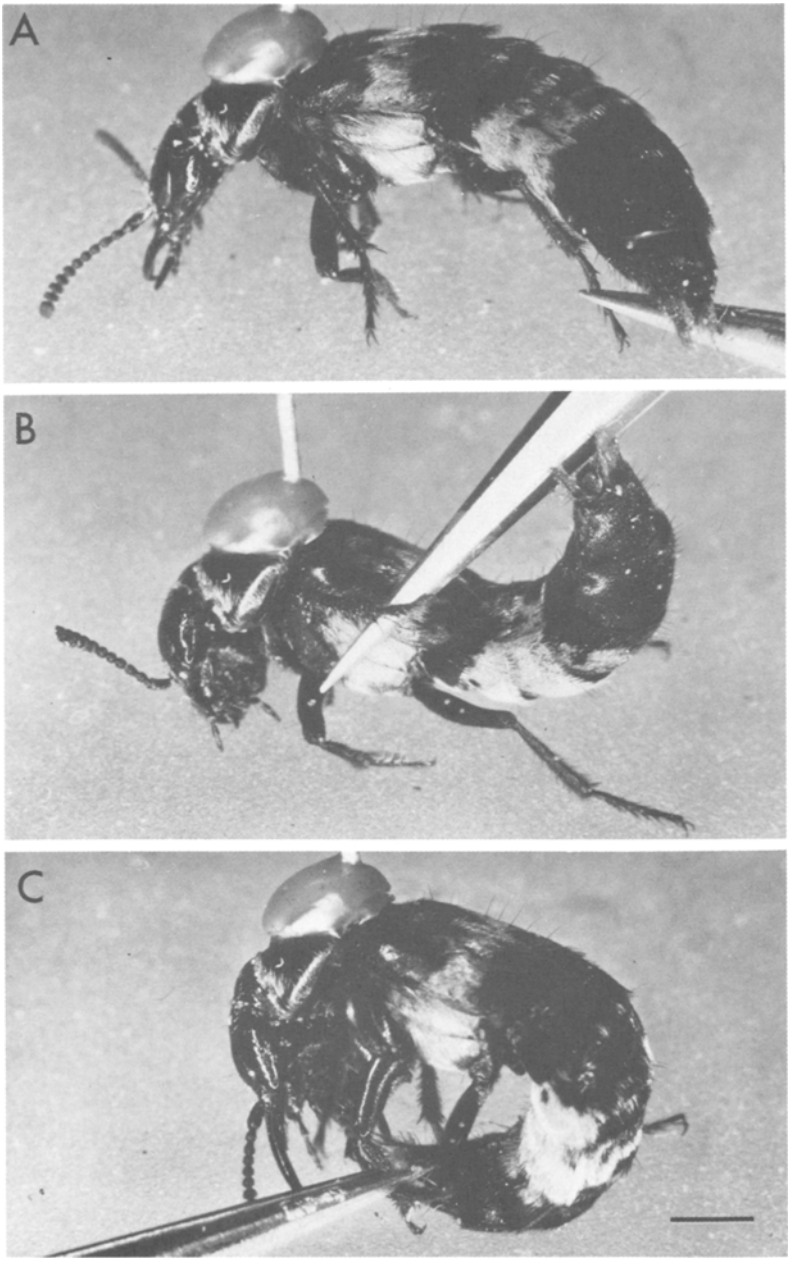


FIG. 4. Tethered *C. maxillosus* responding to pinchings of the left hindleg (A), midsection of body (B), and left midleg (C) with forceps. Note that the beetle revolves the abdomen so as to wipe the abdominal tip against the offending agent. Reference bar = 2 mm.

the anatomical features of the glands. No effort was made to work out the details of the musculature, which is complex, as might be expected given the eversibility of the glands. One set of muscles, which presumably effect glandular retraction, have tergal insertions ahead of the blind ends of the glands and attachments on the glands themselves. Gland eversion might be effected indirectly by body constriction and consequent rise in hemocoel

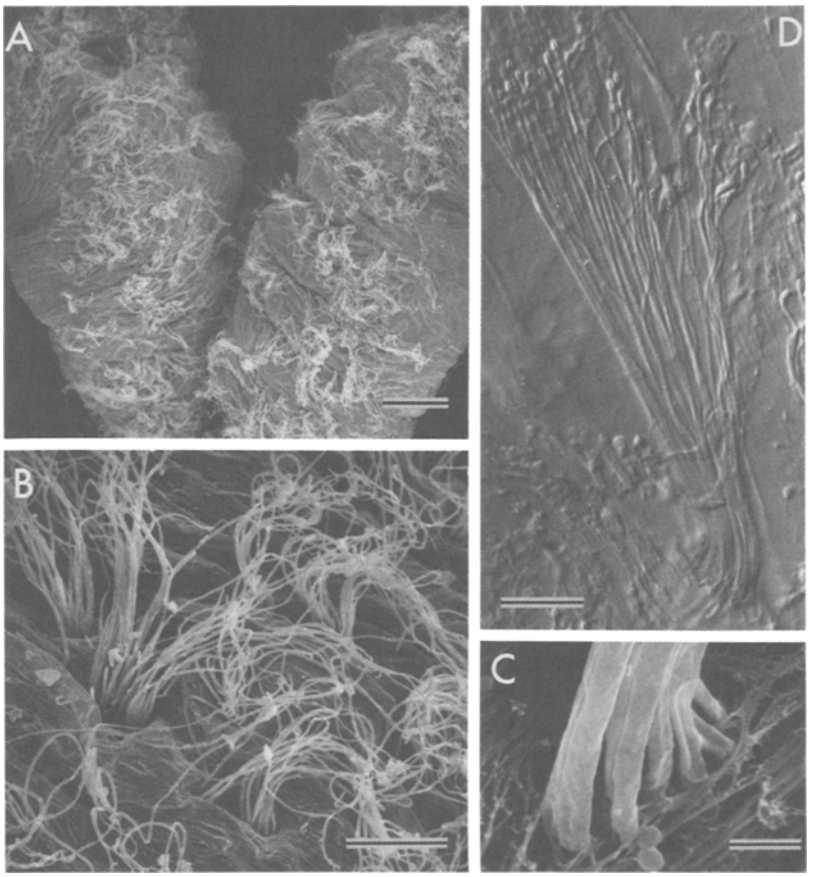


FIG. 5. Secretory tubules of *C. maxillosus* glands, isolated by KOH treatment of glands. (A) Surface view of glands showing clustered arrangement of the tubules (B) Detail of A, showing how the tubules bunch at their sites of junction with the cuticular lining of the gland. (C) Enlarged view of one such junction. (D) Cluster of secretory tubules shown in full length; their swollen distal portions in upper part of figure are ordinarily associated with the secretory cells. Reference bars: A = 100 μm; B and D = 20 μm; C = 2 μm. Figures 5A–C are scanning electronmicrographs; Figure 5D is by Nomarski phase-interference contrast.

pressure. As anticipated, enveloping compressor muscles are entirely lacking in the glands.

The glandular tissue is part of the gland wall itself, but extends over the ventral surface of each gland only (i.e., the surface facing the body cavity in the retracted gland) (Figure 5). It takes the form of an opaque band of tissue which upon microscopic examination (unfixed and unstained whole mounts of glands; phase and Nomarski phase-interference microscopy) was found to be traversed by innumerable tiny cuticular tubules, such as are commonly present as cellular drainage ducts in insectan exocrine tissue (Noirot and Quennedey, 1974). By optical focusing, the trajectory of these tubules was traced from their origin in the midst of the glandular tissue to their ending on the cuticular lining of the glands (Figure 5D). No such tubules were apparent in the dorsal wall of the glands, which is thin and translucent, and overlain by a conventional unspecialized squamous eqithelium.

Examination of KOH-treated glands (Figure 6) showed the tubules to occur in clusters, with tight bunching where these merge with the cuticular gland lining. In the everted gland, where the inner gland surface is exposed, the openings of the ducts are clearly visible (Figure 6D). A series of tiny tufts project from the everted gland surface (Figure 6C, D). These are clusters of cuticular filaments, grouped around the openings of the ducts.

Creophilus vs. Ants. Encounters were staged between *Creophilus* and ants of an aggressive species (*Formica exsectoides*). The beetles were presented singly to groups of five ants that had been preintroduced into small plastic dishes. The ants attacked at once. No sooner had one clamped itself with its mandibles to an appendage of the beetle than the latter responded by revolving its abdomen and touching the abdominal tip to the ant. The latter released its hold and fled and within seconds commenced intensive cleansing activities. As it fled it often dragged portions of its body against the substrate, as ants typically do when contacted by the defensive secretion of intended prey (Eisner, et al., 1961). Events transpired quickly, and it was not always possible to determine with certainty whether gland eversion had accompanied the defensive maneuverings of the beetles. But in some of the more than 20 such encounters that were witnessed, eversion did occur, as did apparently defecation, since there was on occasion a relatively copious output of fluid from the beetle's abdominal tip. The beetles also proved capable of using their mandibles for defense. Ants were often maimed by a beetle's bite, and not infrequently killed by decapitation or transection.

Chemistry of the Secretion. Gas chromatographic (GC) examination of whole-gland extracts showed compositions independent of solvent type and sex. The analytical trace (Figure 1) revealed four major and two trace (A,D) components. These six components were also discernable, albeit against a more "noisy" chemical background, in the extract of the abdominal milkings,

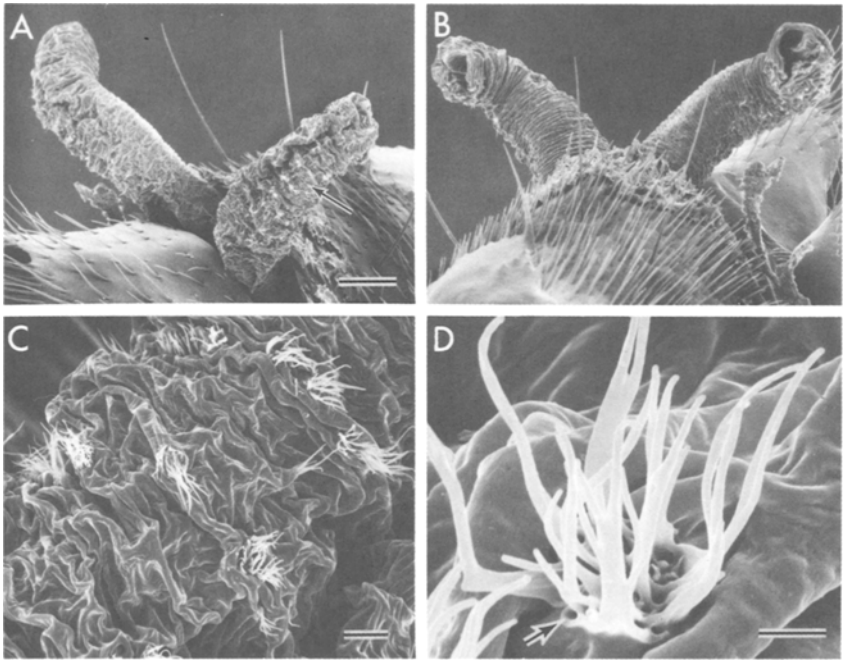
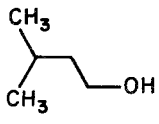


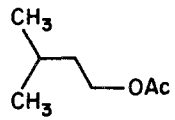
FIG. 6. Scanning electron micrographs of partially everted glands of *C. maxillosus*. Abdominal tip is in left foreground in (A), and right background in (B). The cuticular tufts denoted by arrow in (A), are shown enlarged in (C) and (D). Note openings of secretory tubules (arrow, D) associated with the base of the filaments of the tufts. Reference bars: A, B = 200 μm; C = 20 μm; D = 4 μm.

providing evidence that the compounds are indeed externalized during defense, but in admixture with additional material, stemming presumably from the fecal fluid.

Components A–F were initially characterized by their mass spectra. Thus, A was recognized as isoamyl alcohol (I); the identification was confirmed by direct GC-MS comparison with an authentic sample (Heller and



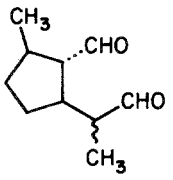
I



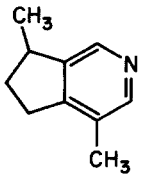
II

Milne, 1978). Similarly, B was proven to be isoamyl acetate (II) by its mass spectrum (Heller and Milne, 1978), by direct GC-MS comparison with an authentic sample, and by the [^{1}H]NMR spectrum of a GC-collected sample.

The mass spectrum of C [*m/e* 168(2), 150(8), 135(43), 111(64), 109(58), 93(45) and 81(100)] showed a parent ion suggesting the molecular formula $C_{10}H_{16}O_2$, and its infrared spectrum showed strong carbonyl absorption at 1720 cm^{-1} along with strong aldehydic C–H absorption at 2720 cm^{-1}. The [^{1}H]NMR showed the presence of two aldehydic protons (δ 9.62, 2H, m). These data suggested that C might be one of the stereoisomeric iridodials (III), a structure previously isolated from several other staphylinid species (Weatherston and Percy, 1978). A synthetic sample of iridodial was prepared from citronellal as described previously (Clark, et al., 1959). This synthesis yields a mixture of several steroisomeric iridodials which were partially separated by GC on a Carbowax 20 M column. The major GC peak from the synthetic material proved indistinguishable [GC-MS, IR, [^{1}H]NMR (in C_6D_6 and $CDCl_3$)] from the natural product.



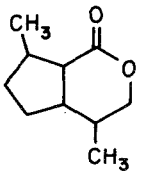
III



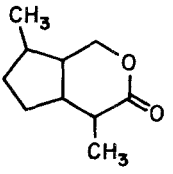
IV

Component D, which was found only in small and variable quantities, showed in its mass spectrum a parent ion at *m/e* 147, corresponding to the molecular formula $C_{10}H_{13}N$. Given the presence of iridodial in this secretion, it seemed likely that D might be the closely related methylcyclopentanoid alkaloid actinidine (IV). This supposition was confirmed by direct GC-MS comparison of D with an authentic sample of actinidine.

The major component of this secretion, compound E, was revealed to be closely related to iridodial and actinidine by its mass spectrum [*m/e* 168(1), 113(65), 110(48), 95(58), 81(100), 67(59)]. A single, strong carbonyl absorption in its infrared spectrum was observed at 1740 cm^{-1}. In its [^{1}H]NMR spectrum, two methyl doublets (δ 1.19, J = 5.8 Hz; 1.00, J = 6.3 Hz) and the AB portion of an ABX pattern (δ 4.38, J = 6, 11 Hz; 3.85, J = 7, 11 Hz) were prominent. These data suggested that E might be either a dihydronepetalactone (V) or an iridolactone (VI). A choice between these two structural alternatives was facilitated on the basis of the observation by Sakan et al. (1965) that dihydronepetalactones undergo a characteristic McLafferty type fragmentation, giving rise to an ion (*m/e* 113) which requires the proximity of the lactonic carbonyl group to the cyclopentanoid methyl group. This ion



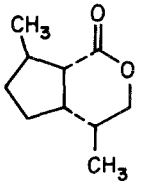
V



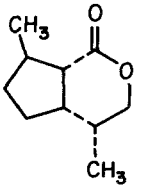
VI

does not appear in the mass spectra of the iridolactones (Stenhagen et al., 1974). Since E shows a prominent *m/e* 113 fragment ion in its mass spectrum, a dihydronepetalactone structure seemed likely.

In order to ascertain the relative stereochemistry of E, the literature describing the dihydronepetalactones was reviewed in detail and was found to contain conflicting data. Syntheses of four of the eight possible diastereomeric



VA



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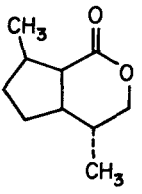
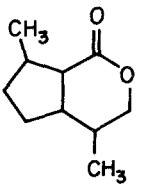


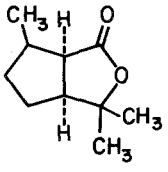
VC



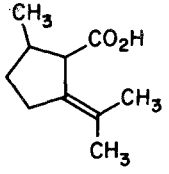
VD

forms of V (VA, VB, VC, and VD) have been described by Wolinsky and Eustace (1972). The spectral data (MS, [^{1}H]NMR, IR) for one of these isomers, designated as "VC" by these authors, appeared to correspond closely to those of our natural product. In a later publication, however, Ficini and d'Angelo (1976) describe an alternative (and unambiguous) synthesis of VC, which gives a product with an [^{1}H]NMR spectrum distinctly different from that reported by Wolinsky and Eustace.

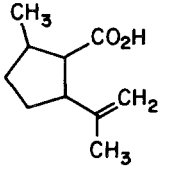
In an attempt to reconcile these differences, we repeated the earlier synthetic work. Starting with *cis,cis*-puleganolide (VII), treatment with potassium *tert*-butoxide in DMF gave a mixture of isomeric unsaturated



VII



VIII

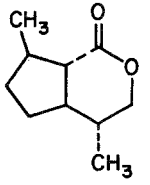


IX

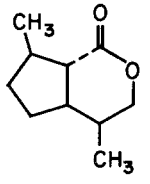
acids, VIII and IX, which was hydroborated (9-BBN) and oxidized (H_2O_2) to give an uncharacterized mixture of hydroxy acids. When this mixture was lactonized thermally (neat, 175° C, 1 hr), three major lactones were obtained, rather than the two ("VC" and VD) previously described. The properties of the additional isomer corresponded closely to those of the Ficini and d'Angelo VC, and direct comparison of this product with a sample provided by these authors proved their identity.

When the lactonization of the hydroxy acid mixture was carried out under milder conditions (benzene, *p*-toluenesulfonic acid at 80° C), only two major lactones were obtained, corresponding to the Ficini VC and the Wolinsky VD. From the latter synthetic sequence, only *cis*-fused lactones would be anticipated, and the designation of these two products as VC and VD appears secure.

Since the Wolinsky "VC" most closely resembled the beetle lactone, and since it appeared likely that this compound must be a *trans*-fused isomer formed by epimerization of a *cis* precursor during the rigorous lactonization experiment, the two authentic *cis*-fused lactones were separated by preparative GC and were subjected to acid-catalyzed epimerization (refluxing xylene, *p*-toluenesulfonic acid, 12 hr). Under these conditions, each *cis* isomer gave rise to an equilibrium mixture of *cis* and *trans* isomers. In this way, the component originally designated as "VC" was revealed to be the *trans* epimer of VD, and we can now assign it the formula VF. While this *trans* lactone resembled the natural product much more closely than either of the *cis*-fused epimers, GC coinjection showed that the compounds were not identical. However, VE, the *trans* lactone obtained by analogous acid-catalyzed epimerization of VC, proved identical to the beetle component ([^{1}H]NMR, IR, GC-MS, and GC coinjection on OV-1 and Carbowax 20 M columns) (Grant et al., 1980).



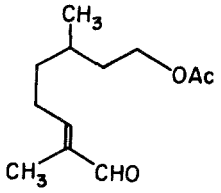
V E



V F

The final component (F) in this secretion showed a molecular weight of 212 (*m/e* 213 in its chemical ionization mass spectrum). In its [^{1}H]NMR spectrum ($CDCl_3$), it shows an aldehydic proton (δ 9.40, 1H, s), an olefinic proton at δ 6.48 (1H, t, 7 Hz), a two-proton triplet at δ 4.12 (2H, t, J = 6 Hz), a two-proton multiplet (δ 2.40, 2H), a three-proton singlet (δ 2.04, 3H), a

three-proton singlet (δ 1.75, 3H, br), and a three-proton doublet (δ 1.00, 3H, J = 5.8 Hz). Irradiation of the J = 6.48 proton reveals coupling to the two protons at δ 2.40. A strong ultraviolet absorption ($\lambda_{max}^{Et_2O}$ = 232 nm) indicated a conjugated enone chromophore. These observations, considered along with the structures of the other components which occur in this secretion, suggested that F might be (*E*)-8-oxocitronellyl acetate (X). This structure was



X

confirmed by oxidation of citronellyl acetate using selenium dioxide in ethanol as described by Robinson et al. (1962), which gave a sample of X that was indistinguishable from the natural product.

Quantitative assay showed the four major components of the secretion to be present in the following relative ratio: dihydronepetalactone (39%), iridodial (27%), isoamyl acetate (21%), and (*E*)-8-oxocitronellyl acetate (13%). The sum total of these was estimated to be 70 μg per beetle.

Repellency of Individual Secretory Components to Ants. When presented with circular drops of dilute honey, foraging workers of *Monomorium destructor* completely encircle the drops and feed to repletion. As individuals depart after satiation, they are quickly replaced by newcomers. If a capillary tube bearing a chemical repellent is introduced in close proximity to the ants, they cease feeding and disperse (Figure 7). The proportion of ants that leave the drop and the rapidity of their dispersal provide a measure of the repellency of the compound.

A standardized test was developed by which the repellency of the four major components of the *Creophilus* secretion was assayed. The two trace compounds, actinidine and isoamyl alcohol, were not tested. For a given test, a microcapillary tube (tip diameter = 0.825 $\pm$ 0.025 mm, N = 10), filled with a particular compound, was abruptly swung into place with a mechanical arm that brought the capillary tip to a point 2 mm above ground just outside the margin of a drop of bait (50% aqueous honey solution). The response of the ants was videotaped for subsequent stop-frame analysis. Counts were made of the numbers of ants initially present at the drop, as well as of the numbers that remained feeding at set times (5, 10, 15, 20, and 25 sec) after presentation of the capillary tube. Four replicate tests were carried out per compound. For comparative purposes, tests were also done with three known repellents of

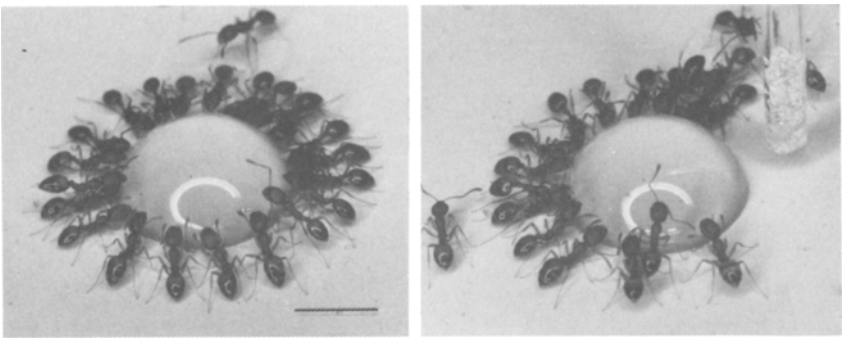


FIG. 7. Ant bioassay scenario (*Monomorium destructor*). Ants initially uniformly distributed around a drop of bait (dilute honey), respond by dispersing when a capillary tube with repellent (camphor crystals) is brought to the margin of the drop. For the actual bioassays, a microcapillary of smaller tip diameter was used. Details in text. Reference bar = 2 mm.

arthropod origin and with two inert compounds (glycerin and H_2O). The data (Figure 8), give the time course of the repellency of the various compounds. Water (data omitted for clarity in Figure 8) proved as inactive as glycerin.

DISCUSSION

Many arthropods that have defensive glands eject their secretions as an aimed spray. Those that are unable to spray often have alternative ways of administering the fluids to the enemy. One way is to transfer secretion from gland to target by means of legs. Such "leg dabbing" is practiced, for example by certain tenebrionid beetles (Tschinkel, 1975), soldier beetles (Eisner et al., 1981), Hemiptera (Remold, 1962), and opilionids (Eisner et al., 1971). *Creophilus*, like other staphylinids (Brand et al., 1973), is unusual in that its glands are on a highly movable emplacement, the abdomen, by which the gland itself is brought into contact with the enemy. The specialization is not without counterparts. Papilionid caterpillars, which also have eversible glands (the osmeteria), albeit anteriorly behind the head, resort to postural adjustments of the front end to brush their glands against an attacker (Eisner and Meinwald, 1965). Abdominal maneuverings essentially similar to those of *Creophilus* are found in earwigs, although these have mechanical pincers in lieu of glands at their rear ends (some do have spraying glands near the base of the abdomen that are aimed by abdominal rotation) (Eisner, 1960). It is interesting that both staphylinids and earwigs have characteristically short front wings, an adaptive concomitant, one would imagine, of their defensive dependence on abdominal mobility.

The glands themselves of *Creophilus* are in no major way unusual. Like other eversible glands of insects, they appear to depend on blood pressure for eversion (Eisner and Meinwald, 1965, 1966), and they possess the typical cuticular drainage tubules that are so commonly found in arthropod glandular tissue (Noirot and Quennedey, 1974). One wonders about the function of the cuticular tufts associated with the tubule openings on the gland lining. One possibility is that the tufts retain secretion as discrete droplets when the glands are everted, thereby facilitating the smeared transfer of fluid when the glands are wiped against the enemy.

Abdominal glands are widespread among staphylinids, vary in structure and function, and seem to have arisen more than once within the family (Jenkins, 1957; Blum et al., 1971; Brand et al., 1973; Schildknecht et al., 1975). Good morphological studies have been done of the glands of some genera, including termitophilous and myrmecophilous forms (Pasteels, 1968a,b, 1969). For free-living species, the chief function of the glands, even in cases where these are not apparently homologous to those of *Creophilus*, seems to be defense. Administration of secretion through abdominal rotation has been demonstrated in some species, as has the deterrence of the secretion to ants

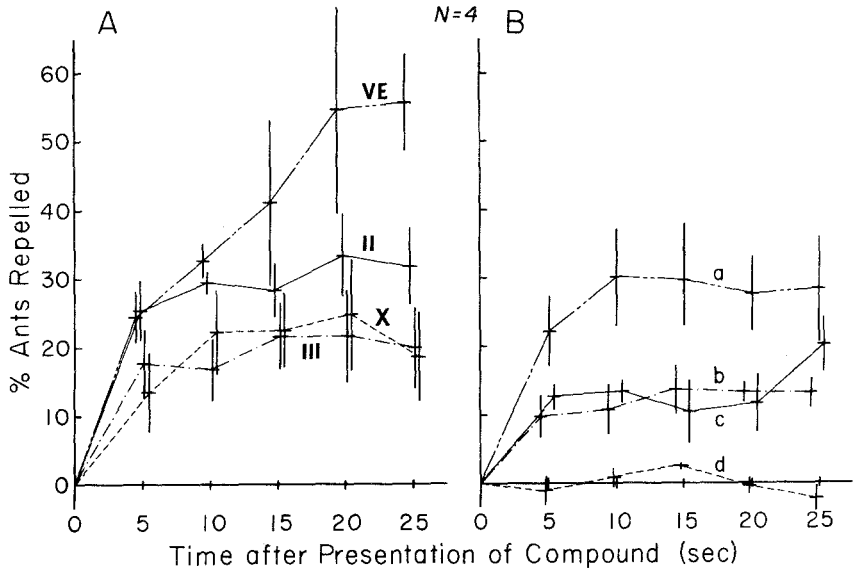


FIG. 8. Repellency of the four principal *C. maxillosus* compounds (A), and of four reference compounds (B), to the ant *M. destructor*. Numbers in (A) correspond to *Creophilus* compound numbers in text; a = *m*-cresol, b = (*E*)-2-hexenal, c = salicylaldehyde, d = glycerin. Horizontal lines = means; vertical lines = one standard error on each side of mean.

(Wheeler et al., 1972; Brand et al., 1973; Kolbe and Proske, 1973). Some exceptional staphylinids use their secretion as a water surface-tension depressant, to propel themselves forward when in need to flee over water (Schildknecht et al., 1975).

The principal component of the *C. maxillosus* secretion, the *trans*, *trans*-isomer of dihydronepetalactone, appears not to have been found in nature, although closely related lactones are known from other sources (Nakanishi et al., 1974). Iridodial has been isolated from other staphylinids, including the Australian *Creophilus erythrocephalus* (Bellas et al., 1974). Isoamyl acetate is a well-known alarm pheromone of honey bees (Boch et al., 1962). Actinidine, one of the lesser compounds in *C. maxillosus*, is also known from other staphylinids (Bellas et al., 1974). The presence of (*E*)-8-oxocitronellyl acetate in a secretion composed largely of methylcyclopentanoid terpenes is of special interest, since this acetate appears closely related to a probable biosynthetic precursor of these compounds.

Methylcyclopentanoid monoterpenes are found in many plants and animals, and it is apparent that the biochemical apparatus for their synthesis is widely distributed (Nakanishi et al., 1974). As is true of many natural products, however, the roles of these compounds have not often been established. In the case of nepetalactone, it was the bizarre effect of catnip oil on the behavior of cats which drew attention to this group of terpenes (Meinwald, 1954). Since that time, a variety of roles, including a defensive one, has been established for these compounds (Eisner, 1964; Meinwald et al., 1977).

Our selection of ants for predation tests was dictated in part by our familiarity with these insects, which lend themselves ideally for bioassays of repellency. The tests with *Formica* showed convincingly that *Creophilus* is well protected against ants, but they left some question about the effectiveness of the secretion per se, since the fluid seemed so often to be delivered to the ants in admixture with feces. The assays with *Monomorium* showed the four principal components of the secretion to be, in themselves, effective, and the major component, dihydronepetalactone, to be the most effective. The reference compounds, *m*-cresol, *trans*-2-hexenal, and salicylaldehyde, which stem from a variety of arthropod sources (Bettini, 1978), were all less effective than dihydronepetalactone.

It would obviously have been desirable to test for the vulnerability of *Creophilus* to a whole series of predators, rather than just ants. One is tempted to predict that the secretion must be active also against other insects, since nepetalactone, the closely related catnip terpene, appears to be a broad-spectrum insect repellent (Eisner, 1964). But whether the secretion is effective also against vertebrates such as birds and mammals, which must figure among the beetle's enemies, remains unknown. And one wonders about the relative

contribution of the fecal additive to the overall effectiveness of the defensive fluid. Emission of enteric fluids is a common defensive reaction in insects (Eisner, 1970), and the rectal effluent of *Creophilus* may well be, in some measure, intrinsically deterrent.

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ETHANOL AND OTHER HOST-DERIVED VOLATILES AS ATTRACTANTS TO BEETLES THAT BORE INTO HARDWOODS[1]

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Abstract—Ethanol, methanol, acetone, and acetaldehyde—chemicals identified in the inner bark of living trees—were used to bait vane traps placed in crowns of oak trees in Connecticut. Ethanol-baited traps caught more cerambycid, scolytid, and clerid beetles than unbaited traps. Buprestidae were not attracted to ethanol. Acetaldehyde and acetone were not attractive to any family. A mixture of ethanol, methanol, and acetaldehyde was no more attractive than ethanol alone. The vane traps were very effective at catching Cerambycidae and Scolytidae, but ineffective compared to sticky panels at catching Buprestidae.

Key Words—Coleoptera, Buprestidae, Cerambycidae, Cleridae, Scolytidae, wood-boring beetles, bark beetles, ethanol, host attractants, hardwood tree insects.

INTRODUCTION

The concentration of ethanol in logs, sapwood, and bark was shown to increase markedly when these tissues were held under anoxic conditions (Cade et al., 1970; Moeck, 1970). The researchers also found that ethanol was an attractant to the ambrosia beetles, *Gnathotrichus sulcatus* LeConte and *Trypodendron lineatum* (Olivier). Ethanol has since been reported as an attractant to several scolytid species (Moeck, 1971; Roling and Kearby, 1975)

[1]Mention of a proprietary product or company name does not constitute endorsement by the USDA Forest Service.

and as a synergist for the aggregation pheromone of *Gnathotrichus* spp. (Borden et al., 1980).

There is circumstantial evidence that ethanol may be attractive to other insects. A major product of fermentation is ethanol. Fermenting baits have long been used to attract insects, particularly Lepidoptera, and have been reported to attract Elateridae and Cerambycidae (Champlain and Knull, 1932). The smell of ethanol from oaks that were declining or infested with two-lined chestnut borer, *Agrilus bilineatus* (Weber), was reported by Coté and Allen (1980).

Wood-boring beetles that attack oak trees following defoliation by gypsy moth caterpillars were of particular interest to us. We suspect that defoliation promotes anoxic conditions that favor production of ethanol. Tree defoliation results in reduced transpiration, which can lead to a marked increase in bole and stem hydration (Stephens et al., 1972). Reduced transpiration also results in higher soil moisture levels and thus prolongs anaerobic soil conditions during periods of high precipitation. Waterlogging of tree roots is known to lead to substantial increase in root ethanol levels (Coutts and Armstrong, 1976). Stem ethanol levels are strongly correlated with ethanol levels in roots (Crawford and Baines, 1977). Defoliation of oak seedlings has been shown to result in increased ethanol content of stems (Wargo, unpublished).

In this paper, we report on the potential of ethanol and other associated host-derived volatiles as baits to attract wood-boring beetles to traps. Vane and sticky-panel traps were tested in the crowns of trees where beetle attack is usually initiated. Our primary objective was to capture *Agrilus bilineatus* and other Buprestidae that attack living oak trees.

METHODS AND MATERIALS

Traps. Vane traps were fabricated from Plexiglas® and common laboratory plastic-ware (Figure 1). The vanes were 25 cm high by 20 cm wide. A 500-ml bottle was fitted to the funnel by drilling a hole through the bottle's lid and holding the lid onto the funnel stem with a neoprene gasket glued to the stem beneath the lid. The bottle served both to collect beetles and dispense test chemicals. Test chemicals also were placed in an inverted 220-ml plastic cup that had its lid glued to the trap top. Chemicals were dispensed from the cup through a capillary pipet onto a cotton wick.

Sticky panels were prepared by coating one side of 25- × 25-cm white paperboards with Tangle-Trap® (The Tanglefoot Company, Grand Rapids, Michigan). The paperboard was backed with fiberboard to provide rigidity. When the sticky panel was raised into the lower tree crown, it was positioned so the sticky white surface faced out, away from the bole of the tree.

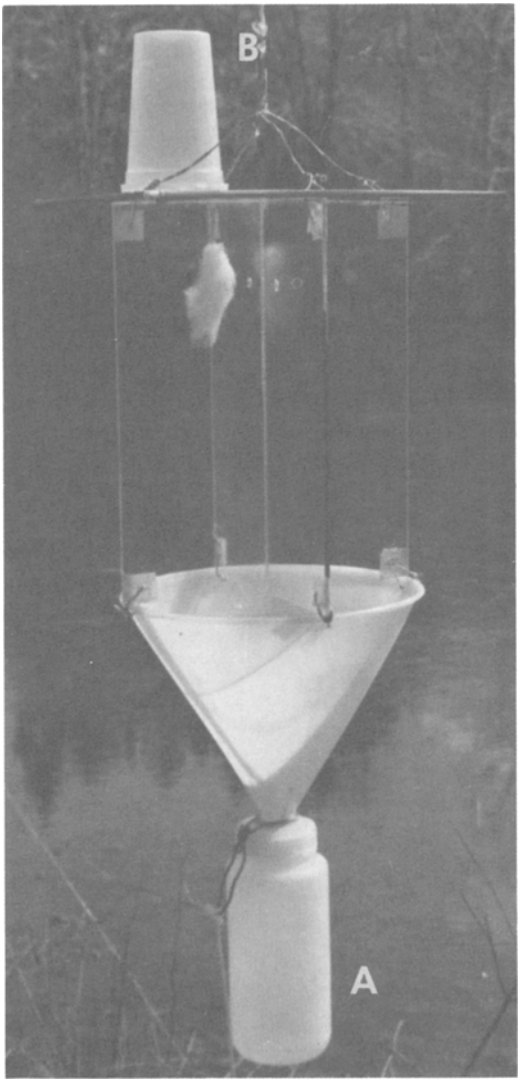

FIG. 1. Vane trap; A is the collecting bottle, which also dispensed treatment chemicals, and B is another dispenser used to release higher rates of chemicals.

TABLE 1. TEST SOLUTIONS[a] USED IN 1979 AND 1980

1979			1980
Treatment	Bottom container[b]	Top container	Treatment[c]
Control	water		Control (water)
Ethanol, low	50% ethanol		50% ethanol
Ethanol, med	50% ethanol	50% ethanol[e]	25% acetone
Ethanol, high	50% ethanol	50% ethanol[f]	25% acetaldehyde
Mixture	50% ethanol	50% ethanol +5% methanol +5% acetaldehyde[e]	bark extract[d] in water bark extract in 50% ethanol

[a]Percentages are v/v.
[b]Bottom containers also had 0.05% sodium hypochlorite aqueous bleach.
[c]Placed in bottom containers which also contained 0.05% Sodium Omidine® biostat.
[d]Equivalent to 1 g bark/10 ml final solution.
[e]Metered through 5-μl capillary.
[f]Metered through 10-μl capillary.

Test Solutions. Selection of test chemicals (Table 1) was based initially on Moeck's (1970) report that ethanol, acetaldehyde, and methanol were present in aged logs. A combination of these three chemicals as well as three levels of ethanol were tested in 1979. During 1979 we (unpublished) vacuum cold-trapped and chromatographed volatiles from inner bark of living trees. In addition to the chemicals reported by Moeck, we found acetone and therefore included it in our 1980 tests. An extract from freeze-dried red oak inner bark also was included in two treatments in 1980. This extract was prepared by grinding the bark to 0.5 mm, homogenizing the powder with water (0.2 g bark/ml), and then filtering. The extract was diluted 1:1 with water or ethanol before placement in the bottom container. Sodium Omidine® (Olin Corporation, Stamford, Connecticut) was added to the bark homogenates and to all other 1980 treatments to prevent fermentation and bacterial growth.

A low rate of ethanol was released by placing 100 ml of 50% ethanol in the catch (bottom) bottle. Higher rates were released by dispensing ethanol from both the catch bottle and top container. By metering top container ethanol through either a 5-μl or 10-μl capillary pipet, medium and high release rates, respectively, were obtained. Approximate ethanol release rates from traps held indoors at 21° C were determined by measuring weight loss and change in concentration. Ethanol loss from the bottom container was about 2 g/day. An additional 8 and 27 g/day were released from the capillaries in the medium and high ethanol treatments, respectively. Total ethanol release at the low, medium, and high rates was thus about 2, 10, and 29 g/day, respectively.

These values do not represent release rates in the field, only relative differences between the treatments.

Experimental Area and Design. Tests were conducted in Madison, Connecticut, in a forest consisting predominantly of oak (*Quercus rubra* L., *Q. alba* L., and *Q. velutina* Lamar) with less than 15% red maple and hickory. Traps were placed 8–15 m from the forest floor in tree crowns. They were raised and lowered by a rope pulley for weekly collection of beetles and renewal of the test solutions. Tests were conducted for 11 weeks each year starting May 30 in 1979 and May 22 in 1980.

In 1979, there were five treatments arranged in five blocks with three replicates of each treatment per block. All traps were placed in red oak trees and were at least 15 m apart. In a separate area, a sticky panel was placed in the crown of each of 48 red oak trees. The capillary system used with the vane traps was fastened to 24 randomly selected panels and used to release 50% ethanol at a medium rate (8 g/day).

In 1980, 36 red oak and 36 white oak trees no closer than 10 m from one another were selected, and six trees of each species were assigned randomly one of six treatments (baits). The treatments were placed in vane traps, and an unbaited sticky panel was placed above each vane trap.

Statistical Analysis. In both years, catch numbers were summed for the 11-week period and transformed to $\sqrt{Y + 0.5}$ for analysis by ANOVA of treatment effects. With the 1980 data, effect of tree species was analyzed along with treatment effects. Comparisons between treatments were made at the 0.05 level using either Scheffe's multiple comparison test or the t test. Data presented are simple means.

RESULTS

1979 Tests. Vane traps containing ethanol caught more cerambycid, clerid, and scolytid beetles than traps containing the water control (Table 2). The three levels of ethanol release did not evoke a typical dosage response. The lowest dosage caught the most Scolytidae, but dosage had little effect on Cerambycidae and Cleridae capture. Traps with the mixture of ethanol, methanol, and acetaldehyde did not catch significantly more beetles of any family than traps with only ethanol released at the same medium rate.

Too few Buprestidae were caught in the vane traps to draw conclusions about the attractancy of ethanol to this family. In a separate test using sticky panels, greater numbers of Buprestidae were captured; however, no significant attraction to ethanol was found: panels baited with ethanol caught a mean of 3.3 Buprestidae/panel, whereas unbaited panels caught 2.7 Buprestidae/panel. In comparison, a mean of 43.0 Scolytidae were caught on ethanol-baited sticky panels, while unbaited panels caught 1.6 Scolytidae.

TABLE 2. MEAN NUMBER/TRAP OF BEETLES CAUGHT BETWEEN MAY 30 AND AUG. 13, 1979, IN VANE TRAPS WITH DIFFERENT BAIT SOLUTIONS

	Treatment[a]				
		Ethanol			
Family	Water	Low	Medium	High	Mixture
Buprestidae[b]	0.4a	0.3a	0.3a	0.4a	0.7a
Cerambycidae	5.6a	8.3b	11.3b	10.1b	10.1b
Cleridae	1.5a	5.0b	5.3b	5.5b	4.9b
Scolytidae	32.8a	223.7c	144.6b	159.9bc	148.6b

[a]See Table 1 for a full description of treatments.
[b]Row means followed by the same letter are not significantly different, $P < 0.05$, Scheffe's test.

1980 Tests. Vane traps baited with ethanol again caught more cerambycid, clerid, and scolytid beetles than control traps with water (Table 3). Neither acetone nor acetaldhyde was attractive to any of the families examined. Adding the bark extract had no significant effect on beetle catch; however, since the extract was equivalent to only 10 g of bark, the lack of significant effect could be due to insufficient potency.

Relative suitability of trap design for each of the four beetle families was examined by comparing capture rates of unbaited vane traps with capture rates of sticky-panel traps placed above the unbaited vane traps (Table 4). Fewer buprestid beetles were caught in vane traps than on sticky panels, whereas more scolytid and cerambycid beetles were captured in vane traps.

TABLE 3. MEAN NUMBER/TRAP OF BEETLES CAUGHT BETWEEN MAY 21 AND AUG. 6, 1980, IN VANE TRAPS CONTAINING DIFFERENT BAIT SOLUTIONS

	Treatment[a]					
Family	Water	Ethanol	Acetone	Acetaldehyde	Bark water	Bark ethanol
Buprestidae[b]	0.67a	0.50a	0.50a	0.42a	0.75a	0.67a
Cerambycidae	0.83a	6.58b	1.83a	1.50a	1.33a	4.67b
Cleridae	0.75a	3.58b	1.33a	1.17a	1.08a	2.58b
Scolytidae	5.67a	114.92b	5.08a	2.17a	2.83a	148.67b

[a]See Table 1 for a full description of treatments.
[b]Means in a row followed by the same letter are not significantly different, $P < 0.05$, Scheffe's test.

TABLE 4. COMPARISON OF VANE TRAPS AND STICKY PANELS USING ONLY CONTROL TRAPS

Family	Mean number captured/trap	
	Vane	Sticky
Buprestidae	0.71	4.98*[a]
Cerambycidae	0.92	0.33*
Cleridae	1.08	1.13 ns
Scolytidae	4.38	2.50*

[a]Asterisk indicates significant difference by paired t test ($N = 24$, $P < 0.05$).

Equal numbers of Cleridae were caught with both traps. Based on area of catching surface, the vane traps would be expected to capture two or three times (two times if the side of the vane trap towards the tree is considered a noncatching surface) as many beetles as the sticky traps.

Sticky panels were placed above the vane traps primarily to monitor possible effects of tree species and tree condition. Beetle capture on sticky panels, however, did not prove to be independent of trap treatment. Sticky panels above ethanol-baited vane traps caught significantly more Scolytidae than those above control traps (224 vs. 60, respectively), and more Cleridae (46 vs. 27, respectively). Buprestidae were caught in near equal numbers (93 vs. 98, respectively) on sticky panels above traps with ethanol compared to sticky panels above water treatments. Very few Cerambycidae were caught on sticky panels regardless of vane trap treatment, 6 vs. 8 on sticky panels above ethanol treated traps and control traps, respectively. The acetone and acetaldehyde treatments in vane traps did not have any signficant influence on numbers of beetles caught on panels.

Tree species had little effect on either vane trap or sticky panel catch of three of the four families. Preference for tree species probably operates at the species rather than the family level. The scolytid beetle caught in greatest number, *Xyleborus dispar* (F.), was caught less frequently in vane traps placed on red oak than in traps placed on white oak, a ratio of 0.6:1, respectively. Differences in preference between *Q. alba* and *Q. rubra* by other species of Scolytidae or by any species of Buprestidae, Cleridae, or Cerambycidae were not detected.

DISCUSSION

Based on the work of Roling and Kearby (1975), who caught over 25 scolytid species in window-pane traps baited with 50% ethanol, we expected to find ethanol attractive to Scolytidae associated with oak forests. However,

Roling and Kearby did not include non-ethanol-baited traps in these tests; hence, it is difficult to gauge the actual attractancy of the ethanol. We found that ethanol-baited traps caught from 5 to 50 times as many Scolytidae as unbaited traps. All of the scolytid species caught in high numbers showed a statistically significant attraction to ethanol. *Xyleborus dispar* (F.) accounted for nearly half the specimens recorded, while *Xyleborinus saxeseni* (Ratzeburg), *Monarthrum mali* (Fitch), and *Pseudopityophthorus minutissimus* (Zimmermann) each accounted for 4–11%. Only female *X. dispar* were captured (males are flightless and do not leave the larval feeding gallery). With *M. mali* and *P. minutissimus*, males predominated. Moeck (1971) also caught *X. dispar* (=*Anisandrus pyri*), which is thought to attack only hardwoods, in great abundance in ethanol-baited traps placed in a Douglas-fir stand in British Columbia, Canada. It may be that ethanol is not only strongly attractive to this beetle but also effective at considerable distance.

Our lowest ethanol release rate, 2 g/day, was more attractive to Scolytidae than higher rates. Moeck (1970) evaporated ethanol from 450-cm pans and found that the lowest concentrations tested, 0.1–0.4%, captured more of the ambrosia beetle, *T. lineatum*, than concentrations of 2–30%. In aged logs that were attractive to the beetles, Moeck (1971) found ethanol concentrations of 0.02 M (<0.1%). It is possible that ethanol release rates lower than those we tested would be more attractive.

Although the Cerambycidae as a family showed attraction to ethanol, the response was not uniform at the species level. Two species, *Aegoschema modesta* Gyllenhal and *Graphisurus fasciatus* (De Geer), were clearly not attracted to ethanol. *Analeptura lineda* (Say), *Clytus ruricola* (Olivier), *Elaphididionides villosus* (Fab.), and *Urgleptes querci* (Fitch) are examples of species strongly attracted to ethanol. We could not discern any relationship between ecology or habits of the cerambycid species caught and attractancy to ethanol. Species that infest wood dead a year or more, as well as those that attack "freshly dead" wood, were attracted to ethanol. However, very low numbers of cerambycid species that attack living trees were caught.

All species of Cleridae caught in sufficient numbers to make statistical tests showed significant attraction to ethanol. These species are: *Cymatodera bicolor* Say, *Enoclerus nigripes* (Say), *Neorthopleura thoracia* (Say), *Phyllobaenus pallipenis* (Say), *Phylogistostermus dislocatus* (Say), and *Placopterus thoracius* (Olivier). Clerid beetles are predators of scolytid, cerambycid, and buprestid beetles, as well as other insects feeding in woody tissue. Ethanol odor likely is used by clerids as an aid in locating prey. It is unfortunate that Cleridae are attracted to ethanol in the sense that these beneficial predators will be caught along with bark- and wood-boring beetles in traps baited with ethanol. Use of specialized traps such as the Scandinavian "drain pipe" that have small holes through which Scolytidae crawl will not entirely exclude

Cleridae. The most abundant clerid beetle in our catches, *P. dislocatus*, is smaller in diameter than the most abundant scolytid beetle, *X. dispar*.

Buprestidae were not attracted to ethanol. Over 65% of the buprestid beetles caught were *Agrilus bilineatus* and *Chrysobothris sexsignata* (Say) with 14 species comprising the remainder. Neither of the two most abundant species considered individually showed attraction to ethanol. Lack of evidence for ethanol attractancy must be tempered because vane traps were very inefficient at catching buprestids, and only limited testing of ethanol was done with sticky panels.

No beetles were attracted to acetone or acetaldehyde. Both of these compounds can be formed in plants by oxidative metabolism of ethanol (Cossins, 1978). Acetone was found by Billings et al. (1976) in fresh ponderosa pine resin, and its loss from resin samples exposed to air was accompanied by a decline in scolytid beetle response to the pheromone-resin complex. They did not establish whether it was acetone or some other highly volatile nonterpene constituent that influenced resin attractiveness. Moeck (1970) found acetaldehyde present in anaerobically treated bark, but the chemical did not prove attractive to *T. lineatum* in olfactometer tests. Evidence regarding the attractiveness of low-molecular-weight volatiles other than ethanol is not conclusive, and they should not be overlooked in future studies.

Initially we theorized that ethanol was produced by trees under stress and consequently was used as a host location cue by beetles such as *A. bilineatus* that attack trees in the initial stages of decline. The beetles attracted to ethanol are those associated with trees that have actually reached the dead stage. It appears that production of ethanol in response to stress would be fairly rapid. Graham (1968) found that peak attractancy of woody tissues to *T. lineatum* occurred at between 20–28 hr of anaerobic processing. Crawford and Baines (1977) found that ethanol levels in the flood intolerant *Picea sitchensis* Carr. increased from 0.5 to 6 μmol/g fresh weight of root in 24 hr.

It is possible that ethanol may also be invoked by beetle infestation. The two families that were attracted strongly to ethanol also tend to seek out beetle-infested wood, the Cleridae in search of prey and the Scolytidae to form aggregations. For the latter, ethanol acts synergistically with aggregation pheromones (Pitman et al., 1975; Borden et al., 1980). Although we did not make ethanol measurements of beetle-infested tissue, we did often note, as did Coté and Allen (1980), a pronounced smell of ethanol from infested tissue. If high levels of ethanol are present in infested wood, it may be produced by microorganisms and decomposition processes connected with beetle activity. Microorganisms associated with bark beetles produce several related alcohols, ketones, and acetates (Brand et al., 1977). Ethanol, in particular, is produced in the initial stages of microorganism growth and thus could serve as a cue by which clerids and scolytids locate recently infested tissue.

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SEX PHEROMONE COMPONENTS OF THE EUROPEAN GOAT MOTH, *Cossus cossus*

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Abstract—Female tip washings of the European goat moth, *Cossus cossus* L., were found to contain the following compounds that are structurally similar to known pheromone components of Lepidoptera (%): decyl acetate (1.5), (*Z*)-5-dodecenol (1), (*Z*)-5-dodecenyl acetate (66), dodecyl acetate (12), (*Z*)-5-tetradecenyl acetate (14), tetradecyl acetate (4), and hexadecyl acetate (1.5). The washes contained an average of 125 ng of (*Z*)-5-dodecenyl acetate per female equivalent. EAG responses to nanogram amounts of the identified products indicated that (*Z*)-5-dodecenyl acetate was the most potent olfactory stimulant followed by (*Z*)-5-tetradecenyl acetate and (*Z*)-5-dodecenol, whereas the saturated acetates caused only weak depolarization. A strong EAG response was also recorded for (*Z*)-3-decenyl acetate, which was not detected in the female tip washings. Field results showed that (*Z*)-5-dodecenyl acetate was essential for the attraction of the males and that (*Z*)-3-decenyl acetate improved the attractiveness of (*Z*)-5-dodecenyl acetate alone or in combination with (*Z*)-5-tetradecenyl acetate.

Key Words—Sex pheromone, *Cossus cossus*, Lepidoptera, Cossidae, (*Z*)-5-dodecenyl acetate, (*Z*)-5-dodecenol, (*Z*)-5-tetradecenyl acetate, (*Z*)-3-decenyl acetate.

INTRODUCTION

The European goat moth *Cossus cossus* L. (Lepidoptera: Cossidae) is a destructive insect pest widely distributed throughout the palaearctic region. In Italy, the larvae inflict serious damage to broad-leaved trees and fruit trees by tunneling into their trunks.

Although the odorous secretion of the mandibular glands of the larvae has been characterized (Garanti et al., 1976), the female sex pheromone has not been identified.

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This paper describes the characterization of the major active components in the washes of pheromone glands from females, in an attempt to formulate a synthetic attractant for developing procedures to survey and control this pest.

METHODS AND MATERIALS

The insects used were collected in the field as last instar larvae (7–9 cm in length). These were maintained on pear wood sawdust till pupation. Pupae were segregated by sex and placed at 17–27° C and 70 ± 5% relative humidity on a 9-hr dark:15-hr light regime until moth emergence. Sex pheromone glands and ovipositors were manually extruded and snipped from the abdomen of female moths when they were 3–4 days posteclosion and 1–2 hr into the scotophase. The excised abdominal tips were each washed with 25 μl of redistilled hexane. Washes of ca. 2–4 female tips were filtered and without further clean-up were reduced to an appropriate volume using N_2.

Gas–liquid chromatography (GLC) was performed on a Carlo Erba 4160 gas chromatograph equipped with an automatic splitless injector system and a flame ionization detector. Glass open tubular capillary (GOTC) columns were used with liquid phases and temperature programing as follows: Carbowax 20 M (50 m × 0.23 mm ID, He carrier at 34 cm/sec), 80–160° C at 15° C/min and then isothermal; OV-101 (30 m × 0.21 mm ID, H_2 carrier at 32 cm/sec.), 50° C for 5 min, 50–100° C at 20° C/min, 100–260° C at 5° C/min, and then isothermal. In GLC analyses designed to detect microozonolysis products, the OV-101 column was held at 50° C for 6 min, and the temperature was then programed to 260° C at 5° C/min. The retention times of heptanal and nonanal were 11.5 and 18.8 min, respectively.

Glass open tubular capillary chromatography–mass spectrometry (GOTC-MS) was performed on a Varian-Mat 112 S instrument equipped with a MAT 200 data system interfaced with an OV-1 GOTC-column (25 m × 0.3 mm ID, He carrier at 30 cm/sec, splitless injection, 1 min 40° C, 5° C/min at 270° C, scan rate 1 dec/sec, reset time 0.7 sec, ion range m/e 29–500, filament current 1.5 mA, ionization voltage 70 eV).

Microepoxidation was carried out by reducing a hexane wash from two abdominal tips to a volume of 40 μl, adding 20 μg of *m*-chloroperbenzoic acid, and allowing it to stand at room temperature for 3.5 hr. The crude reaction mixture was quenched in liquid air and was then analyzed by GLC on the OV-101 column and by GOTC-MS.

The material used for the microozonolysis was obtained by GLC collection on a Carlo Erba 2350 gas chromatograph equipped with an OV-101 packed column on which the retention times of pheromone components had been previously determined in an analytical run. The hexane washings from five abdominal tips were used. The flame ionization detector was removed and

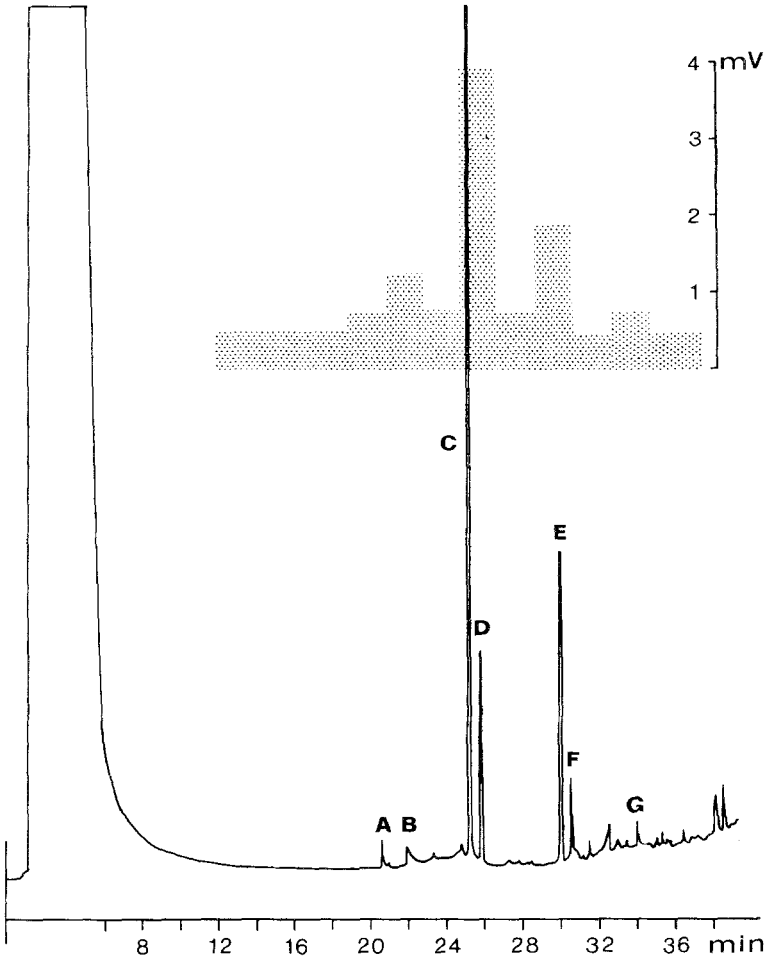

FIG. 1. *Cossus cossus* female ovipositor wash: EAG response of 2-min collections and GLC tracing. OV-101 GOTC column, 30 m × 0.21 mm ID, splitless injection 5 min 50° C, 50–100° C at 20° C/min, 100–260° C at 5° C/min and then isothermal, He carrier gas at 1.7 ml/min. Two female equivalents were used. (A) 10:Ac, (B) *Z*5–12:OH, (C) *Z*5–12:Ac, (D) 12:Ac, (E) *Z*5–14:Ac, (F) 14:Ac, (G) 16:Ac.

components C and D in (Figure 1) were individually collected in CS_2 (1 ml) at −60° C in a small sintered glass filter tube attached directly to the chromatograph outlet by Teflon tubing.

The two fractions were carefully concentrated at approx. 20 μl each with a gentle stream of nitrogen just before the ozonolysis. This was a scaled-down version of the technique described by Beroza and Bierl (1967). The ozone was

conducted into the micro test tube holding the substrate at −80° C through a syringe needle for 15–20 sec. The blue color of excess of ozone in solution was used to indicate the endpoint. The solution, without addition of triphenylphosphine, was analyzed by GOTC (OV-101) and the products compared with ozonized standards by GOTC-MS.

The technique for recording male moth electroantennogram responses (EAGs) of GLC fractions was basically that described previously (Tonini et al., 1982). Antennal responses to complete series of C_{12} monounsaturated alcohols and acetates and C_{14} monounsaturated acetates were determined and normalized against a reference standard as described by Roelofs (1977).

Monounsaturated alcohols and acetates were prepared by an acetylenic route (Henrick, 1977) using sodium 1-alkyn-1-ides in liquid ammonia. All the EAG and GLC standards were purified by high-performance liquid chromatography on a silver ion-silica gel column (10% $AgNO_3$ on Bio-Sil A; 1 cm OD × 1 m stainless steel) using benzene as the eluant. The purified compounds contained no detectable amounts of the opposite isomers (<0.1%), as determined by GLC on Carbowax 20 M column.

Field testing was carried out in poplar groves using Traptest® (Farmoplant) traps during the 1981 European goat moth flight season. All traps were suspended from branches 1.5 m above the ground and positioned about 300 m apart in a randomized complete block design. Synthetic chemicals were dissolved in methylene chloride and the appropriate amount of solution soaked into rubber septa (5 × 9-mm rubber stoppers, sleeve type) containing 1% weight of an antioxidant (Irganox 1010) and an UV-absorber (UV-531).

Since the *C. cossus* male is a heavy-bodied moth, some having a wing spread of 8 cm or more, the sticky surface of the trap (21 cm long × 23 cm wide) and its adhesiveness were often not adequate to catch this species.

RESULTS

Washes of two females tips were injected onto OV-101 GOTC column, collected in 2-min fractions up to 60 min, and the fractions assayed for antennal response by EAG. Three areas of activity at 21–23 min (1.2 mV; other inactive fractions 0.5–0.7 mV), 25–27 min (3.9 mV), and 29–31 min (1.9 mV) were evident, and these corresponded to the retention times of unbranched monounsaturated 12-carbon (C_{12}) alcohols and acetates, and 14-carbon (C_{14}) acetates, respectively. The GLC trace obtained on the same column from another batch of two female tips showed the presence of a peak (B) in the C_{12} alcohol region, two peaks (C, D) in the C_{12} acetate region, and two other peaks (E, F) in the C_{14} acetate region (Figure 1).

Male antennal responses to the series of monounsaturated C_{12} alcohols and acetates and C_{14} acetate standards are shown in Figure 2. Within each

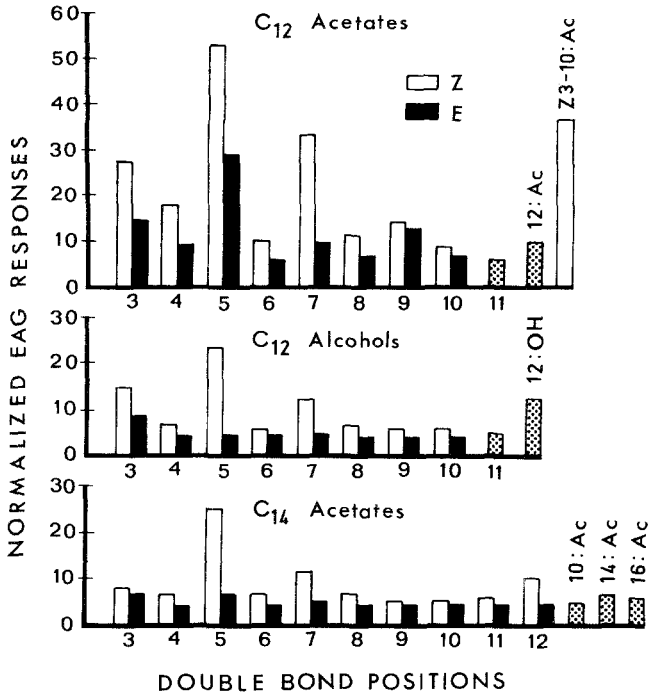

FIG. 2. EAG profiles of male *Cossus cossus* antennal responses to a series of monounsaturated C_{12} acetates, C_{12} alcohols, and C_{14} acetates. The standard, dodecyl acetate, was injected before each test chemical and the responses are compared to the standard, which is converted to a value of 10.

series the largest EAG responses were obtained from (*Z*)-5-dodecenyl acetate (*Z*5–12:Ac), (*Z*)-5-dodecenol (*Z*5–12:OH), and (*Z*)-5-tetradecenyl acetate (*Z*5–14:Ac).

Comparison of the retention times for the active components of female extracts and a complete series of *Z* and *E* isomers of monounsaturated C_{12} and C_{14} standards was made on the Carbowax 20 M column. Peaks B, C, and E consistently cochromatographed with *Z*5–12:OH, *Z*5–12:Ac, and *Z*5–14:Ac, respectively, under different temperature conditions.

GOTC-MS total ion current mass chromatogram of pheromone washes is illustrated in Figure 3. These mass chromatogram data were scanned by the data system for selected ions of saturated, mono- and diunsaturated C_{10}–C_{18} acetates (M^+-60, 61), alcohols (M^+-18), and aldehydes (M^+-1, M^+-18). The selected ions detected are shown in Figure 3, and the mass spectra of the corresponding compounds are listed in Table 1. Peaks A–G had mass spectra that matched those of reference decyl acetate (10:Ac), *Z*5–12:OH, *Z*5–12:Ac,

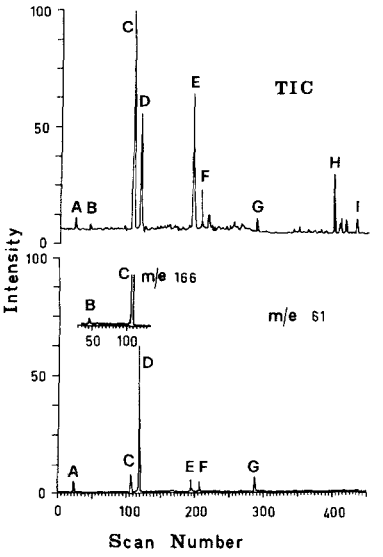

FIG. 3. Mass chromatograms with electron ionization (70 eV) showing the total ion current (TIC) and the selected ions *m/e* 61 and *m/e* 166 (insert) of a *Cossus cossus* female ovipositor wash. Three female equivalents were injected on OV-1 GOTC column [25 m × 0.3 mm ID, He carrier at splitless injection 1 min 40° C, 5° C/min at 270° C, 1 scan/sec (*m/e* 29–500); ion source temperature 260° C, filament current 1.5 m A]. The components were: (A) 10:Ac, (B) Z5–12:OH, (C) Z5–12:Ac, (D) 12:Ac, (E) Z5–14:Ac, (F) 14:Ac, (G) 16:Ac, (H) tricosane, (I) tetracosane.

dodecyl acetate (12:Ac), Z5–14:Ac, tetradecyl acetate (14:Ac), and hexadecyl acetate (16:Ac), respectively.

Interpretation of the GOTC-MS and the EAG data suggested that Z5–12:OH, Z5–12:Ac, and Z5–14:Ac could be three of the pheromone components.

Position of the double bonds in C and E was established by epoxidation and ozonolysis as follows. Epoxidation of a hexane wash from two abdominal tips was carried out with *m*-chloroperbenzoic acid. The reaction could be monitored by GLC on an OV-101 GOTC column. After 3.5 hr at room temperature, two well-resolved peaks (retention times: 29.6 min and 34 min) were observed and the areas of components C and E decreased to about 30% of their initial values. GOTC-MS analysis showed that the two new peaks had retention times and mass spectra that matched those of reference (*Z*)-5,6-epoxy-dodecyl and (*Z*)-5,6-epoxy-tetradecyl acetates, with characteristic fragments at *m/e* 127 [1.5% $CH_3(CH_2)_5CH(O)CH^+$] and *m/e* 155 [2%, $CH_3(CH_2)_7CH(O)CH^+$], respectively.

TABLE 1. MS DATA FOR COMPONENTS OF ABDOMINAL TIP WASH AND STANDARDS

Component	Standard	*m/e* (relative intensity of major ions) [assignment]
A	10:Ac	140(9)[$\overline{M}^+$ – 60], 112(27)[$\overline{M}^+$ – (60 + 28)], 61(55), 43(100).
B	*Z*5-12:OH	166(1)[$\overline{M}^+$ – 18], 138(2)[$\overline{M}^+$ – (60 + 28)], 137(1), 124(1)[$\overline{M}^+$ – (60 + 42)], 123(2.5), 41(100), 31(23).
C	*Z*5-12:Ac	166(2.5)[$\overline{M}^+$ – 60], 138(4)[$\overline{M}^+$ – (60 + 28)], 137(1), 124(1)[$\overline{M}^+$ – (60 + 42)], 123(1.5), 61(2.5), 43(100).
D	12:Ac	168(2)[$\overline{M}^+$ – 60], 140(5)[$\overline{M}^+$ – (60 + 28)], 139(1.5), 126(2)[$\overline{M}^+$ – (60 + 42)], 125(4), 61(37), 43(100).
E	*Z*5-14:Ac	194(1.5)[$\overline{M}^+$ – 60], 166(2)[$\overline{M}^+$ – (60 + 28)], 152(0.5)[$\overline{M}^+$ – (60 + 42)], 151(0.5), 138(3.5), 137(1.5), 61(2), 43(100).
F	14:Ac	196(2)[$\overline{M}^+$ – 60], 168(3.5)[$\overline{M}^+$ – (60 + 28)], 167(0.5), 154(0.5)[$\overline{M}^+$ – (60 + 42)], 140(1.5), 139(2), 61(47), 43(100).
G	16:Ac	224(1)[$\overline{M}^+$ – 60], 196(2.5)[$\overline{M}^+$ – (60 + 28)], 168(1.5)[$\overline{M}^+$ – (60 + 28)], 167(0.5), 154(1), 153(1.5), 61(38), 43(100).

Reductive ozonolysis of GLC-purified C gave a product which cochromatographed with heptanal on the OV-101 GOTC column, whereas component E was found to produce nonanal by ozonolysis.

The above data all support the characterization of C and E as *Z*5–12:Ac and *Z*5–14:Ac, respectively.

Insufficient material was available for ozonolysis of component B. The isomer assignment of *Z*5–12:OH was, therefore, based on EAG data and its retention times on Carbowax 20 M and OV-101 GOTC columns.

Preliminary field tests were carried out around Novara and are presented in Table 2. In these tests the saturated C_{10}, C_{12}, C_{14}, and C_{16} acetates were omitted on the basis of their low EAG activity. On the contrary, (*Z*)-3-decenyl acetate (*Z*3–10:Ac) was included due to its intense EAG response on male antenna (Figure 2). This compound was found by EAG screening conducted on more than 150 synthetic standards related to the known pheromone components of Lepidoptera species. The results indicated that *Z*5–12:Ac was essential for the selective attraction of European goat moths in the field. The 4:1 combination of *Z*5–12:Ac with *Z*5–14:Ac attracted more moths than the *Z*5–12:Ac alone. Addition of 10% of *Z*5–12:OH to this mixture did not seem to improve its attractiveness. Even though *Z*3–10:Ac was not positively identified in the pheromone gland washes, the results showed that it had, at the ratios tested, synergistic activity on the attractiveness of *Z*5–14:Ac alone or in combination with *Z*5–14:Ac.

TABLE 2. FIELD ATTRACTION OF *Cossus cossus* MALES TO VARIOUS CHEMICALS[a]

Chemical components (mg)				Mean no. males/trap/week[b]
*Z*5–12:Ac	*Z*5–12:OH	*Z*5–14:Ac	*Z*3–10:Ac	
1				2 c
10				2 c
	1			0 c
		1		0 c
			1	0 c
0.8		0.2		5 b
0.8	0.1	0.2		4 b
		0.8	0.2	0 c
0.2		0.8		1 c
0.8			0.2	17 a
0.5		0.25	0.25	16 a
2 virgin females[c]				12

[a] Conducted July 7–August 3, 1981. Four replicates, rerandomized 8 times.
[b] Means followed by the same letter are not significantly different from each other according to Duncan's multiple-range test ($P = 0.05$).
[c] Single trap.

DISCUSSION

The analytical data reported here indicate that ovipositor washings from *C. cossus* contain seven compounds that are structurally similar to those of known pheromone components of Lepidoptera. They were present in the following approximate percentages (in parentheses): 10:Ac(1.5), *Z*5–12:OH(1), *Z*5–12:Ac(66), 12:Ac(12), *Z*5–14:Ac(14), 14:Ac(4), and 16:Ac(1.5). The washes contained an average of 125 ng of *Z*5–12:Ac/female equivalent (FE) and 25 ng of *Z*5–14:Ac/FE.

Although the saturated C_{12}, C_{14}, and C_{16} acetates were isolated in significant amounts from female tip washings, none of these compounds caused a meaningful EAG response from the male moth.

The *Z*3–10:Ac was not detected in the female pheromone glands even though it was a synergistic component of the attractant blends tested in the field.

Detailed examination of material derived from female moths failed to reveal any other olfactory stimulants for the male moth, including (5*Z*)–5,13-tetradecadienyl acetate and (3*E*, 5*Z*)-3,5,13-tetradecatrienyl acetate found in the odorous secretion of the mandibular glands of the larvae (Garanti et al., 1976).

Field results indicate that *Z*5–12:Ac is essential in the attraction of *C. cossus* males. The roles of *Z*5–14:Ac and *Z*5–12:OH are not yet clear, and we are planning to determine whether or not these compounds are present in the effluvia emitted by *C. cossus* females. For the time being, the compounds defined in this study provide useful species-specific attractants for monitoring this pest. The formulation to be recommended is a rubber cap containing 250 μg *Z*3–10:Ac, 500 μg *Z*5–12:Ac, and 250 μg *Z*5–14:Ac.

*Z*5–12:Ac is known to be the major component of the sex pheromone of the noctuid *Euxoa ochrogaster* (Struble et al., 1980), and *Z*5–14:Ac has been identified as the major sex pheromone component of the noctuid *Scotia exclamationis* (Bestmann et al., 1980).

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PYGIDIAL DEFENSIVE TITER AND POPULATION STRUCTURE OF *Agabus bipustulatus* L. AND *Agabus paludosus* F. (COLEOPTERA, DYTISCIDAE)

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Abstract—Aromatic pygidial gland constituents of *Agabus bipustulatus* L. and *A. paludosus* F. were quantitatively determined. Concentration fluctuations were found to be dependent on age and season. Both in quantity and quality of the secretion, young beetles differ from mature beetles by storing only small amounts of gland material with different portions of constituents. Seasonal variations are mainly due to the changing population structure of the species.

Key Words—Coleoptera, Dytiscidae, pygidial glands, aromatic defensive secretions, age classes, seasonal variation, *Agabus bipustulatus*, *Agabus paludosus*.

INTRODUCTION

Antimicrobial pygidial gland constituents of adephagous water beetles are chemically well known (Schildknecht, 1970; Dettner, 1979). However, our knowledge concerning their biological function is very poor and based only on some behavioral observations (Maschwitz, 1967), which showed a distribution of pygidial gland material on the cuticle during the cleaning procedure of the beetles. This may prevent epizoic microorganisms, and there may be a drastic change in wettability (Dettner and Schwinger, 1980) of the integument of smaller water beetle species. Further investigations have shown a great annual variability in sequestering of pygidial gland components in two Gyrinidae (Newhart and Mumma, 1979b) and one Dytiscidae species (Newhart and Mumma, 1979a). The following study was intended to find reasons for the spectacular seasonal fluctuations which might indicate

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biological roles of these gland constituents. Preliminary tests showed that recording of population dynamics and age of the beetles together with chemical data might be the first step in interpreting this phenomenon.

METHODS AND MATERIALS

Specimens of *Agabus bipustulatus* L. were collected from a pool with rich water plant vegetation (depth: 1 m; surface: 50 m^2) situated in a heathy ground near Düren, Rhineland. *A. paludosus* F. was found in a warm polluted brook (width: 1 m; depth: 0.5 m) near Aachen, Rhineland. Between April and December 1979 beetles were collected with a water net twice a month. From January to April it was impossible to collect sufficient numbers of water beetles.

The living beetles were carefully transported to the laboratory where they were slowly frozen. By this procedure, approximately 5–15% of the total reservoir volume per beetle was lost. One hundred sixty-one specimens of *A. bipustulatus* and 80 specimens of *A. paludosus* were classified by age after dissection and examination of gonadal development. Both pygidial gland reservoirs were isolated, transferred into a glass capillary, desiccated, and mixed with definite quantities of MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoracetamide) according to species. Derivatization with MSTFA was performed for recording distinct gas chromatographic peaks of more polar minor components which are difficult to detect without derivatization or with other methods. Each beetle extract was submitted to ultrasonic treatment and heated to 70°C for 10 min until silylation was finished. According to species, equal amounts of silylated beetle extracts were separated, using a Carlo Erba Fractovap 2900 capillary gas chromatograph equipped with a FID detector (carrier gas: Helium, 1.2 ml/min). A 7-m glass capillary column coated with OV 101 was used, temperature programed (from 85°C to 130°C: heating rate 8°C/min; from 130°C to 210°C: heating rate 12°C/min). Quantitative concentration data of the gland material were obtained by comparing with injected mixtures of silylated calibration standards (benzoic acid, *p*-hydroxybenzaldehyde, hydroquinone, *p*-hydroxybenzoic acid methyl ester, *p*-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid methyl ester).

RESULTS AND DISCUSSION

Dependence of Pygidial Gland Contents on Age. According to the maturity of their gonads, it is possible to classify both male and female beetles of each species into five age classes. They are characterized by numbers and distinguished as follows (see Figures 1c, 2c):

Male beetles (Figure 1c) were grouped into age class 1/1a (gonads

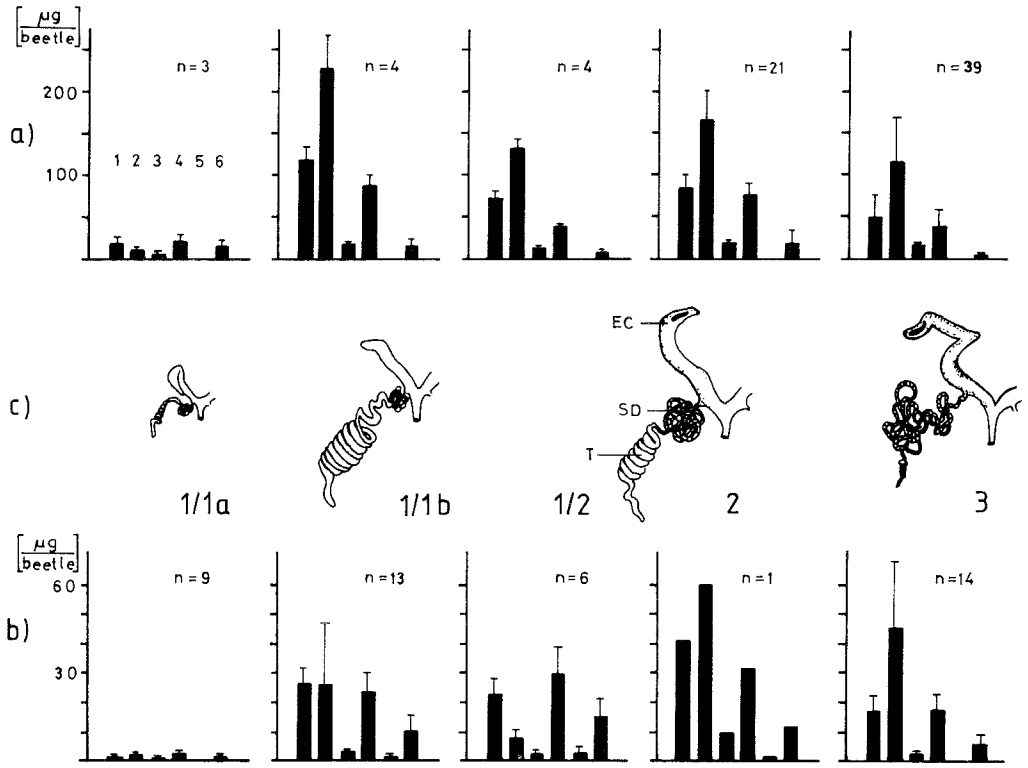

FIG. 1. Absolute concentration of the six main components on the pygidial glands of male water beetles (a: *A. bipustulatus*, b: *A. paludosus*) compared with different age classes (c: 1/1a, 1/1b, 1/2, 2, 3; see test). Abbreviations: 1 = benzoic acid; 2 = *p*-hydroxybenzaldehyde; 3 = hydroquinone; 4 = *p*-hydroxybenzoic acid methyl ester; 5 = *p*-hydroxybenzoic acid; 6 = 3,4-dihydroxybenzoic acid methyl ester; compounds are arranged according to their gas chromatographic retention times; EC = ectadenies; SD = spermatic duct; T = testicle.

transparent and hardly recognizable, no sperm present); age class 1/1b (gonads white, length of ectadenies 6.45 ± 1.73 mm, large testicles and very small, thin spermatic ducts, no sperm present); age class 1/2 (gonads white yellowish, length of ectadenies 10.36 ± 0.88 mm, testicles with sperm, spermatic ducts thickened and disaggregated); age class 2 (gonads yellowish, ectadenies filled with secretion, sperm present in testicles and spermatic ducts); age class 3 (gonads yellowish, length of brownish ectadenies 11.23 ± 2.62 mm, testicles shrunken, sperm only present in widened spermatic ducts).

Female beetles (Figure 2c) were grouped into age class 1 (gonads white, still undifferentiated); age class 2 (ovarioles developing, seminal receptacle filled with sperm); age class 3/1 (ovaries with mature eggs); age class 3/2 (ovaries with mature eggs and corpora lutea); age class 3/3 (ovaries only with corpora lutea).

Investigations of the life histories of the two *Agabus* species have shown that both live for one year and become fertile only once. Therefore the above-mentioned sequence seems to be correct. Figures 1a and b and 2a and b show the absolute concentrations of the six main components of the pygidial gland separated according to species, sex, and age. Comparing the male beetles of the two *Agabus* species (Figure 1), it is notable that the pygidial glands of the youngest beetles (1/1a) are characterized by extremely small amounts of single components. The total contents of the pygidial gland material are given in Table 1. Older specimens however (1/1b–3) contain sevenfold (*A. bipustulatus*) and 17-fold (*A. paludosus*) quantities. Regarding the gland composition with respect to single components, it is evident that not only the total concentration varies wih age but also the relative quantities of the components. While youngest age classes (1/1a) of each species are characterized by nearly equal amounts of benzoic acid, *p*-hydroxybenzoic acid methyl ester, 3,4-dihydroxybenzoic acid methyl ester, and a small quantity of *p*-hydroxybenzaldehyde, the older classes (1/1b–3) contain *p*-hydroxybenzaldehyde as the main component (exception: *A. paludosus*, age class 1/2). Older beetles show a high synthetic capacity for benzoic acid and for *p*-hydroxybenzoic acid methyl ester, whereas hydroquinone and 3,4-dihydroxybenzoic acid methyl ester represent minor components. *p*-Hydroxybenzoic acid is only present in minimal quantities.

Females also show an increasing capacity of storing pygidial gland secretion with age (Figure 2, Table 1), although the contrast between the youngest (1) and the oldest female beetles (2–3/3) is not so great as in males. Within older classes the total concentration varies, especially in *A. bipustulatus* where it shows nearly the same concentration in 3/1 and 3/2 compared to 1 (Table 1). After finishing egg deposition (3/3), females again contain considerable amounts of aromatic compounds. As found in male beetles, the quantitative fluctuation of the single constituents varies with age of females. *p*-Hydroxybenzaldehyde likewise dominates in each class

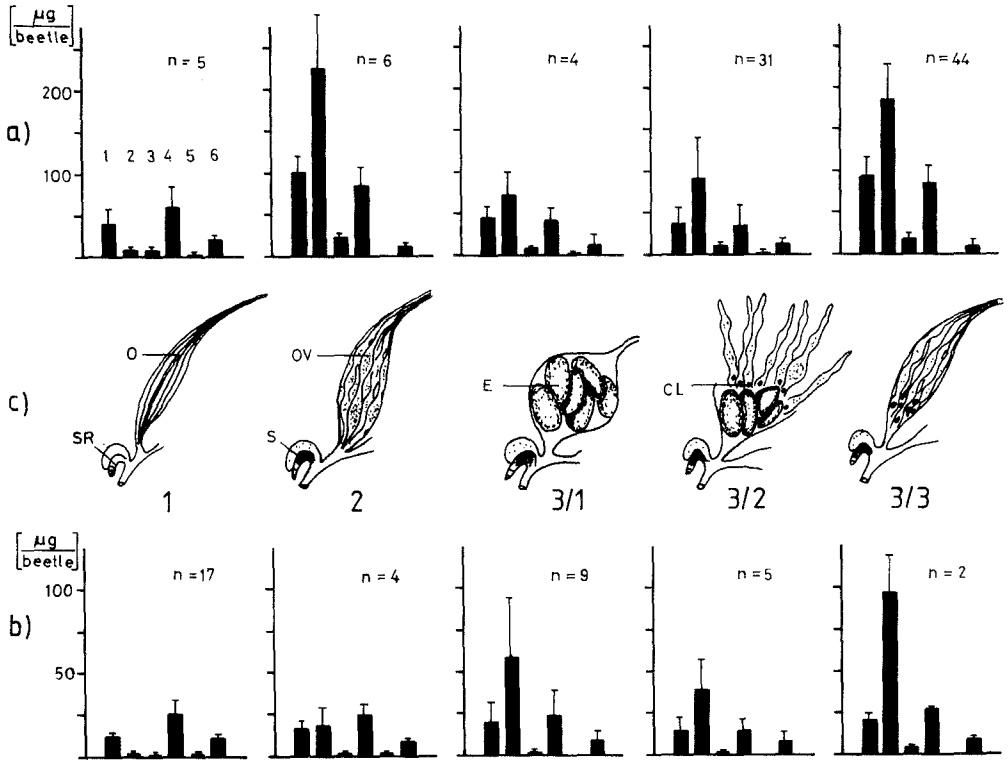

FIG. 2. Absolute concetration of the six main components in the pygidial glands of female water beetles (a: *A. bipustulatus*, b: *A. paludosus*) compared with different age classes (c: 1, 2, 3/1, 3/2, 3/3; see text). Abbreviations: 1-6 see Figure 1; 0 = ovary; SR = seminal receptacle; OV = ovariole; S = sperm; E = eggs; CL = corpora lutea.

TABLE 1. ABSOLUTE CONCENTRATIONS (μg/beetle) OF THE TOTAL PYGIDIAL GLAND SECRETIONS IN DIFFERENT AGE CLASSES OF *Agabus bipustulatus* AND *A. paludosus*.

Age classes		*A. bipustulatus*	*N*	*A. paludosus*	*N*
Males	1/1 a	66.07 ± 47.14	3	4.74 ± 3.43	9
	1/1 b	457.44 ± 140.17	4	95.55 ± 80.50	13
	1/2	260.75 ± 46.35	4	81.42 ± 47.81	6
	2	354.63 ± 131.30	21	156.89	1
	3	220.09 ± 206.21	39	85.32 ± 70.64	14
Females	1	131.93 ± 109.03	5	54.26 ± 39.61	17
	2	439.81 ± 216.36	6	70.52 ± 51.35	4
	3/1	176.89 ± 106.14	4	112.84 ± 129.53	9
	3/2	176.72 ± 192.08	31	76.95 ± 76.14	5
	3/3	397.52 ± 191.25	44	160.00 ± 50.29	2

older than stage 1 (exception: *A. paludosus*, stage 2), and the youngest classes (stage 1) are clearly characterized by *p*-hydroxybenzoic acid methyl ester. In both species in each sex, the total concentrataion as well as the relative composition of pygidial gland secretion of the youngest age class deviates as compared with older classes. Therefore a correlation of synthetic ability with age is obvious. A reasonable assumption could be that the youngest beetles (some of them are still not hardened) have not completely developed glands. However Casper's (1913) observation contradicts this supposition; he found that pygidial glands of water beetles are already present when the third larval stages leave the water to pupate on land.

As concerns the fluctuations of single gland constituents, it seems that these are mainly due to metabolic changes during the beetle's imaginal development. Based on the percentage of each constituent with different age classes, a drastic increase of *p*-hydroxybenzaldehyde and a decrease of *p*-hydroxybenzoic acid methyl ester and 3,4-dihydroxybenzoic acid methyl ester from young to all older beetles (both species and sexes) is obvious. It is well known (Karlson and Sekeris, 1976) that high ecdysone levels during moulting periods have remarkable effects on metabolism of aromatic compounds. Immature, just-hatched water beetles (stage 1) may be still submitted to such hormonal shifts. Since both studies on ecdysone titers of water beetles and detailed biogenetic investigations concerning these aromatic compounds have not been done, it is not possible to find reasons for these concentration changes. Nevertheless similar fluctuations of *p*-hydroxybenzoic acid methyl ester and 3,4-dihydroxybenzoic acid methyl ester may be indicative of a common biogenetic pathway of these two chemically similar compounds.

Seasonal Fluctuation of Pygidial Gland Contents. The total concentration of pygidial gland secretion varies quantitatively with the course of the year. Both *Agabus* species show a concentration decrease from May (*A. bipustulatus*) and from June/July (*A. paludosus*) to November, modified by deviations. *Agabus bipustulatus*, for example, contains in the spring a sevenfold quantity of secretion as compared with fall values. In *A. paludosus* the seasonal titer of *p*-hydroxybenzaldehyde (main constituent of adult beetles) exactly represents the fluctuation of the total secretion concentration per year (Figure 3b). Highest values were found during June/July followed by a drastic decrease to August. From fall to winter, the aldehyde titer of *A. paludosus* shows low, slowly increasing values with minute deviations. The distinct decrease of *p*-hydroxybenzaldehyde from July to August seems to be due to the total change of the population structure which is also characteristic of other water beetle species (Dettner, 1976). Within this time the adult beetles of the preyear generation (age class 3 from May to July; Figure 3c) die while new, immature beetles appear (age class 1 from July to September; Figure 3c). The simultaneous occurrence of both generations in June and July may be responsible for the highest concentration deviations within this time. However, low aldehyde titers during the fall, accompanied by small deviations, might be caused by the presence of only immature beetles. As compared with *p*-hydroxybenzaldehyde, the seasonal titer of the minor compound 3,4-dihydroxybenzoic acid methyl ester shows a contrary trend. This ester, of which considerable concentrations are only found in immature beetles, distinctly increases from July to August, just when population structure is changing and young beetles of age class 1 appear. A second maximum (high deviations) which is reached in December cannot be ascribed to a certain age class since all age classes occur simultaneously within this time.

On the whole, it has been shown that remarkable seasonal concentration differences are due to the age structure of the beetles. However, it is not clear whether the maximum concentration of the main components in spring is alone correlated with the presence of adult beetles. This would be correct if these aromatics were regarded as waste products of metabolism which accumulate with age. Furthermore the total metabolic activity could again arise in spring according to gradually increasing water temperatures. On the other hand, climatic and biotic influences may be involved with the seasonal fluctuations. Based on the result that pygidial gland reservoirs are only partly depleted with 16°C-adapted beetles were cold-shocked to 6°C (depletion in *A. bipustulatus*: 13%, $N = 8$; in *A. paludosus*: 36%, $N = 10$), it is suggested that these gland constituents are not primarily directed against predators. On the contrary, water beetles synthesize toxic secretions within their prothoracic defensive glands which are ejected spontaneously after shocking from 16°C to 6°C (simultaneous depletion of cortexone in

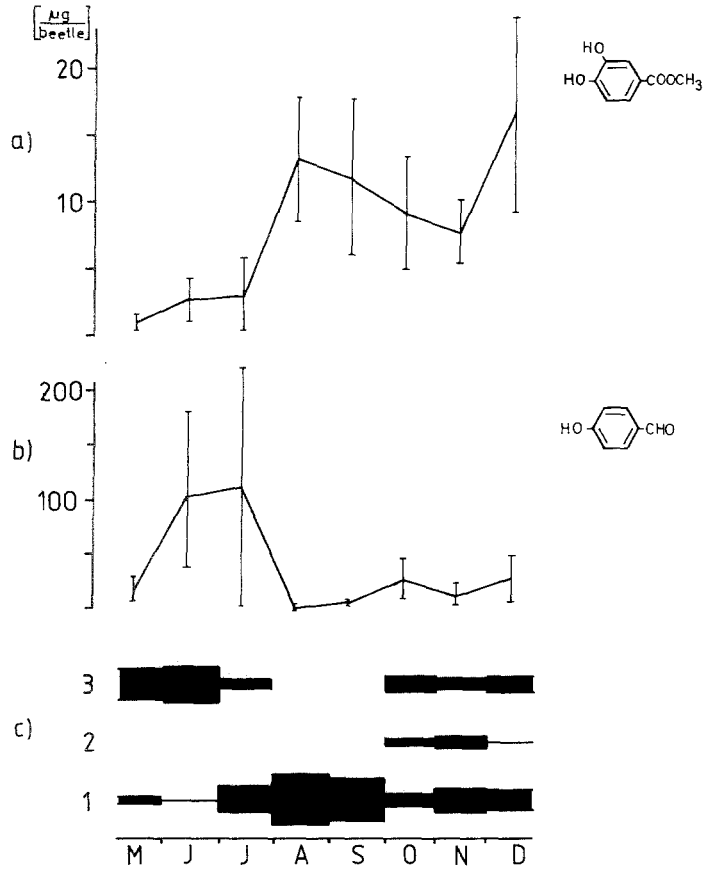

FIG. 3. Seasonal fluctuations of two pygidial gland constituents (a: 3,4-dihydroxybenzoic acid methyl ester; b: *p*-hydroxybenzaldehyde) of *Agabus paludosus* compared with population structure (c) separated according to age classes 1 (stage 1 of females and stages 1/1a, 1/1b, and 1/2 of males are summarized), 2, and 3 (stages 3/1, 3/2, 3/3 of females and stage 3 of males are summarized; see text). Width of horizontal bars in Figure 3c indicates different numbers of beetle specimens caught per age class.

A. bipustulatus: 60%, $N = 8$). Thus, if pygidial gland constituents are primarily used as antimicrobic agents (Maschwitz, 1967), the minimum titer found towards wintertime could result from the lack of epizoic attack. Finally the concentration increase in early summer, which was found in both species, may not be dependent on similar behavior: The ubiquitous *A. bipustulatus* is active, too, during winter months and even deposits eggs, whereas the running water species *A. paludosus* survives this unfavorable season by digging into the mud of the shore.

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SYNTHESIS OF (±)-DISPARLURE

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Abstract—A six-step, convergent synthesis has been developed for racemic (*Z*)-7,8-epoxy-2-methyloctadecane (disparlure), the sex pheromone of the gypsy moth. The key step is a Wittig reaction between 6-methylheptanal and triphenylundecylphosphonium bromide effected by potassium carbonate in the presence of a crown ether.

Key Words—Sex pheromone, sex attractant, *Lymantria dispar*, gypsy moth, (*Z*)-2-methyl-7-octadecene, racemic (*Z*)-7,8-epoxy-2-methyloctadecane, Wittig reaction, crown ether.

INTRODUCTION

Disparlure, the sex pheromone of the female gypsy moth (*Lymantria dispar*), was first identified as (*Z*)-7,8-epoxy-2-methyloctadecane (I) and synthesized a dozen years ago (Bierl et al., 1970). Since that time a number of total syntheses have been reported (Mori, 1981). Although recent pathways have involved strategies such as intramolecular epoxide formation from α-hydroxytosylates (Iwaki et al., 1974; Mori et al., 1976 and 1979) or β-hydroxysulfonium salts (Farnum et al., 1977; Pirkle and Rinaldi, 1979), the epoxide was produced by oxidation of an alkene in the majority of the routes. Two examples of the latter approach utilized a chiral oxidant to convert an allylic alcohol to a chiral β,γ-epoxy alcohol whose side chains were subsequently elaborated to (+)-I (Mori and Ebata, 1981; Rossiter et al., 1981). A dozen other groups, however, focused on the preparation of (*Z*)-2-methyl-7-octadecene (II), which was oxidized to I by peracids. Even this pathway was not without variation as the requisite *cis* alkene has been generated by Wittig reactions (Bierl et al., 1970, 1972; Bestmann and Vostrowsky, 1974; Bestmann

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et al., 1976), α-silylalkyllithium reagents (Chan and Chang, 1974; Mychajlowskij and Chan, 1976), alkyne reductions (Eiter, 1972; Kovalev et al., 1973; Shamshurin et al., 1973; Sheads and Beroza, 1974), disproportionation of alkenes by homogeneous catalysis (Kuepper and Streck, 1976), and cyclooctadiene conversions (Kluenenberg and Shaefer, 1978; Tolstikov et al., 1978).

Although the dextrorotatory enantiomer of I (7*R*,8*S*) showed greater activity in field tests, the racemic mixture offered near equivalency to the natural product at low dispenser dosages (Cardé et al., 1978, and references cited therein). We report here an alternate route to (±)-disparlure, in which the key step is a highly stereoselective preparation of II by an exceptionally mild and convenient Wittig reaction.

METHODS AND MATERIALS

Gas chromatography (GC) analyses were performed on a Varian Aerograph 1400 instrument equipped with a 12-ft × 1/8-in. stainless-steel column packed with 10% Dow-Corning 710 on Chromosorb W (col A) and a 6-ft × 1/8-in. nickel column packed with 3% OV-101 on Chromosorb W (col B) and on a Finnegan 4000/6000 GC-MS system equipped with a 60-m × 0.25-mm fused silica capillary DB-1 Durabond (SE-30 type) column (col C). Column temperatures are described in parenthesis. Mass spectra (MS) were obtained on the above Finnegan instrument and on a Hewlett Packard 5985 GC-MS system. Infrared spectra (IR) were recorded on a Perkin Elmer 237B spectrophotometer. Nuclear magnetic resonance spectra (NMR) were obtained on a Perkin Elmer R 12B spectrometer with chemical shifts reported in parts per million from an internal tetramethylsilane reference. Boiling points and melting points are uncorrected; the latter were determined on a modified Hershberg apparatus with total immersion Anschutz thermometers.

6-Methyl-1-heptanol (IV). To an ice-cold, magnetically stirred solution of 6-methyl-1-heptene (III; 1.12 g, 10.0 mmol; Wiley Organics) in anhydrous Et_2O (4 ml) in a 25-ml round bottom flask fitted with a reflux condenser and $CaCl_2$ drying tube was added borane-dimethyl sulfide complex (0.30 ml, 3.8 mmol; Aldrich Chemical Co.) dropwise from a graduated pipet. The reaction solution was stirred 1 hr at room temperature and then again chilled in an ice-water bath. To the cold stirred solution were added in the following order 95% EtOH (3 ml), 2 N NaOH solution (2 ml), and dropwise 30% H_2O_2 (1.2 ml). The mixture was refluxed 1 hr, cooled, poured into water (10 ml), and extracted twice with Et_2O. The combined ether extract was washed twice with saturated NaCl solution, dried over anhydrous Na_2SO_4, and evaporated to

dryness to yield 6-methyl-1-heptanol (IV; 1.01 g, 77%): bp 94–100° C at 20 mm Hg; IR (neat) 3322 cm^{-1}; GC (col A, 142°, helium 30 ml/min) 6-methyl-2-heptanol (V), 2.5 min; IV, 4.0 min. From the relative areas of the isomeric alcohols a 93:7 was calculated for IV:V.

6-Methyl-2-heptanol (IV). An authentic sample of V for comparison purposes was prepared by the oxymercuration–demercuration method. To a yellow mixture of $Hg(OAc)_2$ (1.60 g, 5.0 mmol), water (5 ml), and tetrahydrofuran (5 ml) contained in a 40-ml polypropylene centrifuge tube (29 × 104 mm) was added 6-methyl-1-heptene (III; 0.56 g, 5.0 mmol), and the reaction mixture was stirred magnetically for 10 min. To the clear colorless solution were added 3 N NaOH solution (5 ml) and 0.5 M $NaBH_4$ in 3 N NaOH solution (5 ml). The aqueous phase was saturated with NaCl, and the mixture was centrifuged to separate the colloidal mercury. The organic phase was withdrawn by capillary pipet, dried over anhydrous Na_2SO_4, and evaporated to dryness to yield 6-methyl-2-heptanol (V; 0.50 g, 77%): IR (neat) 3300 cm^{-1}; GC (see above). From the relative areas of the isomeric alcohols a 3:97 ratio was calculated for IV:V.

6-Methylheptanal (VI). To a 125-ml Erlenmeyer flask containing a magnetically stirred slurry of pyridinium chlorochromate (5.0 g, 23 mmol; Aldrich) in CH_2Cl_2 (30 ml) was added dropwise a solution of IV (1.0 g, 7.7 mmol) in CH_2Cl_2 (10 ml). The reaction mixture was stirred 1 hr at room temperature, diluted with Et_2O (35 ml), and the more mobile phase filtered by suction through a 0.5-cm layer of Florisil. The more viscous phase remaining in the reaction flask was washed with Et_2O, which was decanted and filtered through the Florisil. The total filtrate was treated with decolorizing charcoal, filtered by gravity, and evaporated to dryness to yield 6-methylheptanal (VI; 0.98 g, 99%): bp 55–57° C at 10 mm Hg; IR (neat) 1730 cm^{-1}, no absorption at 3322 cm^{-1} (alcohol), 1718 cm^{-1} (ketone, see below), or 1712 cm^{-1} (carboxylic acid, see below); GC (col A, 142° C, helium 30 ml/min) 4.0 min; 2,4-dinitrophenylhydrazone derivative, mp 84.0–84.8° C ($EtOH-H_2O$).

An authentic sample of 6-methyl-2-heptanone for comparison purposes was prepared from V by the same procedure: IR (neat) 1718 cm^{-1}; GC (col A, 142° C, helium 30 ml/min, 3.0 min. An authentic sample of 6-methylheptanoic acid (Markgraf et al., 1977) exhibited IR absorption at 1712 cm^{-1}.

Triphenylundecylphosphonium Bromide (VII). A mixture of 1-bromoundecane (0.71 g, 3.0 mmol; Eastman Kodak) and triphenylphosphine (1.58 g, 6.0 mmol; Aldrich) contained in a 25-ml round bottom flask fitted with a $CaCl_2$ drying tube was heated 20 hr on a steam bath. The clear viscous liquid was cooled to room temperature and triturated four times with 10-ml portions of anhydrous Et_2O. During these triturations the excess Ph_3P and unreacted 1-bromoundecane dissolved in the solvent and were removed by decantation. The phosphonium salt became viscous, then semisolid, and finally crystalline.

The white solid was collected by suction filtration, washed with Et_2O, and air dried to give triphenylundecylphosphonium bromide (VII; 1.34 g, 90%): mp 91–93° C.

(Z)-2-Methyl-7-octadecene (II). To a 25-ml round bottom flask containing a magnetically stirred slurry of 18-crown-6 (10 mg), anhydrous K_2CO_3 (0.33 g, 2.4 mmol) and triphenylundecylphosphonium bromide (VII; 1.19 g, 2.4 mmol) in anhydrous tetrahydrofuran (10 ml, freshly distilled from $LiAlH_4$) was added 6-methylheptanal (VI; 0.26 g, 2.0 mmol). The flask was stoppered, and the mixture was stirred 5 days at room temperature. The solvent was removed by rotary evaporation at reduced pressure, and the viscous residue was triturated four times with 5-ml portions of hexane. The combined hexane extract was filtered by suction through a layer of Florisil, washed twice with water, dried over anhydrous Na_2SO_4, and evaporated to dryness to give (*Z*)-2-methyl-7-octadecene (II; 0.23 g, 43%): bp 127–135° C at 0.8 mm Hg; IR (neat) 3000, 2933, 2865, 1464, 1383, 1364, 962, and 720 cm^{-1}; NMR (CCl_4) δ 0.89 d + m (9H), 1.27 m (23H), 2.00 m (4H), and 5.25 m (2H); MS *m/e* 266 (M^+); GC (col B, 212° C, helium 30 ml/min) 3.6 min; capillary GC (col C, 120° C for 0.6 min, then 5° C/min to 255° C where held constant) II, 13.17 min, (*E*)-2-methyl-7-octadecene (IX), 13.25 min. From the peak heights of the reconstructed gas chromatogram a 93:7 ratio was calculated for II:IX.

For comparison purposes a sample of II was prepared by a Wittig reaction between VI and VII initiated by *n*-butyllithium in the following manner. The phosphonium salt VII was prepared as above in a tared 50-ml round bottom flask with a side arm capped by a rubber septum. After trituration of the salt with Et_2O, the residual traces of solvent were removed under reduced pressure and the yield of VII was determined. To a magnetically stirred slurry of VII (1.0 g, 2.0 mmol) in anhydrous Et_2O (25 ml) in the same flask fitted with a reflux condenser and $CaCl_2$ drying tube was added dropwise from a syringe via the septum-capped side arm a solution of *n*-butyllithium (0.9 ml, 2.3 M in hexane; Aldrich). The immediate development of a yellow–orange color signified the generation of phosphorane VIII. The mixture was stirred for 10 min at room temperature, and then a solution of VI (0.25 g, 2.0 mmol) in anhydrous Et_2O (10 ml) was added dropwise. The mixture was stirred at reflux for 1 hr, cooled, filtered by suction through a layer of Florisil, and the transfer completed with additional Et_2O. The combined ether extract was worked up as before to yield II (0.44 g, 83%) identical by IR, NMR, and GC to that prepared above. From the capillary GC analysis a 80:20 ratio was calculated for II:IX.

(Z)-7,8-Epoxy-2-methyloctadecane (I). To a 25-ml round bottom flask fitted with a reflux condenser were added (*Z*)-2-methyl-7-octadecene (II; 0.27 g, 1.0 mmol), meta-chloroperbenzoic acid (0.33 g, 1.9 mmol; Aldrich), and

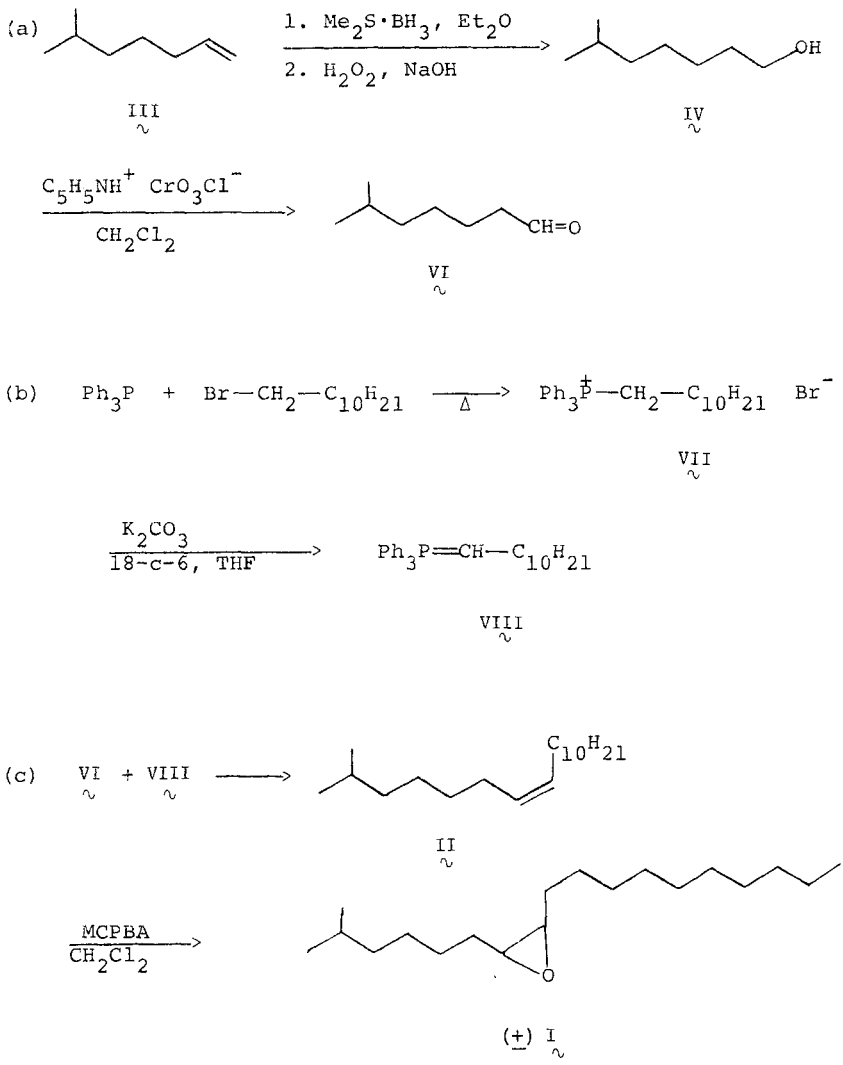
CH_2Cl_2 (10 ml). The reaction mixture was refluxed for 1 hr, cooled, washed twice with 5% Na_2CO_3 solution, twice with water, dried over anhydrous Na_2SO_4, and evaporated to dryness to give (*Z*)-7,8-epoxy-2-methyloctadecane (I; 0.25 g, 89%), identical to an authentic sample of disparlure in all respects: IR (neat) 2967, 2933, 2865, 1471, 1387, 1368, 1263, 1174, and 723 cm^{-1}; NMR ($DCCl_3$) δ 0.86d + m (9H), 1.2–1.6 m (27H), and 2.90 m (2H); MS *m/e* 282 (M^+); GC (col B, 212°C, helium 30 ml/min) 6.0 min.

RESULTS AND DISCUSSION

The present approach to disparlure, patterned after the pioneering work of Bierl et al. (1972), was modified to take advantage of current synthetic methodologies (Scheme 1). Noteworthy aspects included hydroboration of III by borane–dimethylsulfide complex (Lane, 1974), oxidation of IV by pyridinium chlorochromate (Glavos, 1978), and generation of phosphorane VIII under conditions of crown ether catalysis (Boden, 1975).

Hydroboration occurred with the expected regioselectivity to produce the primary alcohol IV. It was found that aldehyde VI must be used shortly after isolation to avoid air oxidation to the corresponding carboxylic acid. Authentic samples of the alcohol and ketone isomeric with IV and VI permitted confirmation of the regioselectivities. Preparation of phosphonium salt VII by heating the reactants without solvent was superior to previously reported methods; the stoichiometry and reaction time were optimized.

The use of potassium carbonate in the presence of 18-crown-6 to effect the Wittig reaction constituted the first application of these conditions to an unactivated aliphatic system. In the previous report by Boden (1975), these reagents were limited to reactions involving either benzaldehyde or benzylphosphonium salts and the observed *Z/E* ratios of the derived alkenes heavily favored the trans product. Although the yield of II in the present work was not optimized, the mild conditions and the high stereoselectivity for the (*Z*)-alkene made this procedure an attractive alternative. The stereochemistry observed in the present case is all the more interesting in light of the recent report that sodium hydride with 15-crown-5 in tetrahydrofuran gave trans alkenes exclusively in the Wadsworth-Emmons modification of the Wittig reaction (Baker and Sims, 1981). Variations in solvent (acetonitrile or dioxane) and in reaction time (2 hr at reflux in THF) produced less than 3% alkene. Similarly, attempts to effect the Wittig reaction using the phosphonium salt itself as a phase-transfer catalyst in a two-phase system of benzene (or dichloromethane) and 50% aqueous sodium hydroxide (Tagaki et al., 1974; Dehmlow and Barahona-Naranjo, 1981) as well as using solid sodium hydroxide and dioxane (Delmas et al., 1981) were unsuccessful.



SCHEME 1. Summary of disparlure synthesis.

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SYNTHESIS OF (*Z*)-13-HEXADECEN-11-YN-1-YL ACETATE Major Component of Sex Pheromone of the Processionary Moth

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Abstract—The synthesis of the major component of the sex pheromone of the processionary moth *Thaumetopoea pityocampa* is described. The synthesis uses the carbocupration of acetylene, followed by the coupling with the appropriate 1-iodoalkyne. This synthetic pheromone was shown to be of 98.8% *Z* purity.

Key Words—Synthesis, sex pheromone, processionary moth, *Thaumetopoea pityocampa*, Lepidoptera, Notodontidae, (*Z*)-13-hexadecen-11-yn-1-yl acetate, acetylene, carbocupration.

INTRODUCTION

The processionary moth *Thaumetopoea pityocampa* (Lepidoptera, Notodontidae) is a severe defoliator pest of pine trees. All of the Mediterranean countries are infested by this moth which causes serious economic damage. Guerrero et al. (1981) have recently isolated and identified the major component of the sex pheromone produced by virgin females. This component, (*Z*)-13-hexadecen-11-yn-1-yl acetate (Scheme 1, I), possesses a conjugated en–yne moiety of *Z* configuration, a new structure in insect pheromone field.

The pheromone and its *E* isomer have been synthesized by Camps et al. (1981). We report here our synthesis, which is based on the carbocupration of acetylene (Normant and Alexakis, 1981). This route affords, preparatively, a product of very high stereoisomeric purity in excellent overall yield and in few steps.

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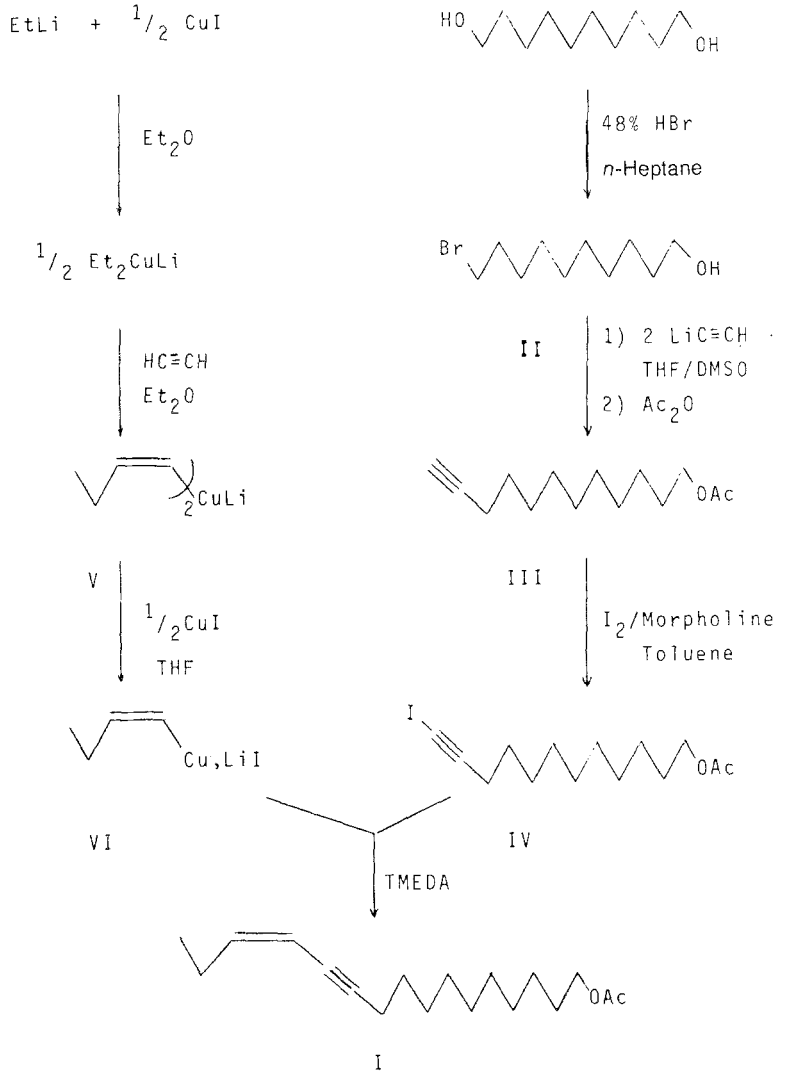

SCHEME I. Synthesis of the main component of processionary moth.

METHODS AND MATERIALS

The [^{1}H]NMR spectra were determined on a Jeol MH-100 spectrometer; [^{13}C]NMR spectra on a Jeol FX 90Q spectrometer. Infrared spectra were measured on a Perkin-Elmer 157 G spectrophotometer. Gas chromatographic analyses were performed on a model 2150 Carlo Erba instrument equipped with capillary glass columns.

10-Bromodecan-1-ol (II). A mixture of 110 g 1,10-octanediol (0.63 mol), 360 ml of 48% aqueous HBr, and 120 ml water is heated at 80° C while continuously extracted by *n*-heptane. After one day the heptane solution is cooled and the precipitate of diol is filtered off and added to the stirred aqueous mixture which is again heated and continuously extracted by *n*-heptane. This operation is repeated on five days after which a total of 137 g (88% yield) of II is obtained (97% purity).

11-Dodecyn-1-yl Acetate (III). A solution of lithium acetylide (0.3 mol) is prepared in 250 ml THF, according to Midland (1975). To this solution are added 23.7 g (0.1 mol) of 10-bromodecan-1-ol (II) at −60° C, then 100 ml of DMSO (formation of a white precipitate). The mixture is stirred overnight at room temperature, whereupon 51 g (0.5 mol) of acetic anhydride are added. The dark mixture is quenched two days later with 100 ml NH_4Cl sat. sol. Then 400 ml of hexane are added to the organic phase which is washed 3 times with water, in order to remove the DMSO, twice with sodium bicarbonate solution, then dried on magnesium sulfate. The solvents are evaporated and the residue distilled, affording 16.46 g of acetate III (yield: 73%); bp 82–83° C (10^{-2} mm Hg). IR (neat 3300 cm^{-1} (m, C≡C), 2120 cm^{-1} (w, C≡C), 1740 cm^{-1} (s, —OAc). [^{1}H]NMR ($CDCl_3$, δ) 4.05 (t, 2H, J = 7 Hz), 1.98 (s, 3H), 1.86 (t, 1H, J = 2.5 Hz).

12-Iodo-11-dodecyn-1-yl Acetate (IV). Iodine (25.5 g, 0.1 mol) is dissolved in 320 ml toluene and 27 ml morpholine in 30 ml toluene are added slowly. After 10 min, acetate III (14.6 g, 0.065 mol) is added to the above orange mixture, and stirring is continued at 45° C for 24 hr. The precipitate is filtered and washed twice with 100 ml ether, and the solutions are washed successively with 10% sodium hydrogen phosphate, 10% sodium thiosulfate, and 5% sodium bicarbonate. The organic phase is dried over magnesium sulfate and the solvents evaporated to afford 21.4 g of nearly pure IV (94% crude yield). IR (neat) 2185 cm^{-1} (vw, C≡C), 1740 cm^{-1} (s, −OAc). [^{1}H]NMR ($CDCl_3$, δ) 4.08 (t, 2H, J = 7 Hz), 2.36 (t, 2H, J = 6.5 Hz) 2.05 (s, 3H). [^{13}C]NMR ($CDCl_3$, δ) 170.9 (C=0), 94.5 (≡C—), 64.5 (CH_2—0), −6.9 (I−C≡).

(Z)-*13-Hexadecen-11-yn-1-yl acetate (I).* A solution of lithium diethyl cuprate is prepared by addition of 80 ml of an ethereal solution (1.25 N) of EtLi (0.1 mol) to a cooled (−40° C) and stirred suspension of cuprous iodide (10.0 g, 0.052 mol) in 100 ml ether. Acetylene (2.4 1, 0.11 mol) is bubbled into this blue solution at −50° C, and after 30 min at −25° C, a greenish solution of (Z)-1-butenyl cuprate (V) is obtained. To this reagent are successively added at −30° C, cuprous iodide (10.0 g, 0.052 mol), 100 ml THF, and 18.5 ml TMEDA (tetramethylethylenediamine) whereupon all CuI dissolved. A solution of iodide IV (21.0 g, 0.06 mol) in 30 ml THF is added dropwise, and the stirred mixture is warmed to 0° C for 30 min. The mixture is then hydrolyzed with 50 ml of ammonium chloride solution and 50 ml 2 N

hydrochloric acid, filtered on celite, and partitioned. The organic phase, to which 200 ml of hexane is added, is washed successively with ammonium chloride solution, 5% sodium thiosulfate, 10% ammonium hydroxide, and a saturated solution of ammonium chloride again. It is then dried over magnesium sulfate and concentrated on a rotatory evaporator. The residue is distilled through a 15-cm Vigreux column to afford 12.6 g of pure pheromone I (76% yield). Bp 105–107° C (10^{-2} mm Hg). $n_D^{20} = 1.4701$. IR (neat) 3020 cm^{-1} (w, HC=CH), 2210 (w, C≡C), 1740 (s, OAc), 730 (m, HC=CH). [^{1}H]NMR ($CDCl_3$, δ) 5.88 (d of t, 1H, $J = 10.5$ Hz and 7 Hz), 5.47 (d, 1H, $J = 10.5$ Hz), 4.12 (t, 2H, $J = 7$ Hz), 2.05 (s, 3H). [^{13}H]NMR ($CDCl_3$, δ) 170.9 (C=0), 143.7 and 109.0 (C=C), 94.4 and 77.3 (C=C), 64.6 (CH_2–0). Analysis—Calc. for $C_{18}H_{30}O_2$: 278.44; C, 77.65; H, 10.86. Found: C, 77.52; H, 10.96.

RESULTS AND DISCUSSION

The synthetic scheme depicted in Scheme 1 shows the simplicity of the four steps in this synthesis. Bromohydrin II is prepared according to the usual procedure in 88% yield. In order to shorten the synthesis, we reacted this crude bromohydrin II with excess (three equivalents) of lithium acetylide, one equivalent for the generation of the lithium alcoholate and the other two for the alkylation. Addition of acetic anhydride before hydrolysis, accomplishes in situ the acetylation. The acetate III is isolated by distillation (73% yield) and then treated with the iodine–morphine complex in toluene to afford the iodoalkyne IV (94% yield)(Lüthy et al., 1978). This crude iodide is then used for the coupling reaction (Normant et al., 1975) with (*Z*)-buten-1-yl copper VI. This reagent is readily obtained by the known addition of lithium dialkyl cuprates to acetylene (Alexakis et al., 1976). The obtained (*Z*)-butenyl-cuprate V, has, in turn, to be transformed into the copper species VI in order to optimize the yield of the coupling reaction (Alexakis et al., 1980).

By this sequence we synthesized ~12 g of pure pheromone I in one batch. The pheromone is easily distilled on a short (10 cm) Vigreux column as a colorless oil. The gas chromatographic analysis (OV-1, 25 m × 0.25 mm, H_2 30 ml/min) showed a stereoisomeric *Z* purity of 98.8% which is consistent with the usual high *Z* purity of the products obtained by carbocupration. Synthetic pheromone was shown to be attractive and specific to male processionary moths in greenhouse and field bioassays in France, Spain, and Israel. This method has been used subsequently on a preparative scale for the synthesis of ~50 g of pheromone which showed a *Z* purity ≥99.5%.

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SYNTHESIS OF (*Z,Z*)-3,13-OCTADECADIEN-1-YL ACETATE
Component of the Sex Pheromone of *Synanthedon tenuis*

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Abstract—The main component of the sex pheromone of the smaller clear wing moth, *Synanthedon tenuis*, was synthesized. This iterative synthesis uses the carbocupration of acetylene, followed by the alkylation of the (*Z*)-alkenyl cuprate or by its reaction with ethylene oxide. (*Z,Z*)-3,13-octadecadien-1-yl acetate, thus obtained, is of 99.8% stereoisomeric purity. An intermediate of the synthesis was (*Z*)-9-tetradecene-1-yl acetate which is obtained in high yield and 99.9% *Z* purity.

Key Words—Synthesis, sex pheromone, smaller clear wing moth, *Synanthedon tenuis* Lepidoptera, Sessiidae (*Z,Z*)-3,13-octadecadien-1-yl acetate, (*Z*)-9-tetradecen-1-yl acetate, carbocupration, alkylation, epoxide opening.

INTRODUCTION

(*Z,Z*)-3,13-Octadecadien-1-yl acetate (Scheme 1, I) has been identified as the major component of the sex pheromone of the female lesser peachtree borer *Synanthedon pictipes* (Tumlinson et al., 1974). Recently Tamaki et al. (1977) pointed to the purity of the pheromone, showing that males of the smaller clear wing moth, *Synanthedon tenuis*, 3,4-(*Z*) double bond was at least 99.5% pure. This fact sets the problem of the stereoisomeric purity of synthetic pheromones. The commonly used method of catalytic partial hydrogenation of the appropriate alkyne is not suited since it affords at least 0.5–1% of the *E* isomer (Henrick, 1977). In the case of 3,13-octadecadiyne-1-ol or its acetate, the usual reduction methods (Lindlar or P-2 nickel or 9-BBN or disiamylborane) gave 97% stereoisomeric purity at best. (Doolittle, 1980). A 99.5% purity product was, however, obtained after HPLC purification on silver

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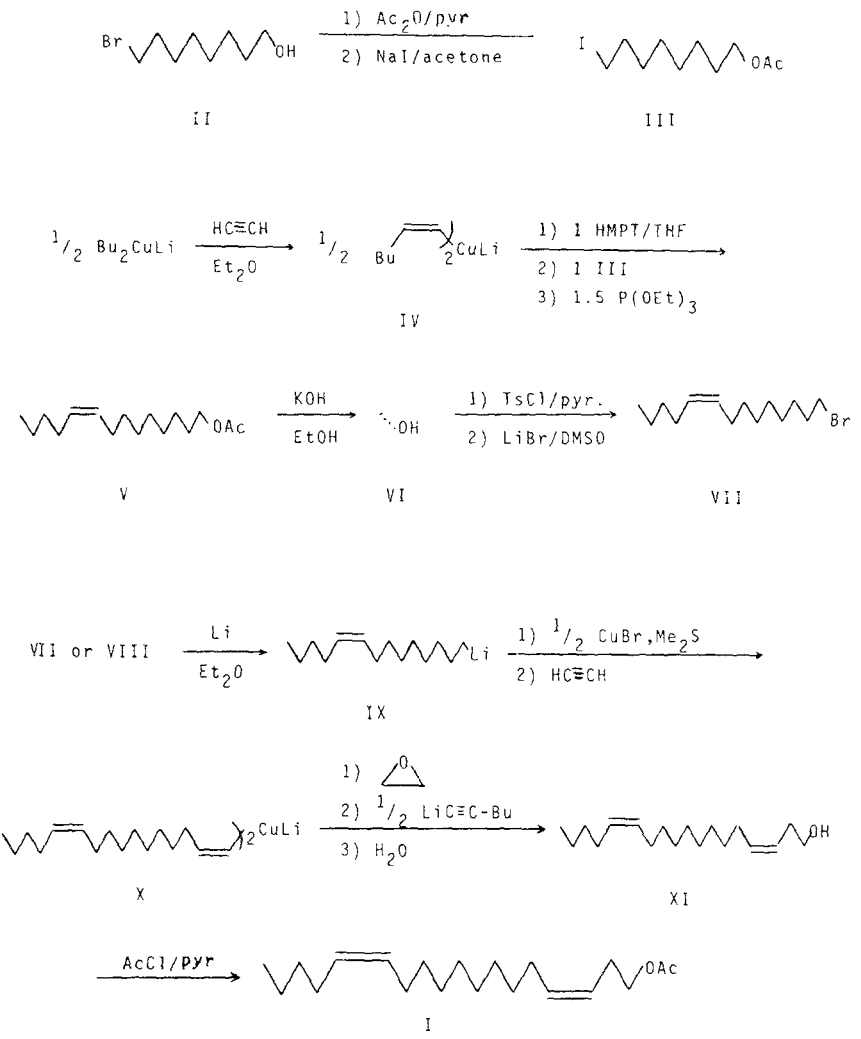


SCHEME 1. Synthesis of the sex pheromone of *Synanthedon tenuis*.

nitrate-silical gel. Uchida et al. (1979) published an elegant synthesis of I, using a cyclic precursor for the elaboration of this sensitive double bond. However, their synthesis suffers from a low overall yield and is ill-suited for preparative purposes. We wish to report here a new preparative synthesis of I of 99.8% stereoisomeric purity without any chromatographic separation. This synthesis is based on the carbocupration of acetylene (Alexakis et al., 1976) which is known to afford products of very high Z purity (Normant and Alexakis, 1981).

Among the intermediates of the synthetic sequence, (Z)-9-tetradecenyl acetate (V) is parpared in high yield and with a Z stereoisomeric purity of 99.9%. This compound is known to be a component of numerous sex pheromones of Lepidoptera.

METHODS AND MATERIALS

[^{1}H]NMR spectra were determined on a Jeol MH-100 spectrometer; [^{13}C]NMR spectra on a Jeol FX 90Q spectrometer. Infrared spectra were measured on a Perkin-Elmer 157 G spectrophotometer. Gas chromatographic analyses were performed on a model 2150 Carlo Erba instrument equipped with capillary glass columns.

8-Iodo-1-octyl Acetate (III). Acetic anhydride (30.6 g, 0.3 mol) is added to 50 ml pyridine. To this ice-cold solution are added, dropwise, 41.8 g of 8-bromo-1-octanol (prepared according to Babler and Invergo, 1979). The mixture is stirred 2 hr at room temperature. Water (200 ml) and ether (300 ml) are added, and the organic layer is washed with 100 ml 1 N hydrochloric acid, then with 100 ml of sodium bicarbonate solution, dried over magnesium sulfate, and concentrated in vacuo. The residue (50.8 g, 100% yield) showed no free hydroxyl group by IR spectrum and was homogeneous by GC. This crude 8-bromo-1-octyl acetate is added, at once, to a solution of 45 g sodium iodide (0.3 mol) in 200 ml anhydrous acetone. A white precipitate is formed which thickens slowly. After stirring 1 hr at room temperature, pentane (200 ml) is added to precipitate inorganic salts, which are removed by filtration. Evaporation of the solvents affords the iodide III with, still, some inorganic salts. Pentane (200 ml) is again added and, after filtration of the precipitate, the solvent is removed under vacuum to afford the crude iodide III (60.1 g, 100% yield) as an oil. This 8-iodo-1-octyl acetate is homogeneous by IR, NMR, and GC analyses, and is used directly in the next step.

(Z)-*9-Tetradecen-1-yl Acetate* (V). Lithium dibutyl cuprate (0.15 mol) is prepared by addition of 215 ml of *n*-butyl lithium (1.4 N in Et_2O) to a stirred suspension of cuprous iodide (30.0 g, 0.157 mol, used as purchased from Prolabo or Merck) in 100 ml ether, at −40° C. Acetylene (7.5 liters, 0.31 mol) is bubbled into this blue solution at −50° C, then the solution is stirred 30 min at −25° C to complete the addition. To the obtained greenish solution of (Z)-1-hexenyl cuprate IV are succesively added at −30° C, 36 ml HMPT admixed with 60 ml THF, then the iodide III (59.6 g, 0.2 mol) in 50 ml THF, and finally 75 g triethyl phosphite. The mixture is stirred at room temperature overnight, then hydrolyzed with 200 ml 5 N hydrochloric acid. After stirring 20 min, the mixture is filtered, the layers separated, and the organic phase (to which 200 ml hexane have been added) is washed succesively with 100 ml 2 N hydrochloric acid, once or twice with 100 ml ammonium hydroxide solution,

then with 100 ml ammonium chloride solution, and finally dried over magnesium sulfate. After evaporation of the solvents, the residue is distilled through a 20-cm Vigreux column to afford pure (*Z*)-9-tetradecenyl acetate (40.6 g, 80% yield), bp 84–85° C (10^{-2} mm Hg). $n_D^{20} = 1.4499$. IR (neat) 3020, 3015 cm^{-1} (w, HC=CH), 1745 (s, —OAc), 1230 (s, —OAc), 725 (v w, HC$\overset{Z}{=}$CH). [^{1}H]NMR ($CDCl_3$, δ) 5.25 (m, 2H), 3.98 (t, 2H, J = 7 Hz), 2.02 (s and m, 7H). [^{13}C]NMR ($CDCl_3$, δ) 170.8 (C$\overset{O}{\underset{O}{<}}$), 129.9, 129.8 (CH=CH), 64.5 (CH_2—O). Analysis: Calc. for $C_{16}H_{30}O_2$: 254.42. C, 75.74; H, 11.89. found: C, 75.51; H, 11.95.

The carbocupration–alkylation sequences depicted in Scheme 2 are performed under exactly the same experimental conditions using the following proportions of reactants:

1. Variation A: BuLi (0.06 mol), CuI (6.3 g, 0.033 mol), HC≡CH (1.5 liters, 0.063 mol), HMPT (5.4 ml), 1,8-dibromooctane (8.16 g, 0.03 mol), triethyl phosphite (15.0 g). Yield: 41%.

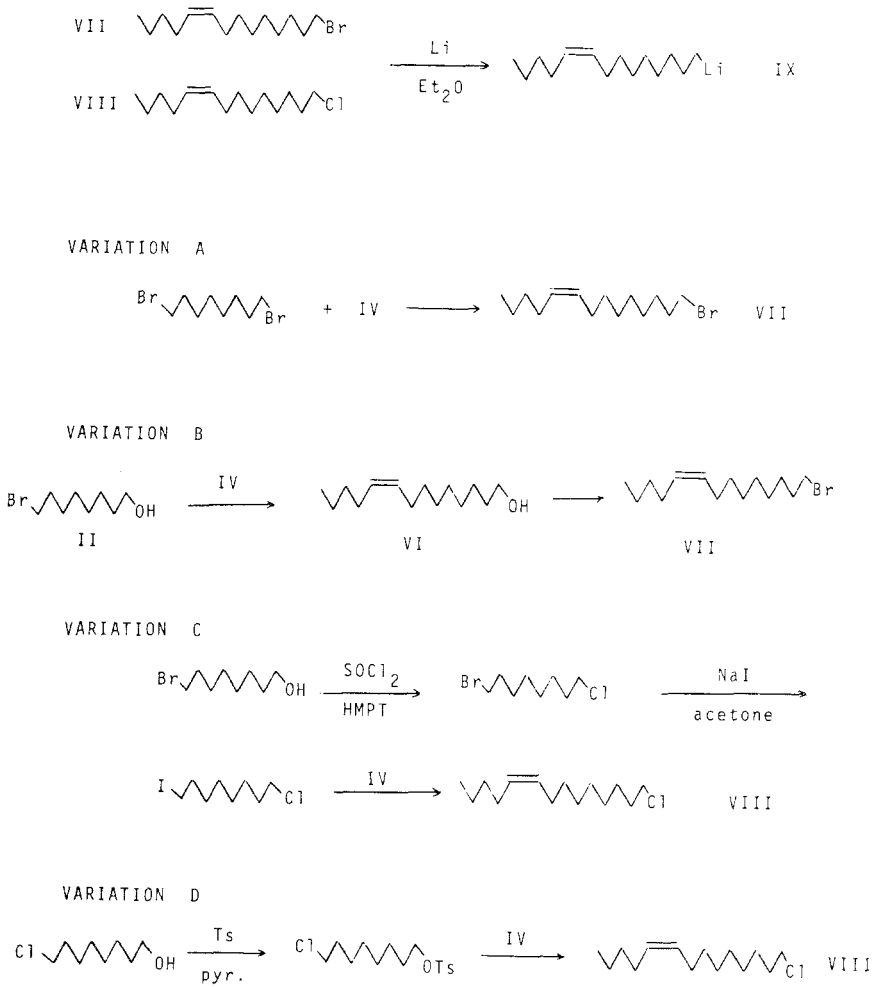
2. Variation B: BuLi (0.3 mol), CuI (30.0 g, 0.157 mol) HC≡CH (7.5 liters, 0.31 mol), HMPT (18 ml), 8-bromo-1-octanol (20.9 g, 0.1 mol), triethyl phosphite (75 g). Yield: 50%.

3. Variation C: BuLi (0.22 mol), CuI (22.8 g, 0.12 mol), HC≡CH (5.5 liters, 0.24 mol), HMPT (27 ml), 8-iodo-1-chlorooctane (41.2 g, 0.15 mol), triethyl phosphite (55 g). Yield: 69%.

4. Variation D: BuLi (0.1 mol), CuI (20.0 g, 0.105 mol), HC≡CH (2.45 liters, 0.10 mol), HMPT (18 ml), 8-tosyloxy-1-chlorooctane (31.8 g, 0.1 mol), triethyl phosphite (25.0 g). Yield: 50%.

(Z)-*9-Tetradecen-1-ol (VI)*. Forty-five milliliters of ethanol, 35 ml water, 23 g (0.4 mol) potassium hydroxide, and 12.7 g (0.05 mol) (*Z*)-9-tetradecenyl acetate are mixed and stirred 6 hr at room temperature. Ether (200 ml) is then added, the layers separated, and the aqueous layer extracted twice with 50 ml ether. The combined organic phases are washed with a solution of ammonium chloride, then dried over magnesium sulfate. After evaporation of the solvents, the residue (10.7 g, 100% yield), which is homogeneous by GC, is used without any other purification. However, the (*Z*)-9-tetradecen-1-ol VI obtained via variation B (see Scheme 2) was distilled, bp 83–86° (10^{-2} mm Hg). $n_D^{20} = 1.4613$. IR (neat) 3300 cm^{-1} (m, —OH), 3010 (w, HC=CH), 725 (v w, HC$\overset{Z}{=}$CH). [^{1}H]NMR ($CDCl_3$, δ) 5.22 (m, 2 H), 3.47 (t, 2H, $J = 7$ Hz). [^{13}C]NMR ($CDCl_3$, δ) 129.8 (CH=CH), 62.5 (CH_2OH).

(Z)-*9-Tetradecen-1-yl Bromide* (*VII*). The crude alcohol VI (10.7 g, 0.05 mol) is added dropwise at 0° C to a stirred solution of tosyl chloride (9.6 g, 0.05 mol) in 15 ml pyridine. The mixture is stirred overnight at room temperature then poured into 100 ml 1 N hydrochloric acid. The tosylate is extracted twice with ether and dried over magnesium sulfate. Evaporation of the solvent



SCHEME 2. Alternative routes to the key intermediate lithium reagent IX.

afforded 17.2 g of crude (*Z*)-9-tetradecen-1-yltosylate. This tosylate is added to a heated mixture (60°C) of 6.5 g (0.075 mol) of lithium bromide and 40 ml DMSO (dimethyl sulfoxide). Heating is continued for 1 hr, and after cooling to room temperature, water (150 ml) and ether (200 ml) are added. The organic layer is washed twice with water (50 ml) and then dried over magnesium sulfate. The solvent is removed under vacuum and the residue distilled through a 10-cm Vigreux column to afford 11.4 g (83% overall yield) of (*Z*)-9-tetradecen-1-yl bromide; bp 88°C (10^{-2} mm Hg). n_D^{20} = 1.4778. IR (neat) 3010 cm^{-1} (w, HC=CH), 725 (v w, HC$\overset{Z}{=}$CH). [^{1}H]NMR ($CDCl_3$, δ)

5.20 (m, 2H), 3.28 (t, 2H, $J = 7$ Hz). [^{13}C]NMR ($CDCl_3$, δ) 129.8, 129.7 (CH=CH), 33.6 (CH_2Br).

(Z)-*9-Tetradecen-1-yl Chloride* (*VIII*). The chloride VIII prepared according to Scheme 2 (see variations C and D) has the following physical and spectroscopic data: bp 71°C (10^{-2} mm Hg). $n_D^{20} = 1.4582$. IR (neat) 3010 cm^{-1} (w, HC=CH), 725 (v w, HC$\overset{Z}{=}$CH). [^{1}H]NMR ($CDCl_3$, δ) 5.29 (m, 2H), 3.48 (t, 2H, $J = 7$ Hz). [^{13}C]NMR ($DCCl_3$, δ) 129.9, 129.7 (CH=CH), 44.8 (CH_2—Cl).

(Z)-*9-Tetradecen-1-yl lithium* (*IX*). A solution of 11.0 g (0.040 mol) of bromide VII in 30 ml anhydrous ether is slowly added, at −15°C, to lithium (0.64 g, 0.088 mol) cut in small pieces and ether (10 ml), to which 2 drops of dibromoethane have been previously added at room temperature (to activate the surface of the metal). After the addition of all the bromide, the solution is stirred 1 hr at 0°C in order to complete the reaction. The greyish solution of reagent IX is titrated by the method of Watson and Eastham (1967) and found to be 0.9 N for a total volume of 34 ml (yield: 75%). The same procedure is used for the preparation of lithium reagent IX starting with (*Z*)-9-tetradecen-yl chloride VIII (yield: 90%). The white precipitate of lithium chloride which is formed does not impede the following carbocupration reaction.

(Z,Z)-*3,13-Octadecadien-1-yl acetate* (*I*). Thirty milliliters of the above-mentioned ethereal solution of (*Z*)-9-tetradecenyl lithium IX (0.9N, 0.027 mol) are added, at −40°C, to a stirred suspension of 3.0 g (0.0146 mol) of the copper bromide–dimethyl sulfide complex in 70 ml ether. Acetylene (0.67 l, 0.03 mol) is bubbled into the resulting solution of cuprate, and the temperature is then raised to −25°C for 15 min. To this (*Z,Z*)-1,10-hexadecadien-1-yl cuprate solution X are succesively added, at −30°C, ethylene oxide (1.5 g, 0.034 mol) in 10 ml ether, then 1-hexyn-1-yl lithium (prepared by addition of 0.015 mol of butyl lithium to 0.015 mol 1-hexyne in Et_2O). The mixture, which turns red, is stirred 1 hr at room temperature, hydrolyzed with 50 ml ammonium chloride saturated solution, and filtered. The organic phase (to which 100 ml of hexane are added) is washed with ammonium hydroxide solution, then with ammonium chloride saturated solution, then dried over magnesium sulfate. After evaporation of the solvents, the crude alcohol XI is added to acetyl chloride (3.15 g, 0.047 mol) in 15 ml pyridine at 0°, then stirred 2 hr at room temperature. The mixture is poured into 30 ml 1 N hydrochloric acid and 100 ml hexane. The layers are separated, the organic phase washed with sodium bicarbonate solution and dried over magnesium sulfate. Evaporation of the solvents afforded the crude pheromone I which is distilled through a 10-cm Vigreux column; 5.3 g of pure pheromone I is thus obtained (63% yield based on the lithium reagent IX); bp: 135°C (10^{-2} mm Hg). $n_D^{20} = 1.4558$. IR (neat) 3010 cm^{-1} (w, HC=CH), 1745 (s, —OAc), 725 (v w, HC$\overset{Z}{=}$CH). [^{1}H]NMR ($CDCl_3$, δ) 5.22 (m, 4H), 3.89 (t, 2H, $J = 7$ Hz), 2.27 (q, 2H, $J = 7$ Hz), 1.93 (s and m, 7H). [^{13}C]NMR ($CDCl_3$,

δ) 170.7 (C=O), 129.8 ($\overset{14}{C}H=\overset{13}{C}H$), 132.9, 124.3 ($\overset{4}{C}H=\overset{3}{C}H$), 63.9 ($CH_2$—O). Analysis: Calc. for $C_{20}H_{36}O_2$: 308.51. C, 77.87; H, 11.76. Found: C, 77.79; H, 11.84.

RESULTS AND DISCUSSION

This iterative synthesis uses two sequential carbocuprations for the elaboration of the two double bonds. In the first step, lithium dibutyl cuprate adds to acetylene to afford the adduct IV, a (Z)-dihexenyl cuprate. This reagent is alkylated in situ (Alexakis et al., 1979) with 8-iodooctyl-1-acetate III (prepared by known methods in 90% yield) giving (Z)-9-tetradecenyl acetate V in 80% distilled yield. The Z stereoisomeric purity of acetate V is very high (>99.9%) as determined by GC on capillary columns (Carbowax 20 M, WCOT 50 m × 0.5 mm, He 30 ml/min). The carbocupration reaction is known to be very stereoselective and this result is consistent with other examples of alkylation of vinylic cuprates. (Z)-9-Tetradecenyl acetate V is a component of numerous sex pheromones of Lepidoptera and it may be prepared competitively, in large scale, as described above.

For the second carbocupration, the acetate V is hydrolyzed quantitatively to the alcohol VI, which in turn is transformed into the bromide VII (83%). This bromide is then used for the preparation of the key lithium reagent IX (75% yield). This reagent may also be prepared from the corresponding chloride VIII in 90% yield. Some variations have been used for the synthesis of (Z)-9-tetradecenyl bromide VII or chloride VIII (see Scheme 2). In variation A, the vinylic cuprate IV reacts with the commercially available 1,8-dibromooctane to furnish 40% (distilled yield) of VII. About 30% of the dibromooctane is recovered unreacted by distillation, the remaining 30% of the mass balance consisting mainly of the dialkylation product. This route is the shortest one for the rapid preparation of lithium reagent IX, but not the most efficient in yield. Variation B uses directly the crude bromohydrin II, which is reacted with (Z)-dihexenyl cuprate IV to afford directly (Z)-9-tetradecenol VI in 60% distilled yield. Variations C and D have been used for the preparation of (Z)-9-tetradecenyl chloride VIII. In variation C, the 1-iodo-8-chlorooctane reacts with reagent IV exclusively at the iodide position, and in variation D, 1-chloro-8-octyl tosylate reacts exclusively at the tosylate position. The respective yields of chloride VIII are 69% and 50%. The second carbocupration, using the lithium reagent IX affords the vinylic cuprate X. In this carbocupration the yield of cuprate X is higher using the copper bromide-dimethyl sulfide complex (House et al., 1975).

Of the two (Z,Z)-hexadecadienyl groups of reagent X, only one reacts initially with ethylene oxide. However, as shown by Alexakis et al. (1980), the second vinylic group may be reactived by addition of a cuprating agent, such

as hexynyl lithium. Thus, both of the (*Z*,*Z*)-hexadecadienyl residues are utilized and after acetylation the total yield of (*Z*,Z)-3,13-octadecedienyl acetate I, based on lithium reagent IX, is 63% of distilled product.

The [^{1}H]- and [^{13}C]NMR spectra showed the pheromone I to be pure. Gas chromatographic analysis of I gave a 99.9% chemical purity (Carbowax 20 M, WCOT 50 m × 0.5 mm, He 30 ml/min). However, as shown by Ebata and Mori (1979), the stereoisomeric purity could not be determined on the acetate I. Upon hydrolysis of a sample of the pheromone I to the alcohol XI a carefull GC analysis (Thermon 1000, 30 m × 0.3 mm, N_2 50 ml/min) showed the presence of 0.1% of (*E*)3–4 isomer, a result consistent with the known high stereoselectivity of the carbocuprate reaction.

Thus, the synthesis depicted above affords preparatively a 31% overall yield (based on bromohydrin II) of pheromone I. The less time-consuming variation A, which uses the commercially available dibromooctane gave a 19.5% overall yield. The authors have synthesized 8 g of this pheromone in two batches (5.5 + 2.5) using the bromide VII obtained by both routes. Additional pheromone (~10 g) was gained by the reaction which used (*Z*)-9-tetradecenyl chloride VIII as synthetic intermediate.

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STABILITY OF CHEMICAL COMMUNICANTS IN URINE: Individual Identity and Age of Sample

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Abstract—Male guinea pigs differentiated between fresh female urine and the same urine aged for 3 hr. Additionally, when tested with an habituation paradigm, they exhibited no evidence of recognizing fresh urine and the same urine aged for 3 hr as coming from the same animal. Males preferred the urine of a strange male compared to their own urine when the urine was fresh, aged for 2 hr or aged for 3 days, but not when the urine was aged for 8 days. These results suggest that the urinary cues of individual identity are not stable and that males have learned to recognize the modifications of their own urine which result from aging. In addition, it was found that changes in urine following aging make it possible for male guinea pigs to discriminate between urine samples aged for different amounts of time.

Key Words—Chemical communication, guinea pig, *Cavia porcellus*, individuality, odors, urine, stability of odors.

INTRODUCTION

Several recent studies involving a variety of mammalian species have demonstrated that information contained in chemical signals is remarkably persistent. For example, the attraction of male hamsters to female vaginal secretions and flank marks persists for at least 100 and 40 days, respectively (Johnston and Schmidt, 1979). The signal in female mouse urine which stimulates males to release ultrasounds persists for 30 days or more (Nyby and Zakeski, 1980). Finally, the attraction of male guinea pigs to female urine in

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preference to male urine is evident for urine aged for at least 40 days (Wellington et al., 1981).

In these recent reports, the sex and species of the donor were critical for producing the response. Other messages, in addition to information on the sex and species of a donor, are also transmitted by chemical signals (Mykytowycz, 1973; Beauchamp et al., 1976). One might predict that the persistence of these other sorts of information might not be so great. Specifically, an important informational component to urine is that which allows the animal to discriminate self from nonself and one individual from another (cf. Yamazaki et al., 1981). If individual identity is coded by a complex pattern of chemical components, as has been speculated (Beauchamp et al., 1976; Bronson, 1971), and if these components are of varying volatilities, the pattern would change rapidly on aging and thus, the information about individual identity would not be very persistent. A rapid loss of differential response following aging of urine from different individuals would support the pattern hypothesis for coding individual identity.

Paradoxically, from an ecological standpoint, it would seem that persistence of cues indicating individual identity would be advantageous in many species. Knowledge of the specific individuals, male and female, which had been in the area during the past several days rather than just during the past few hours could be of considerable value. For example, the ability of the male to recognize his own signal, in spite of aging, could reduce the frequency with which he would need to remark his home area (cf. Mykytowycz, 1973). For such reasons, the persistence of chemical signals has often been suggested as one of their most important characteristics.

The response of conspecifics to the chemical signals of individuals which have aged for varying periods of time has apparently not been previously studied. The tracking ability of dogs, while dependent on more than individual odors, does depend in part on the persistence of odors of individual humans (Kalmus, 1955). Although systematic studies of the temporal constraints on tracking are lacking, the available literature suggests that a track must be less than 7 days old for a dog to reliably follow it (McCartney, 1968).

A related issue is the extent to which the age of a chemical signal can be determined. If the quality and/or intensity of a signal changes with time, it should be possible for an animal to discriminate between samples of different ages. From a practical standpoint it might be advantageous for an individual to be able to determine how recently another individual has deposited a signal.

The following experiments were designed to explore these issues. The first experiment investigated the responses of males to urine of individual females which had been aged for different amounts of time. The next experiment studied the ability of males to discriminate between their own urine and urine of another male when both samples had been aged for equal

amounts of time. In the final experiment, the extent to which males respond differentially to two urine samples from the same female aged for different periods of time was examined.

METHODS AND MATERIALS

Animals. Sexually mature, male domestic guinea pigs (*Cavia porcellus*) bred at the Monell Center were used. The animals were weaned and housed individually at 3 weeks of age. They were maintained on commercial guinea pig chow and water ad libitum.

Urine Collection. Urine for experiments 1a, 1b, and 3 was collected from adult females by placing a clean sheet of aluminum foil beneath the wire floor. Urine for experiments 2a and 2b was obtained by moving the males from their home cage into wire bottom cages for several days prior to collection. They were returned to their home cages a few days prior to testing. Urine which was free from fecal or food contamination was collected every hour, retained as individual samples, and frozen. Previous work indicated that defrosted urine was not differentiated from fresh urine (Beauchamp, unpublished). Estrous state was not determined for female donors since previous studies indicated that males exhibit no differential response to estrous vs. nonestrous female urine under the testing conditions used here (Beauchamp, unpublished).

Testing Procedure. Animals were tested in their home cages (55 × 50 × 35 cm high). Each male was presented with one or two (depending on the experiment) samples (0.1 ml) of urine on a clean glass plate (7.5 × 15 cm). All animals were familiar with the glass plates as repositories of conspecific odors. To prepare aged urine, a fresh sample was placed on a clean glass plate and was retained in an air-conditioned room (approximately 50% humidity) for a prescribed period of time. Under these conditions, samples dried in approximately 30 min. For testing, the plate(s) were placed near the center of the subject's cage and the number of seconds during which the animal had his nose approximately 1 cm from the sample was recorded. The duration of the test varied with experiment (see details below). The observer was not aware of the source of the urine or the predicted results. Data were analyzed with the Wilcoxon sign-rank matched pairs test (T).

Experiment 1. Responses to Female Urine

This experiment was designed to determine if male guinea pigs recognize a sample of urine as that from a particular individual after it has been aged for a short period of time. Males were habituated to an individual female's fresh urine and then tested with aged urine. Two separate treatments (1a, 1b) were used.

Treatment 1a. Twelve male guinea pigs served as subjects. Urine was collected from six adult females over a period of 2 days, divided into four vials per donor and stored at −60° C until the day of testing. Testing made use of a variation on the habituation technique of Schulze-Westrum (1965; Martin and Beauchamp, 1982). Each male was presented with three samples of fresh urine from an individual female, one sample at a time. Each presentation lasted for 1 min with 30 sec between each presentation. Thirty seconds after the third sample, the male was presented for 1 min with a fourth sample from the same female: either fresh or aged for 3 hr. During all presentations, the time spent investigating each sample was recorded. On day 1, half the males received fresh urine and half received aged urine on the fourth presentation. Males in each group were presented with the urine of one of the six females. The test was repeated 2 days later, when each male was exposed to the urine of a different female under conditions opposite to those of the first day.

Treatment 1b. Eighteen male guinea pigs served as subjects. Urine was collected from six female donors over a period of 2 days. Samples from each individual were divided into four vials and stored at −60° C until testing. On the first day of testing, each male was presented with three successive samples of the fresh urine of an individual female. Each presentation lasted 1 min and was separated by 30 sec. Thirty seconds after the third presentation, the males were given a 2-min two-choice test between the aged (3-hr) urine of the same female and aged urine of a different female. Each donor female was randomly assigned to three males for the habituation segment of the testing and to three others for the choice test. Five days later, the test was repeated with the same males. At this test each male was habituated to fresh urine from a female to which he had not been previously exposed. For the choice test following the habituation period, fresh female urine was used: one sample from the familiar (habituated) and one from an unfamiliar donor.

Experiment 2. Response to Self and Nonself Odors

An important informational component in urine is that which allows the animal to discriminate self from nonself (Yamazaki et al., 1981). If urinary signals serve to label an area as home (Johnson, 1973; Mykytowycz, 1973), then it follows that the animal must be able to differentiate his signal from other conspecifics. This was shown to be the case for male guinea pigs; urine from nonself elicits more investigation than urine from self (Beauchamp, 1973).

After the demonstration that components communicating individuality in female urine dissipate rapidly, the persistence of cues for self-identification was tested. In treatment 2a male guinea pigs were given a choice between their own fresh urine and their own urine which had been aged for 3 or 6 hr. It was

hypothesized that a rapid loss of cues for individual identity would result in the aged urine no longer being perceived by males as their own and therefore the males would spend more time with the aged urine than fresh. In Treatment 2b, males were given a series of three two-choice tests between their own urine and that of a strange male. The urines were aged 2 hr, 3 days or 8 days.

Treatment 2a. Eighteen male guinea pigs were subjects and donors. Urine was collected from each individual over a period of 1 day, divided into seven vials and stored at −60°C until the test day. Urine (0.1 ml) was placed on a glass plate and aged at room temperature for the prescribed period of time. The fresh urine was placed on a glass plate immediately before testing. On the first day of testing each subject was given a 4-min two-choice test between two samples of his own urine, one fresh and one aged 3 hr. Two weeks later each male was given a similar two-choice test between fresh urine and urine aged for 6 hr.

Treatment 2b. Fourteen males were tested. Urine was collected from each male over a period of 3–4 days. Each day's sample was frozen separately. Following completion of collection, the urine was defrosted (but kept cold) and all samples taken from one individual were pooled and then divided into six aliquots which were immediately refrozen.

For the first test, one aliquot of urine from each subject was defrosted and a 0.1-ml sample of each was placed in the center of two glass plates. Urine was allowed to dry for 2 hr at which time each male was given a choice between his own urine and urine from another male in the group. This was repeated 3 days later. Two additional pairs of tests, conducted 1 and 2 weeks later, were identical except in these tests the urine was allowed to age for 3 days and 8 days, respectively, on the glass plates prior to testing.

Experiment 3. Response to Differentially Aged Urine

Inspection of the data in experiment 2b as well as our previous studies on gender (Wellington et al., 1981) suggest that the response of male guinea pigs to urine decreases as the urine is aged. Presumably, attractive components are removed by degradation and/or volatilization. Dilution of urine diminishes the time that male guinea pigs spend investigating the samples (Beauchamp et al., 1980); an analogous result probably occurs with aging. If this is the case, it should be possible for guinea pigs to discriminate among different degrees of aging and to detect how long a sample has been aged relative to other samples (Johnston and Schmidt, 1979). To test this ability, we evaluated the response of males to pairs of samples which had aged for different lengths of time. We hypothesized that the sample aged longer would be investigated for shorter periods of time.

Testing Procedure. Female urine was collected, pooled, divided into 2-ml samples and stored at −60°C. Samples (0.1 ml) of urine were aged on

glass plates for prescribed periods of time. Nine males not used in experiments 1 and 2 were given two-choice tests to compare the same urine aged for different periods of time. The tests were separated by 9–15 days and were conducted in the following order: 5 hr (0.21 days) vs. 8 days; 2 days vs. 3 days; 6 days vs. 7 days; 4 days vs. 5 days, and 15 days vs. 16 days.

RESULTS

Treatment 1a. Males presented with fresh female urine spent less time with the samples on each subsequent exposure. When the fourth presentation was a sample which had aged for 3 hr, the males spent significantly more time with this sample than when the fourth sample was fresh urine. Results are summarized in Figure 1.

Treatment 1b. When male guinea pigs were exposed to the fresh urine of one individual and then given a choice between the aged (3-hr) urine of that individual and the aged (3-hr) urine of another, discrimination between the samples was not observed. However, males spent more time investigating the fresh urine of an unfamiliar female than the fresh urine of a familiar female. These results are summarized in Table 1.

These data demonstrate that an individual's urine which has been aged

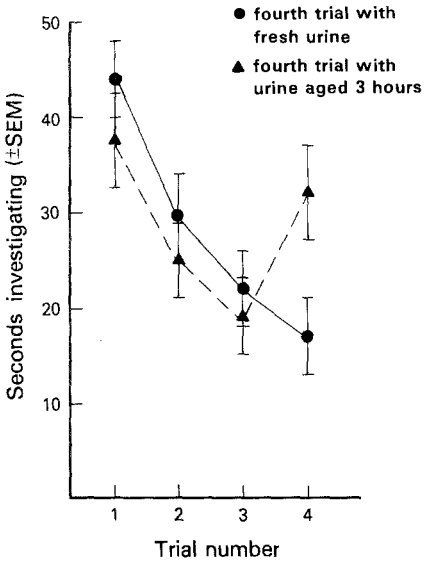

FIGURE 1. Responses of male guinea pigs to samples of urine from individual females. In the first three trials each male was presented with fresh urine from an individual female. In the fourth trial, a sample of fresh urine (circles) or urine aged 3 hr (triangles) from the same female was presented. ($T=11$, $N=12$, $P<0.05$).

TABLE 1. RESPONSE OF MALE GUINEA PIGS TO URINE OF FAMILIAR AND UNFAMILIAR FEMALES

Condition of urine on fourth presentation	Time investigating (sec ± SEM)[a]	
	Familiar	Unfamiliar
Aged 3 hr	27.2 ± 5.1	23.6 ± 3.9
	N.S.	
Fresh	7.75 ± 1.2	31.4 ± 6.8
	$P < 0.01$	

[a]For urine aged 3 hr: $N = 18$, $T = 67.5$; for fresh urine: $N = 18$, $T = 19.5$.

for only 3 hr has changed to the extent that a male guinea pig differentiates it from fresh urine. Males spent more time investigating aged urine than fresh urine after preexposure to fresh urine. This contrasts with experiment 3 which demonstrated that, without habituation, urine aged for a short period of time was preferred to urine aged for a longer period. Further, male guinea pigs habituated to urine from an individual female did not differentiate between urine from this familiar female and urine from an unfamiliar new female when the two samples had been aged for only 3 hr (Table 1). In contrast, males strongly preferred to investigate urine from a novel donor if the urines were fresh during the choice test (Table 1). These results suggest that the components which allow a male guinea pig to identify the fresh urine of an individual female are no longer present or are masked by interfering substances in urine which is aged for as little as 3 hr. At the least, urine aged 3 hr has apparently undergone substantial changes which render components signaling individual identity less effective than if the urine had not been aged.

Treatment 2a. Males did not spend significantly more time with their own urine aged 3 hr ($\bar{X} = 20.5 \pm 3.1$) than their own fresh urine ($\bar{X} = 15.3 \pm 2.8$) ($T = 39.5$, $N = 17$, N.S.). The time they spent with their own urine aged 6 hr ($\bar{X} = 17.3 \pm 3.3$) also did not differ significantly from that spent with their own fresh urine ($\bar{X} = 22.1 \pm 5.0$)($T = 80$, $N = 18$, N.S.).

Treatment 2b. As indicated in Table 2, males spent more time investigating urine from another male compared with their own urine when the samples were aged 2 hr or 3 days, but not when they were aged 8 days.

This set of experiments demonstrates that males discriminate their own urine aged 2 hr or 3 days from the urine of other males which has been aged an equal amount of time. Furthermore, the results suggest that they recognize their own urine as a more familiar stimulus. It is important to emphasize

TABLE 2. RESPONSE OF MALE GUINEA PIGS TO THEIR OWN URINE COMPARED TO URINE OF A STRANGE MALE

	Time investigating (sec ± SEM)[a]	
Condition of urine	Self	Nonself
Aged 2 hr	7.3 ± 1.5	17.6 ± 2.7
	$P < 0.02$	
Aged 3 days	3.7 ± 1.4	9.9 ± 1.9
	$P < 0.05$	
Aged 8 days	2.8 ± 0.9	3.6 ± 0.7
	N.S.	

[a]For urine aged 2 hr: $N = 14$, $T = 15$; aged 3 days: $N = 13$, $T = 15$, aged 8 days: $N = 13$, $T = 27$.

that the subjects have had considerable experience with their own aged urine since, in the cage (and probably in the field), the animals continually return to sniff their old urine. Thus, because the animals are familiar with their own aged urine, this experimental paradigm did not test the hypothesis examined in the first experiment, namely whether fresh and aged urine have significant commonalities to guinea pig subjects.

Experiment 3. Separate Wilcoxon tests were used to test for differences in each of the five comparisons (Figure 2). Urine aged 5 hr was preferred to

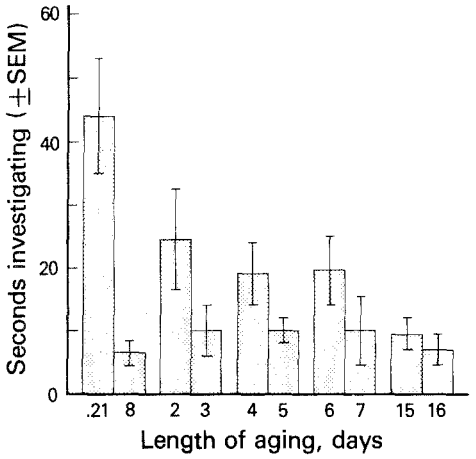

FIG. 2. Responses of male guinea pigs to samples of female urine aged for different lengths of time.

the same urine aged 8 days ($T=0$, $N=9$, $P<0.01$). Similarly, urine aged 2 days was preferred to urine aged 3 days ($T = 1, N = 8, P < 0.02$). Urine aged 4 days was also preferred to urine aged 5 days, although this difference was marginal ($T = 3.5$, $N = 7$, $P < 0.05$ one-tailed). There was no significant difference in response to samples of urine aged 6 compared to 7 days and aged 15 days compared to 16 days.

Thus, males discriminated samples of the same urine aged for different lengths of time. We cannot conclude that the subjects could not, under other circumstances, make the discrimination between the older samples (i.e., 6 days vs. 7 days; 15 days vs. 16 days). However, these data do indicate a potential for time-telling (Johnston and Schmidt, 1979).

DISCUSSION

The data presented here suggest that the signals which communicate individual identity, in contrast to those which communicate gender (Wellington et al., 1981), change rapidly on aging. This adds support to earlier theories (Bronson, 1971; Beauchamp et al., 1976) that individual identity is coded as a complex pattern of volatile molecules. These components, having varying volatilities, seem to be responsible for the change in odor profile with aging. Alternative explanations, such as involvement of a highly labile single molecule, cannot be ruled out, however.

The rapid loss of the individual characteristics of a urine sample with aging is implied by the results of experiment 1, which indicate that males may not be able to identify the aged urine marks of conspecifics. However, guinea pigs are highly social animals (Rood 1972), hence it is possible that, within a social group, males could learn to associate the odor of aged urine of an individual with the fresh urine of the same animal in a manner similar to his learning the characteristics of his own aged urine (experiment 2). As for unfamiliar individuals, the individual identity of these animals may be of minor importance relative to their sex and species, information which we (Wellington et al., 1981) and others (Johnston and Schmidt, 1979; Nyby and Zakeski, 1980) have demonstrated to be remarkably persistent. The paradigm used in these studies to evaluate individual identity takes advantage of the fact that when an animal is habituated to one odor, a different odor elicits more investigation. Other techniques are required to determine whether animals use these differences during natural social interactions (cf. Martin and Beauchamp, 1982).

The data from experiment 2 suggest that male guinea pigs have learned to recognize their own urine as well as modifications in it induced by aging. The alternative explanation, that they are habituated to aged urine present in their cages or on their bodies cannot be ruled out by these experiments. Guinea pigs were able to discriminate their own urine from another male's urine for up to 3 days after voiding. Male guinea pigs have stable overlapping

home ranges and are often aggressive toward strange males. The ability to distinguish one's own urine from another's for an extended period of time has the advantage of allowing the animal to mark his own territory less frequently. Furthermore, with this ability to discriminate, he would be unlikely to confuse his own aged mark with the mark of another individual.

Experiment 3 demonstrated that male subjects were able to discriminate among identical urine samples aged for different periods of time. It is likely that laboratory testing conditions maximized the discriminability of these samples. Such fine distinctions (e.g., between urines aged 4 and 5 days) may be unrealistic. Additionally, the concentration of behaviorally active compounds in urine may vary as a function of a variety of factors including diet, time of day, and context in which the urine is voided. However, these data in conjunction with those of Johnston and Schmidt (1979), suggest that individuals may obtain information on the age of urine marks and hence an indication of how recently other conspecifics have been nearby. This information is relevant to the maintenance of home ranges.

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INVESTIGATIONS OF TRYPSIN INHIBITORS IN LEAVES OF FOUR NORTH AMERICAN PRAIRIE GRASSES

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Abstract—Leaves of four important North American prairie grasses (*Agropyron smithii, Andropogon gerardii, A. scoparius*, and *Bouteloua gracilis*) were examined for the presence of trypsin inhibitors which are thought to protect some plant species from herbivory. Leaves of all four species had significant inhibitor activity at levels comparable to those in tomato leaves. Our evidence suggests that part of the inhibitor activity was proteinaceous and part may have been polyphenolic. Young leaves of *A. smithii* had more inhibitory activity than old leaves, but no leaf age differences were observed in *B. gracilis*. Mechanical wounding of leaves of *A. smithii* caused no consistent increase in inhibitor activity, in contrast to reports for some plant species. Plants of *A. smithii* collected from areas heavily grazed by prairie dogs had trypsin inhibitor levels comparable to those in plants collected from a grazing exclosure. Thus, the ecological role of proteinase inhibitors in these Great Plains dominants remains to be demonstrated.

Key Words—*Agropyron smithii, Bouteloua gracilis, Andropogon gerardii, Andropogon scoparius, Lycopersicon esculentum*, herbivory, trypsin inhibitors, polyphenols.

INTRODUCTION

Plants have developed chemical defense systems against certain herbivores (e.g., Rosenthal and Janzen, 1979; Schoonhoven, 1972). Among many potential defensive chemicals, inhibitors of proteinases such as trypsin have gained recent credence (Ryan, 1978). Numerous investigations, largely with

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seeds, showed that such inhibitors are commonly polypeptides or low-molecular-weight proteins relatively stable to heat and rich in disulfide bonds (Richardson, 1977; Ryan, 1981).

Green and Ryan (1972, 1973) found that chewing by Colorado potato beetles or mechanical wounding of tomato and potato leaves significantly raised the levels of an inhibitor of trypsin and chymotrypsin within 24 hr. Furthermore, either treatment caused inhibitor levels to rise in adjacent noninjured leaves, indicating transport of some factor that induces proteinase inhibitor formation. Evidence for such a factor in leaves from 37 of 39 species representing 20 plant families, including three cereal grains, was subsequently presented (McFarland and Ryan, 1974).

These results suggest that vegetative damage caused by numerous herbivores might enhance production of certain proteinase inhibitors in leaves of many species. If so, such inhibitors could influence evolution of both plants and herbivores. However, Walker-Simmons and Ryan (1977) showed that among 23 plant species, only a few responded either to endogenous proteinase inhibitor inducing factor preparations or preparations from tomato. None of the three cereals they tested responded, a result consistent with those of Kirsi and Midola (1977) and Weiel and Hapner (1976) for barley leaves. Apparently, no native grass species have been tested for the presence of proteinase inhibitors in vegetative tissues.

METHODS AND MATERIALS

Plant Materials. *Agropyron smithii* Rydb. (western wheatgrass), *Andropogon gerardii* Vitman (big bluestem), and *Andropogon scoparius* Michx. (little bluestem) were obtained from sods of plants collected in Wind Cave National Park in southwestern South Dakota (Detling and Painter, 1983). *Bouteloua gracilis* (H.B.K.) Griffiths (blue grama) was obtained from sods collected at the USDA Central Plains Experimental Range near Nunn, Colorado. Tomato plants (*Lycopersicon esculentum* L.) were grown from seeds. All plants were grown in pots in a greenhouse. Because environmental conditions varied between summer and winter, months in which analyses were performed are given in tables. For the grasses, only green portions of leaves from plants without visible florets were used. For tomato, young leaves of plants just forming visible flowers were used.

Extraction of Trypsin Inhibitors. Leaves were excised, weighed, cut into sections about 1 cm long, and homogenized in distilled H_2O (1.0 g fresh wt/12.5 ml) at 5–10° C with a Vir-Tis or Polytron homogenizer. Homogenates were centrifuged at 5° C, either once or twice for 20 min at 20,000 *g*. The green-colored (*A. smithii* and *B. gracilis*), greenish-tan (*L. esculentum*), or tan (*A. gerardii* and *A. scoparius*) final supernatant solutions were used directly

for spectrophotometric protein analysis (595 nm) by the Biorad Coomassie blue dye-binding method (Bradford, 1976; Robinson, 1979) and for trypsin inhibitor analysis.

Trypsin Inhibitor Analyses. A sensitive and specific method (Hummel, 1959) that measures the increase in absorbance at 247 nm as bovine pancreatic trypsin (Sigma Chemical Co.) hydrolyzes the ester bond of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) was used. Assays were performed at 37° C with 0.1 ml of leaf extract (0.04–0.2 mg protein) present in 3.0 ml of a solution also containing 1.65 mM TAME, 8 mM $CaCl_2$, 40 mM Tris HCl, pH 8.1, and 0.18 μg/ml trypsin. Reactions were started by addition of trypsin (prepared and stored at 5° C in 1 mM HCl) and stopped after 20 min with 0.2 ml of cold 5 N acetic acid. Absorption (247 nm) was then measured immediately against corresponding blanks in which H_2O or leaf extract replaced trypsin. Analyses from each extract were performed in duplicate or triplicate. Inhibitory activity is reported on a tissue weight basis (micrograms trypsin inhibited per gram fresh weight) and on a protein basis (specific activity = micrograms trypsin inhibited per milligram protein).

Separation of Inhibitors by Gel-Filtration Chromatography. Sephadex G-25 from Pharmacia was hydrated by boiling for 3 hr in H_2O, then a 1.6 × 15.5-cm column was equilibrated overnight at 5° C with 50 mM Tris HCl (pH 8.1) containing 10 mM $CaCl_2$. The column's void volume (10 ml, determined with blue dextran) and elution volumes for marker proteins of known molecular weights were measured at 5° C according to Pharmacia's instructions. All protein markers and plant extracts were made 10% (w/v) with sucrose before they were added to the column. For *A. smithii*, 1.0 ml of an extract made from 2 g of leaf tissue and 10 ml of H_2O was used. Inhibitor analyses were made with duplicate 0.3-ml aliquots from eluted fractions of 2.0 ml. For *A. scoparius*, 2.0 ml of a similar extract (2 g tissue/10 ml H_2O) were added to the column, and duplicate 0.4-ml aliquots from eluted 2.0 ml fractions were analyzed for trypsin inhibitors. Aliquots (0.3 ml) remaining in each fraction collected were used for protein analysis.

RESULTS

Preliminary results (data not shown) to evaluate the overall inhibitor assay method indicated that leaves homogenized in distilled H_2O had more apparent inhibitor activity than leaves homogenized in 5 mM dithiothreitol. Although dithiothreitol provided greener (less brown) supernatant solutions, such solutions were 20–50% less inhibitory on a leaf weight basis, depending upon the species. Distilled H_2O also yielded slightly more trypsin inhibitor activity than did 50 mM Tris HCl as a homogenizing medium, at least for *A.*

smithii. Inhibition by H_2O extracts was proportional to the volume of extract with aliquot sizes varying from 0.025 to 0.3 ml.

Comparisons of Inhibitor Levels Among Species. Significant inhibitor activity was found in leaves of all four grass species at levels comparable to those in young *L. esculentum* leaves (Table 1). Highest activities on a fresh weight basis occurred in *A. smithii*, while activities in the other three grasses were not significantly different from each other or from *L. esculentum*. On a specific activity (protein) basis, the two *Andropogon* species had the highest activities, because less protein was detected in extracts from them than from all other species. These low protein values likely resulted from precipitation of proteins by phenolics in the tan colored *Andropogon* extracts (Loomis and Battaile, 1966).

Chemical Nature of Grass Trypsin Inhibitors. Part of the inhibitor activity in each grass species is apparently proteinaceous. Evidence that this is true for *A. smithii* and *B. gracilis* was obtained from heat denaturation experiments summarized in Table 2. Heating extracts to 70°C for 10 min (followed by centrifugation but prior to inhibitor assay) caused loss of about two thirds of the inhibitor activity from *A. smithii* and all activity from *B. gracilis*. Concomitantly, about 90% of the protein was made insoluble in each case. For the *Andropogon* species, however, heating made insoluble only about one fourth of the total protein and actually increased apparent inhibitor levels on either a leaf weight basis or a specific activity basis.

TABLE 1. COMPARISON OF TRYPSIN INHIBITOR ACTIVITIES IN FIVE SPECIES[a]

Species	Inhibition (μg trypsin/g fresh wt)	Protein extracted (mg/ml)	Specific activity (μg trypsin/mg protein)
A. smithii ($N = 19$)	59 ± 11 a[b]	1.6 ± 0.6	3.0 ± 1.3 a
B. gracilis ($N = 14$)	43 ± 12 b	2.0 ± 0.2	1.7 ± 0.5 b
A. gerardii ($N = 7$)	41 ± 11 b	0.4 ± 0.1	8.0 ± 2.5 c
A. scoparius ($N = 7$)	44 ± 4.2 b	0.4 ± 0.1	10.0 ± 2.2 c
L. esculentum ($N = 10$)	44 ± 8.7 b	1.7 ± 0.3	2.1 ± 0.3 b

[a] Experiments with *Andropogon* spp. were performed in late summer; others were performed in various seasons.

[b] Means followed by different letters are significantly different at the 95% confidence level (Student's *t* test).

TABLE 2. TYPICAL EFFECTS OF HEATING ON TRYPSIN INHIBITOR ACTIVITIES IN LEAF EXTRACTS[a]

	Agropyron smithii	*Bouteloua gracilis*	*Andropogon gerardii*	*Andropogon scoparius*
Inhibition (μg/g fresh wt)				
Nonheated	63	50	32	38
Heated	22	0	38	48
Protein concentration (μg/ml)				
Nonheated	1.71	1.90	0.40	0.38
Heated	0.18	0.19	0.29	0.32
Specific activity (μg trypsin/mg protein)				
Nonheated	2.7	2.1	6.4	8.0
Heated	9.8	0.0	10.5	12.0

[a]*Andropogon* experiments were performed in July; others were performed in various seasons.

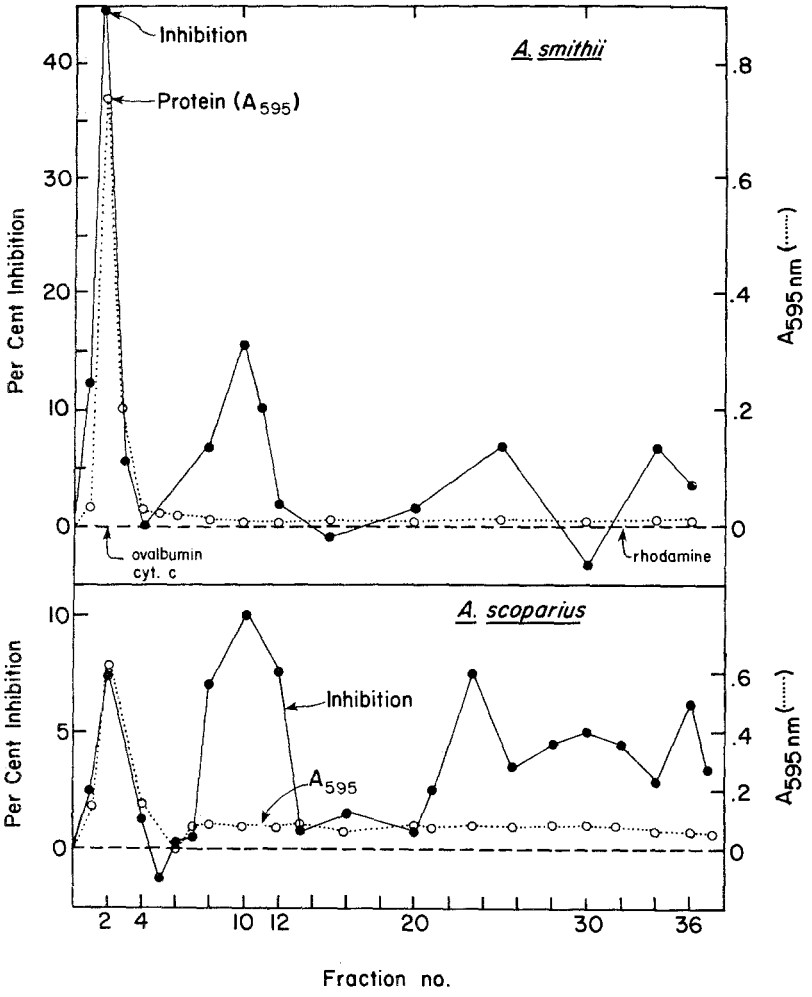

FIG. 1. Separation of trypsin inhibitors on Sephadex G-25 columns. A void volume of 10 ml was eluted before 2-ml fractions were collected. Data points below the zero percent inhibition lines represent apparent stimulation of trypsin.

When an extract from *A. smithii* was fractionated on a Sephadex G-25 column (Figure 1, top), most of the inhibitor activity eluted in a single peak corresponding to marker proteins of molecular weights at least as great as cytochrome *c* (12,400 daltons) and ovalbumin (45,000 daltons). A second inhibitor (fractions 8–12) eluted between those marker proteins and rhodamine D dye (maximum color in fraction 32). Activity in that inhibitory peak is suspected to be polyphenolic in nature. Similar fractionation of an extract from *A. scoparius* (Figure 1, bottom) showed a distinct peak centered in

fraction 2; activity there was probably due to protein(s) but was much lower than the corresponding activity for *A. smithii*. A second peak from *A. scoparius* eluted in fractions similar to those of the second peak from *A. smithii*. In addition, inhibitor activity occurred in fractions 21 to 27. For both species, essentially all protein was probably eluted in the first five fractions. However, *A. scoparius* fractions beyond number 5 were slightly tan colored, and this color contributed to the absorption at 595 nm in protein analyses. We also suspect that the tan color resulted from polyphenolic compounds of diverse molecular weights and that trypsin inhibitor activity in many such fractions might also have resulted from polyphenols.

Inhibitor Levels in Young and Old Leaves. One experiment each for *A. smithii* and *B. gracilis* was performed to evaluate leaf age as a possible factor influencing trypsin inhibitor levels (Table 3). Although young leaves of *A. smithii* contained significantly ($P < 0.05$) greater inhibitory activity than old leaves on a fresh weight basis, no difference on a specific activity basis occurred. For *B. gracilis*, no difference on either basis was found. In a separate study (data not shown) all green leaves from *B. gracilis* plants were compared over a 4-week period; plants grew from about 10 cm tall to about 21 cm tall during this interval. No significant difference in inhibitor activity related to age on a leaf weight or specific activity basis was detected.

Inhibitor Levels in A. smithii *Populations Varying in Herbivory by Mammals.* Clones derived from plants growing within prairie dog colonies or within a grazing exclosure were compared for inhibitor activity (Table 4).

TABLE 3. INHIBITOR LEVELS IN YOUNG AND OLD *A. smithii* LEAVES[a]

Species	Inhibition (μg trypsin/g fresh wt)[b]	Protein extracted (mg/ml)	Specific activity (μg trypsin/mg protein)
A. smithii			
Young	74 ± 9.8 a	1.8 ± 0.3	3.3 ± 0.6
Old	52 ± 14 b	1.3 ± 0.2	3.2 ± 0.8
B. gracilis			
Young	41 ± 5.5 b	2.0 ± 0.2	1.7 ± 0.3
Old	36 ± 5.0 b	1.8 ± 0.3	1.6 ± 0.2

[a] All experiments were performed during February or March using one young and old leaf from 8–16 tillers for *A. smithii* and 11–16 tillers for *B. gracilis*. Young leaves represent those uppermost but completely unfolded. Old leaves represent those closest to the base but still green over three fourths of their lengths; no yellow or brown portions of leaves were used. Each tiller had at least four green leaves. Each value is the mean ± SD of analyses from nine *A. smithii* and four *B. gracilis* plants.

[b] For each species, values followed by the different letters are significantly different from each other at the 95% confidence level (Student's t-test).

TABLE 4. INHIBITOR LEVELS IN *A. smithii* POPULATIONS FROM PRAIRIE DOG TOWNS OR GRAZING EXCLOSURES[a]

Population	Inhibition (μg trypsin/g fresh wt)	Protein extracted (mg/ml)	Specific activity (μg trypsin/mg protein)
Exclosure	54 ± 13	1.6 ± 0.6	2.7 ± 0.9
On town	63 ± 6.9	1.6 ± 0.15	3.1 ± 0.4

[a]Each value represents the mean ± SD of analyses from all green leaves of nine plants made during February–June.

Relative to plants from the exclosure, those subjected to repeated grazing by prairie dogs were much shorter and produced more tillers with shorter, thinner, and less erect leaves (Detling and Painter, 1983). Nevertheless, no significant difference ($P < 0.05$) in trypsin inhibitory levels was detected.

Effects of Wounding on Inhibitor Levels of A. smithii. Two experiments to determine if wounding affects trypsin inhibitor levels in leaves of *A. smithii* were performed. In each experiment, all unfolded leaves on plants with five or more tillers were pressed tightly by mechanical pressure from a pair of pliers. For young, fully green leaves, pressure was applied directly over the midvein 2.5 cm basipital to the tip; for older leaves with senescent tips, pressure was applied 5 cm from the tip. Figure 2 indicates that such wounding treatments caused no consistent increase in inhibitor levels for 4 days after wounding.

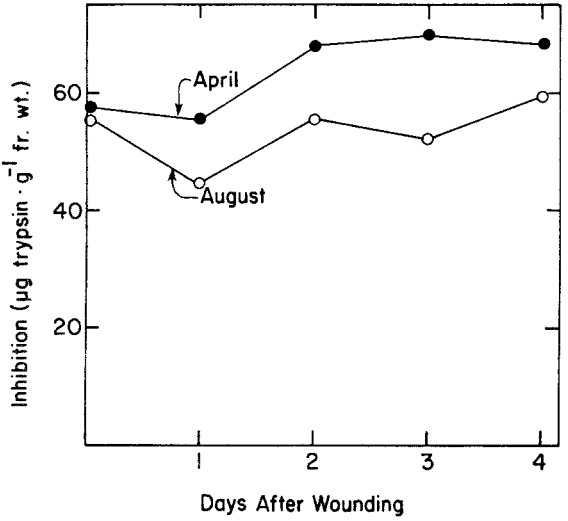

FIG. 2. Influence of wounding upon trypsin inhibitor levels in *A. smithii*. Each point represents analyses from one extract of all green leaves on a single plant.

DISCUSSION

We believe that this is the first demonstration of proteinase inhibitors in leaves of native range grasses. Trypsin inhibitor levels in the species tested were comparable to those in tomato leaves, some of which are proteinaceous and inducible by a factor or factors formed when leaves are injured (Green and Ryan, 1972, 1973; Walker-Simmons and Ryan, 1977; Nelson et al., 1981). Our results suggest that part of the inhibitory activity in each grass species is caused by proteins, although no direct evidence was obtained for *A. gerardii*. First, dithiothreitol lowered the inhibitory activity of extracts from each grass species. Dithiothreitol has two sulfhydryl groups and efficiently reduces disulfide bonds (Cleland, 1964) such as occur in many proteinaceous inhibitors of trypsin and chymotrypsin (Richardson, 1977; Ryan, 1981). Second, *A. smithii* and *A. scoparius* contained one (or more) trypsin inhibitors that elute from a Sephadex column with corresponding leaf proteins and with marker proteins (Figure 1). Third, inhibitor activity in *A. smithii* and *B. gracilis* was reduced by heat denaturation; in fact, heat caused loss of all detectable activity from *B. gracilis* (Table 2). These results suggest that the inhibitors were proteins, but other heat-sensitive compounds or a low molecular weight inhibitor that binds to protein might also have been present.

Heated extracts from each *Andropogon* species actually had more inhibitor activity on either a fresh weight or protein basis (Table 2). The explanation for such increases might relate to the relative abundance of *A. scoparius* inhibitor activity in molecules smaller than proteins (Figure 1) and to the low concentrations of protein (Table 2) and brown color in extracts from both *Andropogon* species. Browning probably resulted from oxidation and polymerization of various phenolic compounds (Bonner, 1950); such compounds remove proteins from solution via H-bonding (Loomis and Battaile, 1966). Perhaps heating of *Andropogon* extracts enhanced formation of trypsin-inhibitory polyphenols and also denatured some plant proteins still in solution. Although we have no direct evidence that polyphenols inhibit trypsin, nonproteinaceous inhibitors apparently contribute to the total inhibitor activity.

Somewhat inconsistent with a suggested role of total trypsin inhibitor activity as a general antiherbivore (Ryan, 1978) system are the similar activities of *A. smithii* plants collected from prairie dog colonies and from uncolonized areas (Table 4). Although persistent morphological and physiological differences emerged during the 12 years of prairie dog colonization (Detling and Painter, 1983), trypsin inhibitor levels apparently did not change. Furthermore, mechanical injury did not appreciably raise inhibitory levels in *A. smithii* (Figure 2). This result differs from the substantial rises in certain inhibitors of trypsin, chymotrypsin, and a metallocarboxypeptidase

after wounding tomato or potato leaves (Graham and Ryan, 1981) but is similar to results from barley leaves (Weiel and Hapner, 1976; Kirsi and Mikola, 1977).

Many additional chemical and ecological data are needed to clarify the importance of trypsin inhibitors in these native grasses that are preferred foods for herbivores such as grasshoppers (Campbell et al., 1974; Hewitt, 1978), prairie dogs (Summers and Linder, 1978), and bison (Peden et al., 1974). Furthermore, there is no compelling reason to assume that the bovine trypsin inhibitors we measured in vitro would be similarly effective against comparable digestive enzymes in native herbivores (Gatehouse et al., 1979).

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VENOM
Source of a Sex Pheromone in the Social Wasp *Polistes fuscatus* (Hymenoptera: Vespidae)

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Abstract—Females of *Polistes fuscatus* possess a sex pheromone in the venom gland and sac. The pheromone attracts males from short distances and releases male copulatory behavior.

Key Words—Social wasp, *Polistes fuscatus*, Vespidae, venom, sex pheromone, male reproductive behavior.

INTRODUCTION

Female sex pheromones occur in many insect orders (Shorey, 1976). Among the social Hymenoptera they have been demonstrated in ants and social bees, where the source of the pheromone is the venom gland (Hölldobler, 1971, 1976; Buschinger, 1972, 1974, 1976; Buschinger and Alloway, 1979; Buschinger et al., 1980), Dufour's gland and the bursa pouches (Hölldobler and Wüst, 1973), or a tergal gland (Hölldobler and Haskins, 1977) in ants, and the mandibular gland in bees (Gary, 1962; Butler, 1971). Sex pheromones in social wasps have not been reported. In this paper we provide the first experimental evidence of the existence, morphological source, and action of a female sex pheromone in a social wasp. The venom of females of the paper wasp *Polistes fuscatus* (F.) contains a chemical that both attracts males and releases copulatory behavior. This is the second communicative function discovered for *Polistes* venom; the venom of a tropical congener *P. canadensis* (L.) possesses an alarm pheromone (Jeanne, 1982).

The annual colonies of *P. fuscatus* produce reproductive males and females during late summer. The males leave the nest several days after they

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emerge and either establish and defend territories at female hibernation sites or patrol female foraging sites, where they intercept and attempt to mate with females. Males are attracted visually to conspecifics of both sexes flying through their territories, but they attempt to copulate only with females (Post and Jeanne, 1983).

METHODS AND MATERIALS

Response to Visual and Chemical Cues. Males were obtained both from the field around Madison, Wisconsin, and from laboratory-reared colonies. To determine whether a chemical or a visual cue releases mating behavior, we released 35 males into a rearing room (1.4 × 2.1 × 2.8 m). Three levels of shelves on the walls of the room held up to 30 Plexiglas nesting cages (20 cm on a side) with screen tops, each containing a nest and females. Six to eight of the males used the shelves and the screen tops of the nesting cages as perches, around which each defended a territory approximately 0.5 m^2 in area. The territorial behavior of the males in the rearing room was identical to that observed in field populations (Post and Jeanne, 1983).

Dried or fresh freeze-killed (FFK) females were tethered by a 30-cm-long thread to a wire pole and waved 15–25 cm in front of each territorial male. The numbers of copulatory attempts elicited by the two types of treated females were tallied. Dried specimens were dried for at least six months and were assumed to be odorless. FFK specimens were gently placed (so as not to alarm them) in a refrigerator at 0° C until immobilized, and then killed by freezing at −20° C for 20 min. They were used in the experiment immediately after killing. FFK females were obtained from the laboratory nesting cages and had little or no prior contact with males.

Bioassay of Female Body Parts. Males reared from nests in the laboratory rearing room and obtained from nests collected from the field in August and September were placed in a holding cage (120 × 60 × 30 cm). The number of males maintained in the cage fluctuated between 100 and 300 individuals. Males housed under these crowded conditions frequently attempted to mate with each other and with dead males. When young males (4–5 days old) recently removed from the natal nest were introduced to the holding cage, the caged males showed a marked increase in flight activity, aggression, and frequency of homosexual (male–male and male–dead male) copulation attempts. These same responses also were obtained on introduction of old (>5 days) males which had been removed from the cage and placed for more than seven days in a 20-cm^3 or a 30-cm^3 cage containing females. A homosexual copulatory attempt was indistinguishable from a heterosexual one: the male grasped the dorsum of the wasp, extruded and probed with the genitalia, and vibrated the antennae (for a description of copulatory behavior, see West-Eberhard, 1969). This suggested that males in contact with females acquired

an odor from them, and that this odor, along with a suitable object (in this case, a live or dead male), elicited copulatory behavior in other males.

We took advantage of these homosexually directed copulatory attempts to develop a bioassay that eliminated the visual cue of the female in our search for the morphological source of the pheromone. Each of several body parts tested was rolled into a 3-cm^2 piece of clean cheesecloth and placed in a wire screen (18 × 14 mesh) cage (1.3 × 2.2 × 3.8 cm). A control cage contained an empty roll of cheesecloth. Individually tested internal organs and glands were macerated on a 1-cm^2 piece of white filter paper which was then rolled into cheesecloth. The control cages for these each contained a clean piece of filter paper rolled into cheesecloth. The cages were placed in the center of a circle (20 cm in diameter) on the floor of the male holding cage. Each trial consisted of presenting a control cage in the circle for 5 min and then replacing it for the next 5 min with a cage bearing a test object. The effect of each on male sexual behavior was quantified by recording the number of homosexual copulatory attempts occurring within the circle during each 5-min interval.

Paired tests were conducted because the activity of the males varied with time of day and temperature. The flight cage was kept in the laboratory and was exposed to natural lighting near a window during the tests conducted from September to November. The greatest activity occurred between 1100 and 1400 hr; after 1700 hr the males formed clusters in the cage and did not respond to the tests. Therefore, all trials were conducted between 1100 and 1430 hr.

Bioassay of Hexane Extract. The venom glands and sacs of ten FFK females were squeezed with forceps in 500 μl hexane. For each trial 50 μl (one female equivalent) of the extract was placed on a 2-cm^2 piece of white filter paper and allowed to dry for 3 min. For presentation to males, the preparation was placed at the center of the 20 cm circle in the holding cage, as described previously. Controls consisted of pure hexane presented similarly. Each trial began with a 3-min presentation of a control and was followed by presentation of the extract for the next 3 min. The number of homosexual copulation attempts and the number of times the males antennated the filter paper were recorded for each control and test paper.

Attraction of Males to Female Sex Pheromone. Two 10-cm-diameter circles were drawn, 45 cm apart, on the floor of the holding cage. Into one circle we placed a test object and into the other a control object. The test and control objects were not placed in a small cage as above. The tested objects and corresponding controls were a FFK head vs. dried head, a FFK thorax vs. dried thorax (both with legs and wings removed), a FFK gaster vs. dried gaster, venom gland and sac crushed onto 1-cm^2 white filter paper vs. clean white filter paper, and 50 μl (one female equivalent) hexane extract of venom sacs and glands prepared as above vs. pure hexane. The males in each circle were counted at the beginning of each trial, and at 10 and 30 sec after the

simultaneous introduction of the test and its control. During consecutive trials with the same type of object, the test and control were alternated between the two circles. Trials were separated by at least 15 min, enough time to allow the males to disperse from the test circle.

RESULTS

Response to Visual and Chemical Cues. The dried females (visual cue alone) did not elicit copulatory attempts ($N = 16$ males) from the males holding territories in the rearing room. On the other hand, 9 of 14 males attempted to copulate with tethered FFK females (visual plus odor cues). These results suggest that the females possess a pheromone which releases copulatory behavior in males.

Bioassay of Female Body Parts. FFK females caused a significant increase in homosexual copulatory attempts among males in the holding cage, while dried females did not (Table 1, a and b), corroborating the results with territorial males described above. The tests of isolated body tagmata showed that the responsible odor is localized in the gaster (Table 1, c–e), and specifically in the venom sac and gland (Table 1, f–j).

Bioassay of Hexane Extract. The extract of the venom gland and sac clearly contained an odor which released male copulatory behavior (Table 2).

Attraction of Males to Female Sex Pheromone. Significant numbers of

TABLE 1. BIOASSAY OF FEMALE BODY PARTS[a]

Test object	No. of trials	Control	Test	T/C ratio	t	P value
a. Dried female	10	92	80	0.87	0.69	$0.5 < P < 0.75$
b. FFK female	10	100	161	1.61	4.38	$0.001 < P < 0.01$
c. Head	10	52	61	1.17	1.08	$0.2 < P < 0.4$
d. Thorax	10	69	60	0.87	0.79	$0.4 < P < 0.5$
e. Gaster	10	59	135	2.29	2.90	$0.01 < P < 0.02$
f. Venom gland and sac	10	66	185	2.80	5.78	$P < 0.001$
g. Dufour's gland	10	91	83	0.91	0.75	$0.4 < P < 0.5$
h. Sixth sternite gland	10	93	88	0.95	0.60	$0.5 < P < 0.9$
i. Ovaries and hindgut	10	109	102	0.94	0.94	$0.2 < P < 0.4$
j. Gaster minus f–i	10	104	106	1.02	0.25	$0.5 < P < 0.9$

[a]Total numbers of homosexual copulatory attempts during 5 min within a 20-cm circle on the floor of a cage of *Polistes fuscatus* males. Data evaluated using paired t test.

TABLE 2. BIOASSAY OF HEXANE EXTRACT[a]

No. of trials	Control	Test	T/C ratio	t	P value
Antennate filter paper					
8	10	60	6.00	7.20	< 0.001
Homosexual copulation attempt					
8	26	123	4.73	6.90	< 0.001

[a]Data are the numbers of times a male antennated filter paper containing a hexane extract of the venom sac and gland and the numbers of homosexual copulatory attempts during 3 min within a 20-cm circle on the floor of a cage of *Polistes fuscatus* males. Data evaluated using paired t test.

males were attracted into the circles containing the FFK gaster, crushed venom sac and gland, and hexane extract of the venom sac and gland (Table 3). Thus the venom contains a pheromone that attracts males from short distances.

DISCUSSION

Our results show clearly that the venom of females of *Polistes fuscatus* contains a sex pheromone that attracts males and releases male copulatory behavior. It is not known, however, how the sex pheromone is released by the females. Females may either actively control venom release specifically to attract and excite males, or the venom may continually leak out in small amounts on the sting apparatus. The latter appears to be more likely, for males are attracted to and attempt to copulate with females on the nest, where females are unreceptive to males and mating typically does not occur (Noonan, 1978). The attractiveness of females to males in this nonreproductive context suggests that active release of venom need not occur to make females attractive in a reproductive context.

The venom clearly attracts males over distances of a few centimeters, but appears to play no role in long-range attraction of males. In contrast to some species of myrmicine ants possessing a sex pheromone in the venom (Buschinger, 1972, 1974, 1976; Buschinger and Alloway, 1979; Buschinger et al., 1980), *P. fuscatus* females do not exhibit a calling behavior in which they hold the gaster upright and extrude the sting (D.C.P., personal observation). Moreover, since in the field males are frequently attracted to various insects, such as bees, flies, and butterflies, as well as conspecifics (Post and Jeanne, 1983), males probably respond more to visual than to chemical cues for the initial detection of females.

For social insects, the presence of a sex pheromone in the venom is not

TABLE 3. ATTRACTION OF MALES TO SEX PHEROMONE[a]

Test object	No. of trials	Time after introduction (sec)	Control	Test	T/C ratio	t	P value
Head	10	0	35	46	1.31	1.11	$0.2 < P < 0.4$
		10	50	53	1.06	0.29	$0.5 < P < 0.9$
		30	36	48	1.33	1.72	$0.1 < P < 0.2$
Thorax	10	0	39	51	1.31	1.25	$0.2 < P < 0.4$
		10	43	42	0.98	0.16	$0.5 < P < 0.9$
		30	54	51	0.94	0.37	$0.5 < P < 0.9$
Gaster	10	0	47	52	1.11	0.46	$0.5 < P < 0.9$
		10	36	106	2.94	4.42	$0.001 < P < 0.01$
		30	40	125	3.13	5.61	$P < 0.001$
Venom gland and sac	10	0	32	25	0.78	1.30	$0.2 < P < 0.4$
		10	24	92	3.83	3.89	$0.001 < P < 0.01$
		30	22	93	4.23	5.80	$P < 0.001$
Hexane extract	8	0	31	27	0.87	0.12	$0.9 < P$
		10	29	53	1.83	2.61	$0.02 < P < 0.05$
		30	28	65	2.32	13.02	$P < 0.001$

[a]Total numbers of males within 10-cm-diameter test and control circles on the floor of a male holding cage at 0, 10, and 30 sec after the simultaneous introduction of test (FFK ♀) and control (dried ♀) objects into the circles. Data evaluated using paired t test.

unique to *P. fuscatus*. The venoms of the myrmicine ants *Xenomyrmex*, *Pogonomyrmex*, *Harpagoxenus*, *Leptothorax*, and *Formicoxenus* also possess a sex pheromone (Hölldobler, 1971, 1976; Buschinger, 1972, 1974, 1976; Buschinger and Alloway, 1979; Buschinger et al., 1980). Venom as the medium of sex pheromone may represent a primitive state. Venom is preadapted as a male sex attractant, since it is the most abundant female-specific exocrine product. Our finding that active release of the venom by females need not occur to elicit a response from males suggests that *Polistes* females have evolved little in the way of refinement in signal production, such as the calling behavior evolved by ants. In the context of their social behavior, uncontrolled release of sex pheromone leads to continual harassment of females by males, causing a disruption in the functioning of the colony. One might expect that in higher social wasps females have evolved the ability to control the release of sex pheromone, leading to greater colony cohesion during male production.

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FIELD ATTRACTION OF *Agrotis segetum*[1] MALES IN FOUR EUROPEAN COUNTRIES TO MIXTURES CONTAINING THREE HOMOLOGOUS ACETATES

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Abstract—(*Z*)-5-Decenyl, (*Z*)-7-dodecenyl, and (*Z*)-9-tetradecenyl acetate were tested as single compounds and blends for field attraction to the turnip moth in Denmark, France, Hungary, and Switzerland. Best attraction was obtained when all three components were present. Attractants for other noctuids are described, e.g., *Meristis trigrammica*, *Hoplodrina alsines*, *Erastria trabealis*, *Euxoa nigricans*, *Axylia putris*, and *Autographa gamma*, which were caught with some of the components and their blends.

Key Words—(*Z*)-5-Decenyl acetate, (*Z*)-7-dodecenyl acetate, (*Z*)-9-tetradecenyl acetate, sex attractant, sex pheromone, moth, *Agrotis* (*Scotia*) *segetum*, *Meristis trigrammica*, *Hoplodrina alsines*, *Erastria trabealis*, *Euxoa nigricans*, Lepidoptera Noctuidae.

INTRODUCTION

The turnip moth or white-line dart moth, *Agrotis* (*Scotia*) *segetum* Schiff. (also called "Saateule" in German, "Agerugle" in Danish, "ver gris" in French, and "vetési bagoly" in Hungarian), is one of the most destructive noctuid pests

[1]Lepidoptera: Noctuidae.

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in vegetable and root crops in Europe. Several reports have dealt with its biology, detection, and control (Jermy, 1952; Poitout and Bues, 1977; Surján and Herczig, 1978; Esbjerg et al., 1980, 1982; Mikkelsen and Esbjerg, 1981).

In order to develop an attractant to monitor the pest, several researchers have concurrently studied the chemistry of its sex pheromone. Bestmann et al. (1978) identified (*Z*)-5-decenyl acetate (*Z*5–10:Ac) as the EAG-active component in extracts of turnip moth females. They were able to attract males to the synthetic chemical in field cage experiments, but dispenser loads above 0.5μg proved to be inhibitory.

In independent studies, Arn et al. (1980) found evidence for the presence of several components whose structures, from the evidence available, were consistent with *Z*5–10:Ac, dodecyl acetate, (*E*)-5-dodecenyl acetate, (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac), (*Z*)-8-dodecenyl acetate (*Z*8–12:Ac), and (*Z*)-9-dodecenyl acetate (*Z*9–12:Ac). A mixture of these components excluding *Z*8–12:Ac was as attractive as virgin females; however, only *Z*5–10:Ac and *Z*7–12:Ac were found to be essential for attraction. *Z*5–10:Ac alone was attractive to field populations in France, but not in Denmark or Switzerland; *Z*7–12:Ac was not attractive. Later studies (Arn, Bues, Esbjerg, and Städler, unpublished) showed that, with both essential components present, catches could be improved by increasing the dosage. A synergistic effect of *Z*9–12:Ac observed in earlier tests could not be confirmed.

Meanwhile, Tóth et al. (1980) identified the two homologous compounds *Z*7–12:Ac and (*Z*)-9-tetradecenyl acetate (*Z*9–14:Ac) in a 4:1 ratio in females of the same moth. Field tests were conducted in two locations in Hungary. In one case, the mixture with the natural ratio was the most attractive, in the other, *Z*7–12:Ac was just as attractive as the blend; *Z*9–14:Ac alone was not attractive.

A fourth study of interest in this context was undertaken in Japan by Wakamura (1980) on *Agrotis fucosa*, a closely related species. He identified *Z*5–10:Ac and (*Z*)-7-decenyl acetate (*Z*7–10:Ac), two compounds of the same chain length, in female extracts. Each compound alone was unattractive; best attraction was obtained at ratios of 1:9 and 1:99.

The contradictions in the reports on *A. segetum* suggest that either some of the identifications of moths or chemicals were wrong or incomplete, or that sex pheromone communication in this species is subject to intraspecific variation. In the present study we have attempted to clarify the problem by testing various compounds for which field activity was previously demonstrated—three homologous acetates for the major part of the experiment—as components of attractants for *A. segetum* males.

METHODS AND MATERIALS

*Z*5–10:Ac was kindly provided by Dr. D.L. Struble, Agriculture Canada, Lethbridge, Alberta, and *Z*7–10:Ac by Dr. S. Voerman, Institute of

Pesticide Research, Wageningen, Netherlands. Z7–12:Ac and Z9–14:Ac were purchased from Farchan Chemicals (now ChemSampCo, Columbus, Ohio). All chemicals were purified by liquid chromatography on $AgNO_3$/silica gel and contained less than 0.1% of the *E* isomer. Overall purity was 99% or better as observed by capillary GLC on Silar 10C.

Dispensers were made up at Wädenswil; Tetra traps with flaps and rubber caps used as dispensers were the same as used previously (Arn et al., 1979). The caps as well as traps with caps attached, of the same treatment, were kept in sealed bags of polyethylene–polyvinylidene chloride-coated paper (Perlamix TR 504018, Papierfabrik Perlen, 6035 Perlen, Switzerland). In preliminary tests, the bags retained pentane for several weeks before loss was complete, and it was assumed that they would minimize evaporation of attractant during transport and storage. While the same caps were left in the traps for the duration of the preliminary test and the dosage test, caps in the three-component test were replaced once a week.

Traps were placed in vegetable fields near the top of the foliage, i.e., between 35 and 80 cm above ground, or in orchards at 150 cm. Host plants and distances between treatments were those given in Table 1 unless indicated otherwise. Distances between replicates were generally wider where space was available. To balance position effects, different sequences of traps were chosen for every replicate. In the three-component test, the 21 traps were placed in three parallel rows or, exceptionally, in one long row, and new positions assigned when the caps were replaced or more often.

Catches were recorded once a week or more. Identifications were made by the laboratory concerned on the basis of wing pattern and, if necessary, genitalia. Numbers were transformed to $\log(x + 1)$ and submitted to analysis of variance followed by Duncan's multiple-range test.

RESULTS

Preliminary Tests. First field tests incorporating the compounds used by Wakamura (1980) and by Tóth et al. (1980) were carried out in France during the first generation flight of 1981. The standard used was a 2:1 blend of Z5–10:Ac and Z7–12:Ac which was optimal in the previous tests in Denmark, France and Switzerland.

The results (Table 2) show that Z7–10:Ac, when added to the standard at the same amount as Z5–10:Ac, had no effect on *A. segetum* catch, whereas a 1:10 mixture of the two decenyl acetates, similar to that used for *A. fucosa* (Wakamura, 1980), was significantly more attractive than the standard. These results may suggest similarities in sex attraction of *A. segetum* and *A. fucosa*

A significant increase in catch was also obtained when Z9–14:Ac was added to the standard blend, both at high and low dosage. A mixture of Z7–12:Ac and Z9–14:Ac alone, at the level used by Tóth et al. (1980), was not attractive. Traps baited with this mixture caught *Macdunnoughia confusa* to

TABLE 1. TRAP PLACEMENT IN THREE-COMPONENT TEST

Country	Denmark	Switzerland	France	Hungary
Commencement	12 June	16 June	7 July	21 July
Duration (weeks)	2	3	6	3
Replicates	4	5	1	7
Culture	Carrot, potato, cucumber	Sugar beet, carrot, others	Apple	Peach
Distance between traps (m)	25–30	15–25	5	15

which the other traps were not attractive. However, a test for *Agrotis ipsilon* conducted in the same area showed that *A. segetum* males are indeed attracted to binary mixtures of Z7–12:Ac and Z9–14:Ac when these chemicals are present at ratios of 2:1 to 4:1 and a total amount of 4–25 μg/cap. These results indicated that *A. segetum* is attracted to both binary mixtures of Z7–12:Ac with Z5–10:Ac or Z9–14:Ac and that catches can be higher when all three homologs are present. This strongly suggested the necessity for systematic tests over the entire mixture range of these components.

Attraction of A. segetum *Males in a Three-Component System.* All one- to three-component formulations were made up containing Z5–10:Ac, Z7–12:Ac, and Z9–14:Ac at 0, 20, 40, 60, 80, and 100% with a total amount of 25 μg/cap. These 21 treatments were tested almost concurrently in the four countries during 1981; the first flight in Denmark and Switzerland coinciding with the second flight in France and Hungary.

The results are given in Figure 1. In all countries, catches are seen to be highest in the inner part of the diagram with blends containing all three

TABLE 2. *A. segetum* CATCHES WITH BLENDS OF ACETATES[a]

Composition of lure (μg chemical/cap)				Total no. males caught
Z5–10:Ac	Z7–10:Ac	Z7–12:Ac	Z9–14:Ac	
10		5	(standard)	28b
10	100			65a
10	10	5		31b
10		5	1	63a
10		5	10	71a
		800	200	1c

[a]Montfavet, April 14–June 18, 1981, 6 replicates. Numbers followed by the same letter are not significantly different at $P = 0.05$.

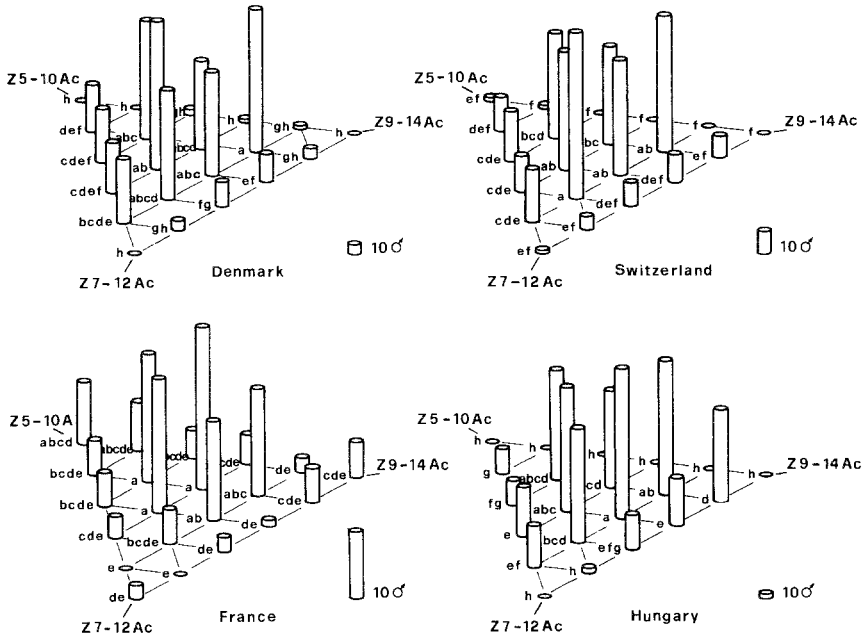

FIG. 1. Catches of *A. segetum* males in four countries with (*Z*)-5-decenyl acetate, (*Z*)-7-dodecenyl acetate, and (*Z*)-9-tetradecenyl acetate as single compounds and as binary and ternary mixtures. Bars flanked by the same letter are not significantly different at $P = 0.05$.

components. Differences can mainly be observed with one- and two-component systems. In Denmark, Switzerland, and Hungary almost no catches were made with single components or with mixtures lacking *Z*7–12:Ac. In these countries, attraction can be observed for both binary mixtures of *Z*5–10:Ac and *Z*9–14:Ac with *Z*7–12:Ac, confirming earlier results by Arn et al. (1980) and Tóth et al. (1980). In Hungary, peak attraction for mixtures of *Z*7–12:Ac and *Z*9–14:Ac is at a ratio of 1:4 which is the reverse of the previous result. This may be due to the fact that the cap load in those tests was 40 times higher (Tóth et al., 1980). In France, surprisingly, *Z*5–10:Ac alone was a good attractant, as observed earlier (Arn et al., 1980).

It can also be noted that significant differences are found among the ternary blends and that positions of maxima differ from one country to another. This may not necessarily reflect a true difference between populations. Release rates of the three homologs from rubber are very different (Butler and McDonough, 1979), and despite the precautions taken by replacing the caps every week, the composition of the attractant vapor can be expected to vary with age and weather conditions. Nevertheless, a blend

containing approximately equal amounts of the three homologs should provide a good attractant for *A. segetum* monitoring throughout Europe.

Dosage Test. As soon as the first results of the three-component test were obtained, an additional experiment was set up to establish the optimum dose of a mixture of equal amounts of each homolog. The results (Table 3) show that doses of 30 and 300 μg of chemical are in the optimum range while catches drop off at either end of the scale. Caps of a 3000-μg dosage attracted *Cucullia* sp. in France and *Cucullia umbratica* in Denmark and Hungary.

Captures of Other Species. A number of other noctuid species were attracted to traps containing one or more of the three homologs. Figure 2 gives the results for the country in which highest catches were obtained.

Meristis trigrammica Hufn. was caught in Denmark and Switzerland with mixtures containing all three components; maximum catch was observed at 60% Z5–10: Ac and 20% of each of the other two components. Mixtures of Z7–12: Ac and Z9–14: Ac, but not the single components, attracted *Hoplodrina alsines* Brahm. in Hungary and Switzerland.

In several species, attraction was apparently caused by only one of the components. Traps containing Z5–10: Ac and mixtures of this compound caught *Erastria trabealis* Scop. in Hungary and France. Z9–14: Ac alone and in combination with Z5–10: Ac attracted *Axylia* (*Rhyathia*) *putris* L. in all four countries, in accordance with Descoins et al. (1978) and Szőcs et al. (1981). In Hungary, traps attractive to *A. putris* also attracted *Hoplodrina blanda* Schiff. *Euxoa nigricans* was attracted to traps with Z7–12: Ac alone and in combination with Z5–10: Ac. *Autographa gamma* L. was caught with Z7–12: Ac, the other two acetates acting as inhibitors, confirming Priesner (1980). Eight males of *Caradrina clavipalpis* Scop. were caught in Switzerland with the same attractant (cf. Szőcs et al., 1981). Results with *E. trabealis*, *E.*

TABLE 3. *A. segetum* CATCHES AT DIFFERENT DOSAGE LEVELS OF Z5–10: Ac, Z7–12: Ac AND Z9–14: Ac (1 : 1 : 1)[a]

	No. males caught between dates given (1981)			
Amount of blend per cap (μg)	Denmark July 5–25	Switzerland June 29–July 22	France July 15–August 26	Hungary July 15–September 14
3	0 c	2 a	37 b	61 b
30	10 ab	8 a	67 ab	98 a
300	18 a	10 a	75 a	111 a
3000	3 bc	2 a	72 a	17 c

[a]Totals of 4 replicates. In each column, numbers followed by the same letter are not significantly different ($P = 0.05$).

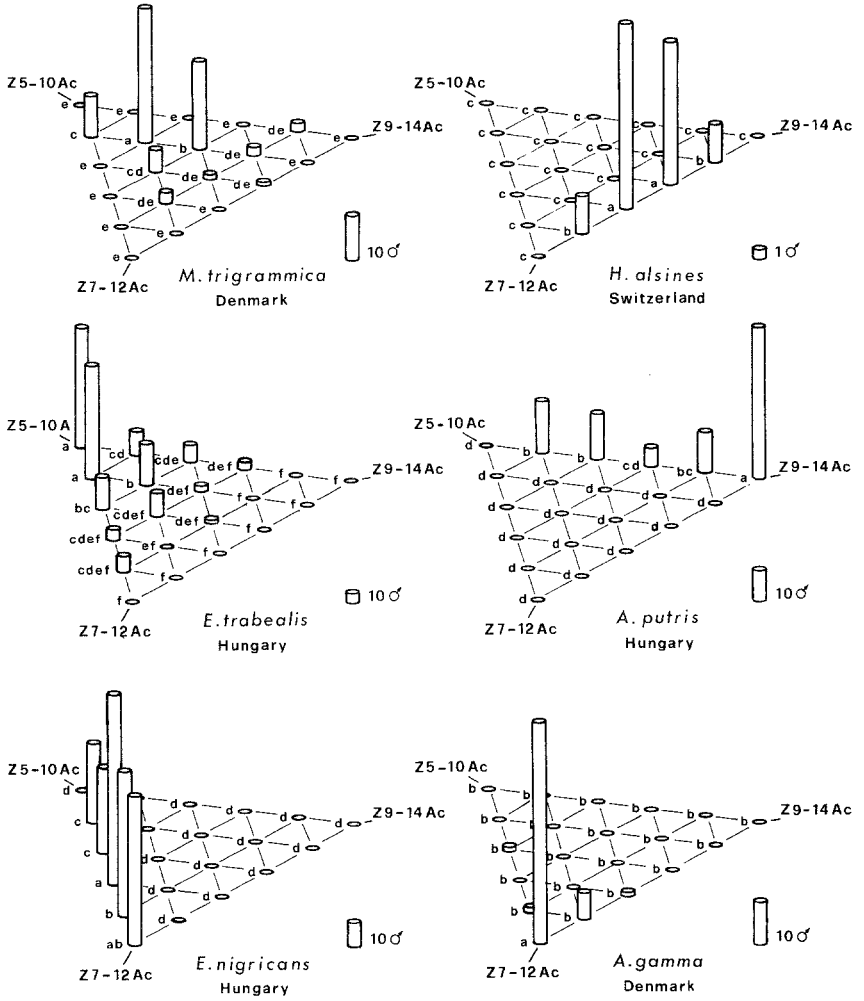

FIG. 2. Catches of other noctuids in the three-component attractant test for *A. segetum* shown in Figure 1. Bars flanked by the same letter are not significantly different at $P = 0.05$.

nigricans, and *H. alsines* are in accordance with previous findings which will be reported elsewhere (G. Szőcs and M. Tóth, in preparation).

Depending on the species, a chemical can lead to trap catch increase (+), decrease (−), or have no effect (0) at a particular dose. It is interesting to consider that with three components of which at least one is required for attraction, 19 combinations are possible, each giving rise to a different type of

attraction diagram. *A. segetum* and *M. trigrammica* both belong to the +++ type since all three components are required for attraction. *H. alsines* follows a −++ scheme since Z5–10:Ac is inhibitory, whereas *A. gamma* can be classified as a −+− type, Z5–10:Ac and Z9–14:Ac being inhibitors. A clear distinction between an inhibitor and an inert material cannot always be made since no dose–effect curves on single compounds or binary mixtures were established. It appears, however, that *A. putris* can be classified as a 0−+ type and *E. trabealis* as a +00 or an intermediate between that and +−−. *E. nigricans* which, from the present results, appears to be of the 0+− type, was reclassified as ++− in more detailed studies (G. Szőcs and M. Tóth, in preparation). It can be expected that most of the species caught in this experiment will respond to additional components which could give rise to other attraction diagrams. The present examples illustrate the multitude of messages which can be transmitted with a limited chemical vocabulary and point out one of the many ways by which reproductive isolation can be accomplished.

DISCUSSION

In this study we have established a common denominator for previous reports on sex attraction in *A. segetum* by finding a three-component blend which was generally more attractive than (in one case as attractive as) any of the one- or two-component systems so far described. Whether this blend represents the true pheromone of the species remains in question. The three homologs could be formed through a common biosynthetic pathway. It is conceivable that all three components occur in the female gland and that previous analyses were incomplete due to the small amounts present. A logical explanation would seem to be that Bestmann et al. (1978) did not detect the two higher homologs by concentrating on the peak obtained by electroantennographic detection. Likewise, Tóth et al. (1980) might have overlooked Z5–10:Ac because its amount was too small to be seen without electroantennographic detection. Arn et al. (1980) did not systematically search for a 14-carbon acetate.

On the other hand, the possible existence of *A. segetum* strains using different pheromones cannot be excluded. Bues and Poitout (1982) point out differences in development within and between *A. segetum* populations from various geographical regions. Our results may indicate differences in the functions of the three compounds between populations in the south of France and those of other parts of Europe.

Intraspecific variation may also account for the open questions regarding additional components. Ernst Priesner (personal communication) finds cells on *A. segetum* antennae which are specific for the perception of Z5–10:Ac,

Z7–12: Ac, Z9–14: Ac and, in addition, (Z)-5-dodecenyl acetate and (Z)-7-dodecen-1-ol (Z7–12: OH). The latter two components are not among those previously reported in female extracts, and Z7–12: OH had no effect in field tests (Arn et al., 1980). The possible involvement of Z7–10: Ac, the compound found in *A. fucosa* (Wakamura, 1980), also needs reinvestigation. We have not undertaken any new analytical studies to date; they will be meaningful only if done with insects from defined field sources.

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EXOCRINE SECRETIONS OF BEES
V. Terpenoid Esters in the Dufour's Secretions of *Panurginus* Bees (Hymenoptera: Andrenidae)

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Abstract—The volatile components of Dufour's gland extracts were analyzed in two species of Holarctic *Panurginus* bees: *Panurginus pontentillae* and *P. atramontensis*. Two terpenoid esters, citronellyl citronellate and citronellyl geranate, were identified in *P. pontentillae*, whereas only the latter was in *P. atramontensis*. Citronellyl citronellate was identified in pollen ball extracts of *P. pontentillae*. Mandibular gland extracts from male and female *P. pontentillae* contained neral and geranial. The significance of the Dufour's gland secretion in andrenid systematics and its function in the Andrenidae are discussed.

Key Words—Bees, exocrine products, mass spectrometry, citronellyl citronellate, citronellyl geranate, geranial, neral, Dufour's gland, mandibular gland, *Panurginus*, Hymenoptera, Andrenidae.

INTRODUCTION

The chemistry and function of Dufour's gland secretions in Apoidea have recently been studied intensively. The Dufour's gland, or "alkaline gland," is associated with the sting apparatus (Lello, 1971). It varies in size and shape from species to species and among the families of bees. The chemistry of the Dufour's gland secretions is known for several dozen holarctic species of *Andrena* (Andreninae) (Bergström and Tengö, 1974; Tengö and Bergström, 1975; Fernandes et al., 1981). These secretions typically contain either: (1) farnesyl hexanoate or geranyl octanoate; (2) esters of octanoic and/or hexanoic acids and aliphatic alcohols; or (3) mixtures of these two types.

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The holarctic genus, *Panurginus*, is represented in the United States by seventeen small sized species (Hurd, 1979), many of which are oligolectic. Nests are constructed in the ground, usually in aggregations. Few biological data are available on this group.

The chemistry of panurgine Dufour's gland secretions has not been reported. Here we show that the Dufour's gland of *Panurginus potentillae* contains citronellyl citronellate and citronellyl geranate, whereas that of *P. atramontensis* contains only citronellyl geranate. Mandibular gland exudates of *P. pontentillae* contain neral and geranial. The function of the Dufour's gland secretions in these bees and their significance in apoid systematics are discussed.

METHODS AND MATERIALS

Panurginus pontentillae and *P. atramontensis* were collected at separate nesting sites at the Quantico, Virginia, Marine Training Base during May 1981. Individual specimens were placed in glass shell vials and stored in an ice chest until dissection, which was completed within 24 hr. Dufour's and mandibular glands were excised under water and extracted with methylene chloride. Twenty-five Dufour's glands or 20 male and 20 female *P. pontentillae* mandibular glands were pooled per extract. In addition, separate extracts were made of male and female heads.

Pollen balls and cell linings from ten brood cells were extracted for *P. pontentillae*. As much dirt as possible was removed from the cell linings, which were then washed in water before extraction with methylene chloride for 48 hr. When this extract showed no volatiles, the solvent was removed, and the dried cell wall linings were hydrolyzed with 0.5 ml 6 N hydrochloric acid for 6 hr at 100°C before extraction with methylene chloride.

Extracts were analyzed on a Finnigan 3200 computerized gas chromatograph–mass spectrometer (GC-MS) utilizing a 1.5-m × 1-mm 3% OV-17 on Supelcoport 60/80 column, temperature programed from 60 to 300°C at 10°C/min. Individual compounds were identified by comparison of their mass spectra and retention times with those of standard compounds (Wheeler et al., 1982).

While specimens were being collected at the nesting site of *P. pontentillae*, male patrolling behavior and mating was observed on several different days.

RESULTS

Analysis of Dufour's gland extracts of *P. pontentillae* indicated the presence of two terpenoid components in addition to odd carbon-containing alkanes and alkenes (Figure 1). These terpenes, with apparent molecular

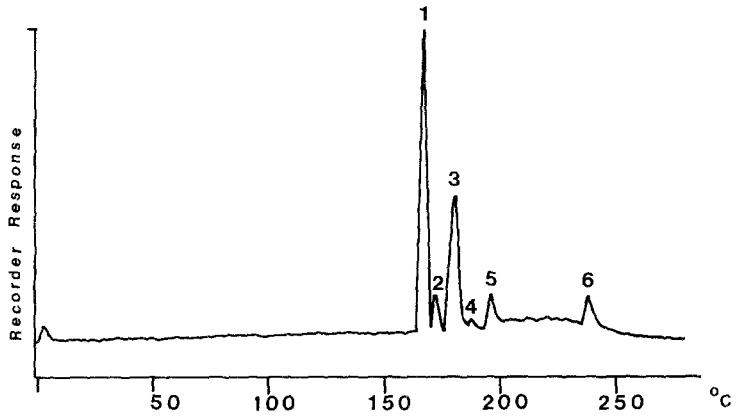

FIG. 1. Gas chromatogram of *Panurginus pontentillae*. 1 = Citronellyl citronellate (mol wt 308), 2 = citronellyl geranate (mol wt 306), 3 = C_{23} alkane and alkene (mol wt 324 and 322), 4 = C_{24} alkane (mol wt 338), 5 = C_{25} alkane and alkene (mol wt 352 and 350), 6 = C_{27} alkane (mol wt 380). Drawing after the original.

weights of 308(1) and 306(2), both exhibited base peaks of m/z 69 with other important ions at m/z 41(83), 55(38), 81(42), 95(35), 109(28), 123(25), 138(18), 152(6), 153(5), 170(1), 171(0.6) and 308(0.5) for (1) (Figure 2); and m/z 41(78), 55(15), 81(35), 82(30), 95(28), 100(5), 109(10), 123(40), 138(22), 151(20), 168(1), 169(1), 306(0.5) for (2) (Figure 3). These compounds were identified as citronellyl citronellate (1) and citronellyl geranate (2) on the basis of their mass spectra and GC retention times when compared with those of standard compounds (Wheeler et al., 1982). Dufour's extracts of *P. atramontensis* (Figure 4) showed only citronellyl geranate (2) in addition to hydrocarbons.

Analyses of an extract made from ten pollen balls of *P. pontentillae* indicated the presence of one volatile compound, identified as citronellyl

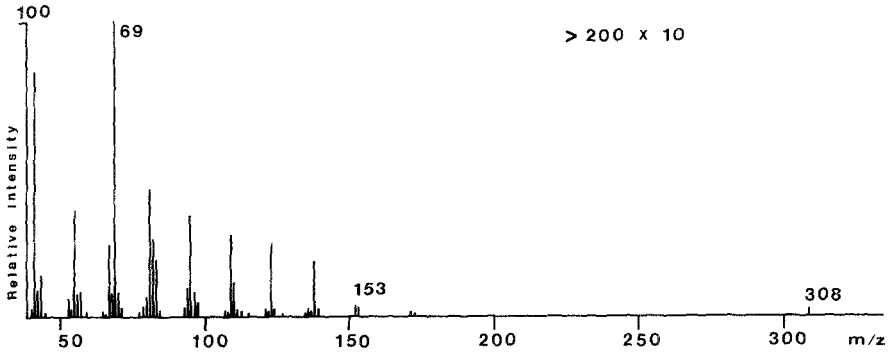

FIG. 2. Mass spectrum of citronellyl citronellate.

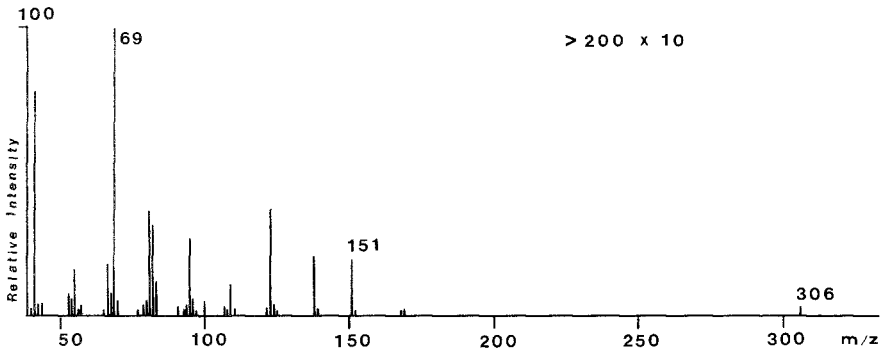

FIG. 3. Mass spectrum of citronellyl geranate.

citronellate. Similar analyses of methylene chloride extracts of the cell linings and cell lining hydrolysates of *P. pontentillae* did not show any volatiles.

GC-MS analysis of head extracts of *P. pontentillae* indicated two volatile constituents, apparently terpenoid from the pronounced peak at *m/z* 69. The compounds were identified in both male and female head extracts as neral and geranial in a ratio of 1:2. Analyses of the mandibular gland extracts confirmed that the compounds originated there.

On several consecutive days in May 50–75 *P. pontentillae* males were observed flying approximately 1–3 cm above a nesting site. Males frequently landed on the ground to investigate and dig at what seemed to be locations of

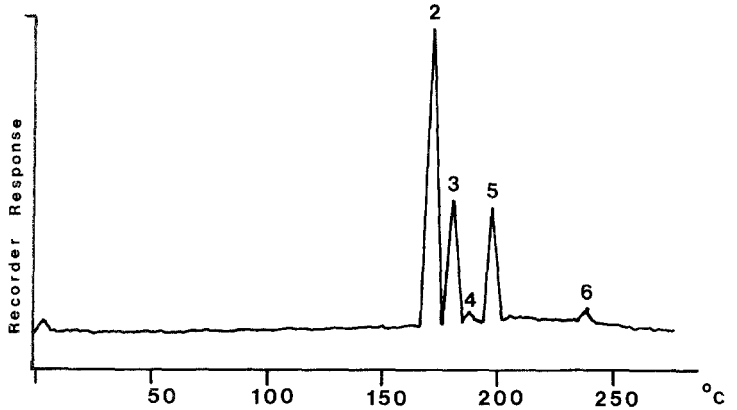

FIG. 4. Gas chromatogram of *Panurginus atramontensis*. 2 = Citronellyl geranate (mol wt 306), 3 = C_{23} alkane and alkene (mol wt 324 and 322), 4 = C_{24} alkane (mol wt 338), 5 = C_{25} alkane and alkene (mol wt 352 and 350), 6 = C_{27} alkane (mol wt 380). Drawing after the original.

future female emergence holes. Frequently, six to eight males were observed trying to mate simultaneously with one female on the ground.

DISCUSSION

This is the first report of citronellyl citronellate and citronellyl geranate as exocrine products of bees. Both compounds have been recently identified in the 7th sternal gland of European hornet workers, *Vespa crabro* (Wheeler et al., 1982). The function of these compounds in the hornets remains to be determined. In both cases citronellyl nerate is absent.

Although Rozen (1967) speculates that *P. pontentillae* mates while visiting flowers, our observations show that mating takes place at the nesting site. *Panurginus* mandibular gland secretions may play an integral role in mating behavior. *Colletes* females, engaged in digging and defensive behaviors at nesting sites, release their mandibular gland secretions (Cane and Tengö, 1981). Hefetz et al. (1979a) report that *Colletes* males are attracted to synthetic mixtures of the female mandibular gland constituents. Mandibular gland secretions of *P. pontentillae* may function similarly. These bees have such behaviors in common with *Colletes* as male patrolling of the nesting sites and mating at nest sites.

Functional Implications. Overlapping chemistries of Dufour's secretions and brood cell linings in the Colletidae—*Colletes* (Hefetz et al., 1979b; Albans et al., 1980), Halictidae—*Augochlora* (Duffield et al., 1981) and *Lasioglossum* (Cane, 1981), Andrenidae—*Andrena* (Cane, 1981), and Anthophoridae—*Anthophora* (Norden et al., 1980), have demonstrated Dufour's secretions are used to line brood cell walls. It has also been shown that the Dufour's secretions are incorporated into the pollen/nectar provisions of *Colletes* (Duffield and Wheeler, unpublished), *Augochlora* (Duffield et al., 1981), and *Andrena* (Cane, 1981).

Brood cells of *P. pontentillae* have a conspicious, shiny lining as do those of most panurgine bees (Rozen, 1967). Unlike the waxlike ones of *Andrena*, these linings are like cellophane and can be teased away from the earthen wall. The *P. pontentillae* linings are not soluble in such organic solvents as methylene chloride, even though those of *Andrena* are. Although hydrolysis of the cell linings showed no volatile compounds, the linings may still be polymers of citronellyl citronellate involving the double bonds. The hydrolysis products, however, were either not stable, further fragmented, or insufficient to detect. Since larger sample sizes are needed to clarify this point, the question of the source of the *P. pontentillae* cell linings remains unanswered.

Unlike the panurgine genera, *Calliopsis*, *Perdita*, and *Nomadopsis*, which coat the surface of the provisions (Rozen, 1967), *Panurginus* appar-

ently does not. It is important to note that *P. pontentillae* incorporates its Dufour's secretions into the provisions. The chemicals may serve: (1) as a food source for the developing larvae; (2) for moisture control; and (3) as a fungal growth inhibitor for the sugar-rich provisions. These functions remain to be proven.

Systematic Implications. The exocrine chemistry of bees is useful for recognizing some of the higher taxa of the Apoidea. All species examined in the Halictidae, Halictinae, Nomiinae (Duffield et al., 1981, 1982) and Colletidae (*Colletes*: Hefetz et al., 1979b; Albans et al., 1980; Cane, 1981; and *Hylaeus*: Duffield et al., 1980) contain macrocyclic lactones in their Dufour's glands. Esters characterize the Dufour's secretions of representatives of *Andrena* (Andreninae; Fernandes et al., 1981) and *Melitta* (Melittidae; Tengö and Bergström, 1976). Triglycerides characterize the Dufour's gland secretions of *Anthophora* (Anthophoridae; Norden et al., 1980) and *Megachile* (Megachillidae; Duffield and Wheeler, unpublished). Chemical analyses of representatives of the remaining subfamilies should help clarify their systematic relationships.

The Andrenidae is composed of two subfamilies, the Andreninae and Panurginae (Michener, 1979). The panurgine bees are believed to share a common ancestor with the holarctic Andreninae (Rozen, 1967). Dufour's extracts have been analyzed in several dozen species of *Andrena* (Andreninae). This is the first chemical analyses of a species in the Panurginae. *Panurginus* Dufour's esters contain a terpenoid acid as well as a terpenoid alcohol component. In contrast, the secretions of *Andrena* are of three main types: (1) those dominated by farnesyl hexanoate or geranyl octanoate; (2) esters of octanoic and/or hexanoic acid and aliphatic alcohols; or (3) mixtures of these two types. No terpenoid acid moieties have been found in *Andrena*.

This preliminary chemical data on the Dufour's secretions can be employed to distinguish between *P. pontentillae* and *P. atramontensis* and to separate these two species of *Panurginus* from other andrenids.

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TANNIN ASSAYS IN ECOLOGICAL STUDIES
Precipitation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase by Tannic Acid, Quebracho, and Oak Foliage Extracts

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Abstract—Tannic acid and quebracho precipitate many times their weight of the abundant leaf protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC). The use of this protein in protein precipitation assays for tannin content is described. Extracts of mature foliage of pin, bur, and black oak precipitate 2.01, 0.69, and 0.09 mg RuBPC/mg (dry wt) of leaf powder extracted, respectively, at pH 6.1. From these measurements it can be calculated that all three of these oak species have sufficient tannins to precipitate all of the RuBPC present in their foliage. At mildly alkaline pHs, however ($pH \geqslant 7.5$), RuBPC is not precipitated by tannins. Since RuBPC is the most abundant protein present in photosynthetic tissues, often constituting as much as 50% of the soluble proteins and 25% of the total proteins in leaf tissue, the interactions of this protein with tannins are highly relevant to an evaluation of the role of tannins as antiherbivore, digestibility-reducing substances. Our measurements provide no basis for arguing that differences in tannin levels in different species reflect differences in the digestibility of leaf proteins or that tannins have any effect whatsoever upon the digestibility of leaf protein under conditions which normally prevail in most insects' guts. These findings emphasize the need to test more of the assumptions underlying contemporary interpretations of the importance of tannins in plant herbivore interactions.

Key Words—Herbivory, chemical defense, allelochemics, tannins, digestibility reducing substances, RuBPC, *Quercus*.

INTRODUCTION

Tannins are water-soluble phenolic compounds which occur widely in vascular plants (Bate-Smith, 1957; Swain, 1979a). They are known to have

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adverse effects upon organisms as diverse as viruses, bacteria, fungi, insects, reptiles, birds, and mammals (Swain, 1979a). They have been accorded an important role in protecting plant tissues from herbivore attack (Feeny, 1976; Rhoades and Cates, 1976), although Bernays (1981) has recently stressed the variability of the effects of tannins on insect herbivores and has cautioned against premature generalizations concerning their evolutionary and ecological significance.

Defining the status of tannins as defensive chemicals in plants requires suitable procedures for measuring tannin content. The assay procedures which have been most frequently used in ecological studies have been the Folin-Denis assay for total phenols, the butanol–HCl assay for proanthocyanidins, and the vanillin–HCl assay for catechins. Unfortunately, these methods depend upon the presence of functional groups which are neither unique to tannins nor invariant features of tannin structure. Dissatisfaction with chemical, functional group assays has led to the development of other procedures based upon the ability of tannins to form insoluble complexes with proteins. Since it is the capacity of tannins to precipitate proteins which is postulated to be responsible for the adverse effects of these substances, these assays would seem to be particularly appropriate ones in studies of the significance of tannins in herbivory. Reliable assays for tannin content, based upon the precipitation of hemoglobin (Bate-Smith, 1973; Schultz et al., 1981), β-glucosidase (Becker and Martin, 1982) and bovine serum albumin (BSA) (Hagerman and Butler, 1978, 1980; Martin and Martin, 1982) have been described.

Since these assays use proteins which are not present in the diets of foliage-feeding insects, it is prudent to question whether the measure of protein-precipitating capacity they provide is of any relevance to the study of the role of tannins in plant–herbivore interactions. Proteins differ in the extent to which they are precipitated by tannins (Mandels and Reese, 1963; van Sumere et al., 1975; Griffiths, 1979; Hagerman and Butler, 1981), and a tannic extract which precipitates 1 mg of BSA or hemoglobin will not necessarily precipitate 1 mg of leaf protein or insect digestive enzyme.

In this study we have compared the precipitation of the abundant leaf protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), with the precipitation of BSA by solutions of tannic acid and bisulfited quebracho and by extracts of the mature foliage of three oak species (pin oak, *Quercus palustris*; bur oak, *Q. macrocarpa*; black oak, *Q. velutina*). As expected, the two proteins differ in the extent to which they are precipitated by a given tannic solution. However, the rank order of protein-precipitating capacity of a series of tannic extracts established using the nonleaf protein, BSA, correctly predicts the rank order using the leaf protein, RuBPC. Since RuBPC often makes up as much as 25% of the total protein and 25–50% of the soluble protein in leaf tissue (Singer et al., 1952; Akazawa, 1970; Lyttleton, 1973;

Jensen and Bahr, 1977), it is a major dietary protein for any foliage-feeding insect. Its interactions with tannins are, therefore, particularly relevant to an evaluation of the potential role of tannins as digestibility-reducing substances. Our measurements of the amounts of RuBPC precipitated by oak leaf tannins over a range of pHs call into question some of the current interpretations of the role of tannins as antiherbivore defensive chemicals and emphasize the need for direct experimental investigations of interactions between tannins and relevant dietary proteins under conditions which prevail in an herbivore's gut.

METHODS AND MATERIALS

Processing of Plant Materials. Foliage was processed in a manner calculated to minimize chemical alterations in leaf constituents (Swain, 1979). Whole, mature undamaged leaves were frozen and lyophilized immediately after collection and then stored at −15° C in a desiccator. Shortly before the leaves were to be extracted, the midribs were removed, and the remainder of the leaf was ground to a powder (60-mesh) in a Wiley mill. The leaf powder was temporarily stored in a desiccator in the dark at room temperature. At no time were the leaves or leaf powder exposed to any preservatives or to temperatures above 25° C. Although we have noted a gradual decrease in phenol and tannin content during the storage of leaf tissue processed in this way, these changes cause no alteration in the rank order of phenolic content in the foliage of six oak species, suggesting that parallel and roughly comparable changes occur in the different samples (Martin and Martin, 1982). Likewise Gartlan et al. (1980) showed that when foliage samples were sun- or oven-dried (60° C), there was an apparent decrease in phenolic content, but there was still a very strong correlation between the phenol values from fresh and dried samples. Lyophilization would be expected to result in much less chemical modification of leaf phenols than sun- or oven-drying, and we assume that what limited changes do occur are roughly comparable in all foliage samples.

Preparation of Extracts. Leaf powder (30–150 mg) was extracted twice for 8 min with 2 ml of boiling 50% (v/v) aqueous methanol in a centrifuge tube (capped with a marble) placed in a heat block at 95° C. After centrifugation (9600 rpm, 19000 *g*, 15 min, 5° C), the pellet was resuspended in a small volume of 50% methanol and centrifuged as before. The volume of the combined supernatants was adjusted to 5 or 10 ml, and dilutions appropriate to the assay were prepared from aliquots of the stock solution. Extracts were prepared immediately prior to use.

Protein-Precipitation Assay. The procedure is a variant of the method of Martin and Martin (1982). To a solution of 1.2 mg of protein (BSA, Sigma

A-4378, Lot 70F-9350; or spinach RuBPC, Sigma R-8000, Lot 98C-7140) in 1.8 ml buffer (0.1 M sodium succinate, pH 4.1, with BSA; 0.1 M sodium 2-(*N*-morpholino)-ethanesulfonate, MES, pH 6.1, with RuBPC) containing 0.17 M sodium chloride was added 0.3 ml of a 50% aqueous methanolic solution of tannic acid (Sigma T-0125, Lot 40F-0253, 6.4% moisture), bisulfited quebracho (Pilar River Plate Corp., 18% moisture), or an aliquot of foliage extract. After vortexing, the mixture was centrifuged (9600 rpm, 19000 *g*, 15 min, 5° C). Separate experiments demonstrated that the precipitation of the tannin–protein complex occurs rapidly and that waiting as long as 30 min before centrifugation does not increase the amount of protein precipitated. Separate experiments also demonstrated that the presence of methanol in the tannin solution, which produced a final mixture containing 7% methanol, does not affect the amount of protein precipitated.

After centrifugation, the pellet was rinsed very gently with 0.4 ml of buffer and centrifuged as before. The combined supernatants were applied to a 1.7 × 5.0-cm column of Sephadex G-25 (Pharmacia Fine Chemicals, PD-10 Columns), which had been equilibrated with buffer containing 0.17 M sodium chloride and 6% methanol. Proteins were eluted completely in 3.5 ml of the same solvent mixture. This step removes all materials from the supernatant which absorb at 595 nm. The amount of protein in the eluent was determined by mixing a 50-μl aliquot with 2.5 ml of Coomasse brilliant blue G-250 dye reagent (Bio Rad Protein Dye Reagent), and determining A_{595} after 6 min (Bradford, 1976) using a blank consisting of 50 μl of buffer containing 6% methanol plus 2.5 ml of the dye reagent. The absorbance at 595 nm was transformed into mg of BSA or RuBPC by the use of a calibration curve constructed on the same day as the assay. From a determination of the amount of protein in the original solution, the amount precipitated by the addition of the tannin or foliage extract could be calculated. Separate experiments verified that the presence of 6% methanol, which is the final concentration of methanol in these experiments, does not interfere with the determination of protein using the Bradford procedure.

The protein-precipitating capacity was measured as the slope of the linear regression of amount (mg) of protein precipitated on amount (mg dry wt) of tannin or leaf powder extracted. By obtaining measurements at several concentrations of tannin or foliage extract, it is possible to ensure that the determinations are being performed under conditions which generate a linear relationship between the amount of protein precipitated and the amount of tannin or extract being used and to determine how close the *y* intercept is to zero. Some protein-precipitation assays are characterized by significant negative *y* intercepts, indicating a threshold concentration of extract below which no protein is precipitated (Bate-Smith, 1973; Schultz et al., 1981; Becker and Martin, 1982; Martin and Martin, 1982). In this study, the relationship between the amount of BSA or RuBPC precipitated and the

amount of tannin added to the test solution of protein was linear over a wide range of concentrations of tannic acid, quebracho, and foliage extracts which brought about the precipitation of 1.5–89% of the protein present. Correlation coefficients greater than 0.96 were obtained in every assay. In most cases the y intercepts were not significantly different from zero (Table 1). Regression coefficients (slopes) and y intercepts were calculated assuming that the independent variable (dry weight of tannin in solution or leaf powder extracted) was measured without error, employing data in which there was more than one value of the dependent variable per value of the independent variable (Sokal and Rohlf, 1969). In calculating standard errors of regression coefficients, mean squares were not pooled.

Buffers used in the pH studies were 0.1 M sodium citrate (pH 3.1), 0.1 M sodium succinate (pH 4.1, 5.6), 0.1 M sodium acetate (pH 5.0, 5.1), 0.1 M sodium 2-(*N*-morpholino)-ethanesulfonate (MES) (pH 6.1), 0.1 M sodium piperazine-*N*,*N*′-bis-2-ethanesulfonate (PIPES) (pH 6.6, 7.1), 0.1 M sodium *N*-2-hydroxyethylpiperazine-*N*-ethanesulfonate (HEPES) (pH 7.6), and 0.1 M sodium *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonate (HEPPS) (pH 8.0). All buffers contained 0.17 M sodium chloride. The ionic strengths of the succinate (pH 5.6) buffer and both PIPES buffers were between 0.35 and 0.40. All of the other buffers had ionic strengths between 0.22 and 0.24. Ionic strengths in this general range have been reported for the gut fluids of representative Lepidoptera (Giordana and Sacchi, 1978) and Ortoptera (Dow et al., 1981).

RESULTS AND DISCUSSION

As expected, BSA and RuBPC differ in the extent to which they are precipitated by Tannins (Table 1). At favorable pHs for precipitation (4.1 for BSA, 6.1 for RuBPC), a given amount of tannin or foliage extract precipitates much more RuBPC than BSA. The rank order of protein-precipitating capacity of the various tannin solutions or foliage extracts is the same whether measured with BSA or RuBPC. Using either BSA or RuBPC, tannic acid (a hydrolyzable tannin) precipitates more protein than quebracho (a condensed tannin), and the relative protein-precipitating capacities of the three oak foliage extracts are in the sequence, pin oak > bur oak > black oak. However, the actual numerical values for the relative protein-precipitating capacities of the different tannins or foliage extracts depend upon which protein is used in the measurement (Table 2). For example, pin oak foliage has a protein-precipitating capacity 22.6 or 8.3 times greater than black oak foliage, depending upon whether it is measured using RuBPC or BSA. Furthermore, the disparity between relative protein-precipitating capacities of different tannins or different foliage samples measured using the two different proteins is not a constant factor. Thus, while the activity of pin oak

TABLE 1. AMOUNTS OF BSA AND RuBPC PRECIPITATED BY SOLUTIONS OF TANNIC ACID, BISULFITED QUEBRACHO, AND EXTRACTS OF MATURE FOLIAGE OF THREE OAK SPECIES[a]

Precipitating solution or extract	BSA pptd (mg/mg)[b] (pH 4.1)	RuBPC pptd (mg/mg)[c] (pH 6.1)
Tannic acid	4.92 ± 0.47 (4,6,20)	20.84 ± 0.99 (4,5,13)
Quebracho	1.92 ± 0.04 (2,6,12)	4.00 ± 0.21 (2,6,11)
Q. palustris (pin)	0.357 ± 0.007 (2,6,12)	2.01 ± 0.11 (2,5,10)
Q. macrocarpa (bur)	0.257 ± 0.008 (2,6,12)	0.691 ± 0.064 (2,5,10)
Q. velutina (black)	0.043 ± 0.005 (2,6,12)	0.089 ± 0.006 (2,6,11)

[a] Entries are regression coefficients (slopes ± SE) of mg protein precipitated vs. mg (dry weight) of tannin or of leaf powder extracted. The number of separate solutions or extracts used, the number of different concentrations examined, and the total number of measurements performed are indicated, in that order, in the parentheses.

[b] y intercepts: tannic acid, −0.08; quebracho, −0.04; pin oak, −0.008; bur oak, −0.014; black oak, −0.015. None of the y intercepts are significantly different from zero ($P > 0.01$).

[c] y intercepts: tannic acid, −0.58; quebracho, −0.09; pin oak, −0.44; bur oak, −0.015; black oak, −0.063. Only the y intercepts for tannic acid and pin oak are significantly different from zero ($P < 0.01$).

relative to black oak foliage is 2.7 times higher when determined with RuBPC than when determined using BSA (22.6 vs. 8.3), the activity of bur oak relative to black oak foliage is only 1.3 times higher (7.8 vs. 6.0). Considering the extraordinary complexity of tannin–protein interactions (Goldstein and Swain, 1965; Calderon et al., 1968; Van Buren and Robinson, 1969; Haslam, 1974; van Sumere et al., 1975; Becker and Martin, 1982), it comes as no surprise that relative protein-precipitating capacity is not a simple, invariant property of a tannin.

These results help to define the value of protein-precipitation assays in ecologically oriented studies designed to probe the role of tannins in plant–herbivore interactions. Measurements of the protein-precipitating capacity of a set of foliage extracts using BSA, almond emulsin, or hemoglobin as the test

TABLE 2. RELATIVE PROTEIN-BINDING CAPACITIES OF DIFFERENT TANNINS AND FOLIAGE EXTRACTS USING BSA AND RuBPC AS TEST PROTEINS

	Tannins	Oak foliage
Test protein	Tannic acid/quebracho	Pin/bur/black
BSA	2.6/1.0	8.3/6.0/1.0
RuBPC	5.2/1.0	22.6/7.8/1.0

proteins provide consistent rank orders of tannin contents which can be very useful in correlative ecological studies of plant–herbivore interactions. However, none of the above-named proteins occur in leaves. Consequently, these measurements cannot be used to estimate the actual fraction of dietary protein which might be precipitated in an herbivore's gut by the tannins in their food plants. The use of the abundant leaf protein, RuBPC, as the test protein is a step in the direction of making measurements of protein-precipitating capacity more useful in assessing the likelinood that tannins function as digestibility-reducing substances.

It is revealing to examine the implications of the RuBPC-precipitating potentials of the three oak extracts. Lawson et al. (1982) have reported that the mature foliage of pin, bur, and black oak contain 2.27, 2.92, and 2.11% nitrogen, respectively. Using the factor 6.25 to convert "% nitrogen" into "crude protein," and assuming that 25% of the total leaf protein is RuBPC, it follows that mature pin, bur, and black oak foliage contain 0.035, 0.046, and 0.033 mg RuBPC/mg (dry wt), respectively. Thus, all three oak species have sufficient tannins to precipitate all of the RuBPC present in their foliage (Table 1). Even black oak, the species with the lowest potential for precipitating proteins, has sufficient tannins to precipitate more than 2.5 times the amount of RuBPC present. Thus the more than 20-fold greater protein-precipitating potential of pin oak relative to black oak foliage does not reflect a corresponding difference in the extent to which the RuBPC in the two species might be precipitated by the tannins present. This finding clearly provides no basis for arguing that the more tannin-rich pin oak foliage is less digestible than black oak foliage. Of course it is still possible that high levels of tannins are required to precipitate RuBPC in the presence of other tannin-binding leaf constituents, to precipitate other leaf proteins which are important dietary components, or to precipitate insect digestive enzymes. At the very least, however, we urge ecologists to be wary of the assumption that differences in tannin levels necessarily reflect differences in nutritive value or differences in extent of protection against herbivores.

The amount of BSA or RuBPC precipitated from an aqueous solution by the addition of a solution of tannic acid or quebracho depends upon pH (Figure 1). Maximum precipitation of BSA by tannic acid occurs at a pH around 4. Hagerman and Butler (1978) have also reported an optimal pH between 4 and 4.5 for the formation of an insoluble complex between BSA and sorghum tannins. The dependence of RuBPC precipitation by tannins on pH (Figure 1) raises significant questions about the potential efficacy of these substances as antiherbivore defensive chemicals. Extensive precipitation occurs between pH 5.6 and 7, but there is very little precipitation at pHs above 7.5. At pH 8 no RuBPC was precipitated from a solution by the addition of an extract of pin oak leaf powder which would have precipitated all of the RuBPC at pH 6.1. Midgut pHs in excess of 9 are not uncommon in foliage-

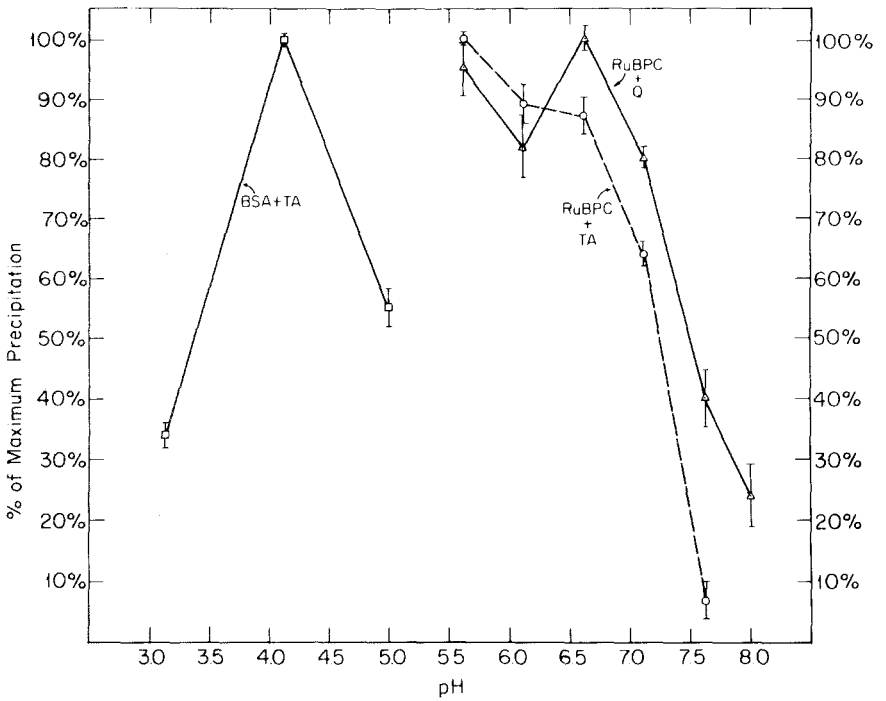

FIG. 1. Effect of pH on the precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) and bovine serum albumin (BSA) by tannic acid and quebracho. Incubation mixtures contained 1.2 mg of protein and either 180 μg of tannic acid (with BSA), 90 μg tannic acid (with RuBPC), or 300 μg quebracho (with RuBPC). Tannic acid precipitated 0.806 mg of BSA at pH 4.1 and 0.999 mg of RuBPC at pH 5.6, while quebracho precipitated 1.04 mg of RuBPC at pH 6.6. All points are the mean of five measurements; bars give the standard error of the mean. Abbreviations: BSA, bovine serum albumin; RuBPC, ribulose-1,5-bisphosphate carboxylase/oxygenase; TA, tannic acid; Q, quebracho.

feeding Lepidoptera (Berenbaum 1980), and Feeny (1970) and Berenbaum (1980) have suggested that the high gut alkalinity in herbivores could be an adaptive mechanism to prevent or reverse the binding of proteins by tannins. While it is certainly true that the maintenance of modestly alkaline conditions in the gut would be a useful mechanism to prevent the precipitation of dietary proteins by tannins, our results show that extremely high pHs are not necessary. Indeed, many insects, including some non-tannin-adapted species, have sufficiently alkaline guts to prevent the precipitation of RuBPC by tannins. Of course, it is still possible that highly alkaline conditions are required to prevent the precipitation of some other important dietary proteins by tannins or that the RuBPC in oak foliage binds more tenaciously to tannins

at high pHs than does spinach RuBPC. However, until those possibilities have been tested experimentally, it is prudent to take cognizance of possible alternative explanations for the adaptive significance of an alkaline gut which have no connection with a need to overcome the presumed digestibility-reducing properties of tannins.

Correlative studies have generated many important and original hypotheses concerning strategies of chemical defense and the role of secondary metabolites in influencing interspecific interactions. In this paper we have emphasized how few of the assumptions underlying these hypotheses have been tested. Experiments to test them are quite feasible, and it is our opinion that the next major advances in clarifying the role of tannins in plant-herbivore interactions will come from chemically oriented studies of interactions of proteins and tannins actually present in an insect's food plant under conditions which might reasonably be expected to prevail in the gut.

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ALLELOPATHIC EFFECTS OF JUGLONE ON GERMINATION AND GROWTH OF SEVERAL HERBACEOUS AND WOODY SPECIES[1]

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Abstract—Laboratory experiments were conducted to determine juglone sensitivity of 16 species (*Trifolium incarnatum, Coronilla varia, Vicia villosa, Lespedeza stipulacea, L. cuneata, Acer ginnala, Caragana arborescens, Elaegnus angustifolia, E. umbellata, Lonicera maackii, Quercus alba, Fraxinus americana, Liriodendron tulipifera, Alnus glutinosa, Pinus strobus*, and *P. sylvestris*) being considered for mixed plantings with *Juglans nigra* (black walnut). All species were sensitive to juglone, but seed germination and radicle elongation were less affected than shoot elongation and dry weight accumulation. Seed germination and radicle elongation were affected by juglone in 6 and 11 species, respectively, mainly by the higher concentrations (10^{-3} M and 10^{-4} M). Shoot elongation and dry weight accumulation of all species were affected by juglone; many species were sensitive to concentrations as low as 10^{-6} M. Seedlings of all species were severely wilted and eventually killed by 10^{-3} M juglone, and most were chlorotic and severely retarded by 10^{-4} M juglone. Seedlings inhibited by 10^{-6} M and 10^{-5} M juglone did not show any visible signs of injury. Based on the effects on seedling shoot elongation and dry weight accumulation, the five species found to be most sensitive to juglone were: *Lonicera maackii, Lespedeza cuneata, Trifolium incarnatum, Alnus glutinosa, and Elaeagnus umbellata*.

Key Words—Seed germination, radicle elongation, shoot elongation, dry weight accumulation, juglone, *Juglans nigra, Trifolium incarnatum, Coronilla varia, Vicia villosa, Lespedeza stipulacea, L. cuneata, Acer ginnala, Caragana arborescens, Elaeagnus angustifolia, E. umbellata, Lonicera maackii, Quercus alba, Fraxinus americana, Liriodendron tulipifera, Alnus glutinosa, Pinus strobus, P. sylvestris*.

[1]This article was written and prepared by U.S. Government employees on official time; it is therefore in the public domain.

INTRODUCTION

Black walnut (*Juglans nigra*) is the most notorious of allelopathic trees. According to Gries (1943), the first written record of walnut toxicity can be traced back to the first century AD when Pliny and Elder wrote: "the shadow of walnut trees is poison to all plants within its compass." Walnut has been reported to be toxic to a wide variety of organisms, including herbaceous and woody plants (Brooks, 1951; Rietveld, 1979).

The principal chemical responsible for walnut allelopathy is juglone (5-hydroxy-1,4-naphthoquinone) (Davis, 1928). In living plant tissues, a colorless, nontoxic reduced form called hydrojuglone is abundant, especially in leaves, fruit hulls, inner bark, and roots (Gries, 1943; Lee and Campbell, 1969). When exposed to the air or some oxidizing substance from the roots of other plants, hydrojuglone immediately is oxidized to its toxic form, juglone. Rain washes juglone from living leaves and carries it to the soil. It is also released to the soil from decaying leaves and fruits. Little is known about the mode of action of juglone in affected plants; however, Perry (1967) found that juglone restricted respiration of leaf discs of tomato and bean by inhibiting oxygen uptake.

Juglone has been isolated from many plants in the walnut family (Juglandaceae) including black walnut, butternut (*J. cinerea* L.), Persian walnut (*J. regia* L.), Siebold walnut (*J. ailantifolia* Carr.), Manchurian walnut (*J. mandshurica* Maxim.), shagbark hickory [*Carya ovata* (Mill.) K. Koch], mockernut hickory (*C. tomentosa* Nutt.), Caucasian walnut [*Pterocarya fraxinifolia* (Lam.) Spach], and pecan [*C. illinoensis* (Wangenh.) K. Koch] (Thomson, 1971; Graves et al., 1979).

Recent research has shown that mixed plantings of certain nitrogen-fixing species with black walnut can substantially boost the growth and possibly the quality of the walnut trees (Funk et al., 1979a; Van Sambeek and Rietveld, 1981). Other species are being considered as possible cocrops with walnut to diversify vegetation and increase overall yields of products and amenities. However, only a few plant species have been tested for their sensitivity to juglone (Funk et al., 1979b). The laboratory experiments reported here were conducted to assess the allelopathic potential of black walnut juglone to several herbaceous and woody species being considered for cocrops, nurse crops, and cover crops in intensively cultured black walnut plantations.

METHODS AND MATERIALS

The study consisted of two parts: (1) seed germination and radicle elongation in response to several juglone concentrations on two media, and

(2) shoot elongation and dry weight accumulation in hydroponic cultures containing the same juglone concentrations.

The following species were tested:

Herbs—crimson clover (*Trifolium incarnatum* L.), crown vetch (*Coronilla varia* L.), hairy vetch (*Vicia villosa* Roth.), Korean lespedeza (*Lespedeza stipulacea* Maxim.), and sericea lespedeza [*Lespedeza cuneata* (Dumont) G. Don].

Shrubs—ginnala maple (*Acer ginnala* Maxim.), Siberian peashrub (*Caragana arborescens* Lam.), Russian olive (*Elaeagnus angustifolia* L.), autumn olive (*Elaeagnus umbellata* Thunb.), and amur honeysuckle (*Lonicera maackii* Maxim.).

Trees—white oak (*Quercus alba* L.), white ash (*Fraxinus americana* L.), yellow poplar (*Liriodendron tulipifera* L.), European black alder [*Alnus glutinosa* (L.) Gaertn.], eastern white pine (*Pinus strobus* L.), and Scotch pine (*Pinus sylvestris* L.).

Certain species were excluded from some tests because of problems in obtaining seed, stratification, or germination. The pines were omitted from the growth tests because they had been tested previously (Funk et al., 1979b). Seed were collected and stored until needed. Pregermination treatments used were those specified for each species in *Seeds of Woody Plants in the United States* (USDA Forest Service, 1974). Test conditions and germination criteria used were those specified for each species in *Rules for Testing Seeds* (Association of Official Seed Analysts, 1970) and *International Rules for Seed Testing* (International Seed Testing Association, 1976). Seed of all species were germinated with seed coat and cotyledons intact, except for white oak where the basal one-third of each acorn was removed and then germinated standing on end.

Germination and radicle elongation tests were run on two media—blotter paper and soil. The soil used was the "A" horizon from a forested site demonstrated to be excellent for walnut growth. The soil was screened, sterilized with methyl bromide, oven dried, ground, and stored in sealed containers until it was used. The relatin between soil matric potential and soil water content was determined so that soil moisture could be maintained at field capacity (0.3 atmosphere) by maintaining total weight at a determined value.

An aqueous 10^{-3} M stock solution of juglone (Sigma Chemical Co.) was prepared by constantly stirring at 40° C for 24 hr; 10^{-4} M, 10^{-5} M, and 10^{-6} M concentrations were prepared by serial dilutions from the stock solution. Tests were run in a Stults laboratory germinator. For each of the 16 species, four trays of 100 seeds each were germinated in each of the four juglone concentrations plus one control (distilled water).

When germination was occurring at a rapid rate, 10 randomly selected germinants were set aside on each tray for measurement of radical elongation.

Radicle length was measured initially and again after radicles in the controls had elongated from 10 to 15 mm, usually from 2 to 6 days.

In the second part of the experiment, seedlings were grown in hydroponic culture to test the effects of juglone on shoot elongation and dry weight accumulation. Seedlings were grown in sand culture to the first true leaf stage and then transferred to half-strength Hoagland nutrient solutions (Hoagland and Arnon, 1938) containing the same juglone concentrations used in the germination tests. A specially designed static solution hydroponic system (Rietveld, 1982) installed in a growth chamber was used for the growth tests. The growth chamber schedule was set for a 16-hr, 30° C day:8 hr, 20° C night. Each of the four nutrient-plus-juglone solutions and control (nutrient solution) was represented by four 400-ml culture vessels (replications) containing three seedlings each. The locations of vessels were completely randomized in the growth chamber. Solutions were changed weekly. Tests lasted from 4 to 6 weeks, depending on rates of growth and development of treatment effects. Seedling height was measured initially and repeatedly during the tests. Seedling shoot and root dry weights were determined at the end of each test.

Data were analyzed for significant differences by factorial analysis of variance and Duncan's multiple-range test using the 5% significance level.

To evaluate the effect of juglone concentration on the concurrent time and amount of shoot elongation that occurred during the tests, the speed of shoot elongation (SSE) was calculated as follows:

$$\text{SSE} = \sum \frac{\text{increment since last measurement (mm)}}{\text{time since beginning of test (days)}}$$

A large SSE value indicates early, rapid growth, and a low SSE value indicates late, slow growth. SSE was calculated and summed for each seedling for the duration of the test or period it was alive. Mean SSE of the three seedlings in each replication was subjected to analysis of variance and Duncan's multiple-range test using a 5% significance level.

RESULTS

Seed Germination. Juglone significantly affected percent germination in only 6 of the 14 species tested. Germination was inhibited in four species (Korean lespedeza, sericea lespedeza, autumn olive, and amur honeysuckle) by the 10^{-3} M concentration (Table 1). However, in five other instances (Korean lespedeza and ginnala maple on blotter, amur honeysuckle on blotter and soil, and white oak on soil) germination was enhanced by 10^{-6} M or 10^{-5} M juglone. In most species, germination was similar on the two media; however, germination of Korean lespedeza and autumn olive was inhibited on

TABLE 1. GERMINATION (PERCENT) OF SEED OF 14 SPECIES AT 5 JUGLONE CONCENTRATIONS AND ON 2 GERMINATION MEDIA (EACH VALUE IS THE MEAN OF 4 TRAYS OF 100 SEEDS EACH)

Species	Juglone concentration (M) on blotter					Juglone concentration (M) in soil				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Crimson clover	89	89	91	94	78	76	77	73	80	82
Crown vetch	58	62	58	61	56	68	63	62	63	60
Hairy vetch	78	78	84	79	81	84	84	95	86	86
Korean lespedeza	67b[a]	77ab	81a	66b	31c	58	62	63	66	66
Sericea lespedeza	66a	72a	69a	70a	52b	85a	72bc	77ab	77ab	61c
Ginnala maple	51b	47b	52b	55b	74a	45	52	54	53	53
Siberian peashrub	71	79	85	84	76	83	87	84	80	81
Russian olive	95	90	92	84	83	64	61	53	51	53
Autumn olive	53a	51a	44a	50a	26b	50	47	47	40	53
Amur honeysuckle	75b	92a	61b	63b	43c	63bc	80a	71b	55c	53c
White oak	18	16	24	13	18	72b	83a	65b	63b	71b
European black alder	25	26	25	22	26	23	21	26	27	24
Eastern white pine	79	60	62	49	60	70	67	60	61	63
Scotch pine	27	36	30	31	24	41	34	40	42	38

[a]Values within each row (one test consisting of five juglone concentrations on blotter or soil) followed by the same letter are not significantly different at the 5% level. Tests not followed by a letter showed no significant differences.

TABLE 2. MEAN RADICLE ELONGATION (mm) OF GERMINATING SEED OF 15 SPECIES IN PRESENCE OF VARIOUS JUGLONE CONCENTRATIONS ON 2 MEDIA (EACH VALUE IS THE MEAN OF 10 SEEDS ON EACH OF 4 TRAYS)

Species	Juglone concentration (M) on blotter					Juglone concentration (M) in soil				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Crimson clover	14b[a]	17a	16ab	17a	5c	6	5	4	5	4
Crown vetch	12a	13a	11a	10a	6b	13a	11ab	10bc	12ab	8c
Hairy vetch	14a	13ab	14a	11bc	10c	13a	13a	10b	13a	7c
Korean lespedeza	34a	29b	27b	26b	9c	22	19	10	18	21
Sericea lespedeza	19a	21a	22a	20a	10b	11	12	13	15	11
Ginnala maple	9a	6b	4c	4c	5bc	8a	8a	3b	4b	4b
Siberian peashrub	16	20	17	17	18	21	18	16	23	21
Russian olive	4	4	4	4	2	7	6	8	5	9
Autumn olive	9a	9a	10a	6b	5b	8a	7a	7a	6ab	4b
Amur honeysuckle	8a	6b	6b	8a	0c	8b	11a	6bc	6bc	5c
White oak	36	34	31	32	30	25	24	24	27	24
White ash	14a	13a	8bc	10ab	4c	8	10	9	9	8
European black alder	14	13	11	11	10	11b	12ab	13a	11b	10b
Eastern white pine	12	10	11	10	12	7	8	7	8	6
Scotch pine	21a	13b	17ab	14b	16ab	9	9	9	11	9

[a]Values within each row (one test consisting of five juglone concentrations on blotter or soil) followed by the same letter are not significantly different at the 5% level. Tests not followed by a letter showed no significant difference.

blotter paper but not on soil and germination of white oak acorns was much higher on soil than on blotter paper.

Radicle Elongation. Radicle elongation was more sensitive to juglone than was seed germination—radicle elongation for 11 of the 15 species tested was significantly affected by juglone (Table 2). The higher juglone concentrations (10^{-3} M and 10^{-4} M) were responsible for inhibited elongation in 10 of the 11 species affected (crimson clover, crown vetch, hairy vetch, Korean lespedeza, sericea lespedeza, white ash, scotch pine, ginnala maple, autumn olive, and amur honeysuckle). In three instances (crimson clover on blotter, European black alder on soil, and amur honeysuckle on soil) radicle elongation was stimulated by 10^{-6} M or 10^{-5} M juglone. The herbs as a group were more sensitive to juglone than the woody species. Inhibition of radicle elongation was more common on blotter paper than on soil; however, for unknown reasons, elongation was generally lower on soil than on blotter paper.

Shoot Elongation. All seedlings of all species (except hairy vetch) were killed by 10^{-3} M juglone within a few days after exposure, and seedlings of some species were eventually killed by 10^{-4} M juglone (Table 3). Most seedlings were severely inhibited by 10^{-4} M juglone solutions (Table 3). Shoot elongation of six species (crimson clover, sericea lespedeza, white ash, white

TABLE 3. SHOOT ELONGATION (mm) OF SEEDLINGS OF 13 SPECIES GROWN IN AERATED NUTRIENT SOLUTIONS CONTAINING 5 JUGLONE CONCENTRATIONS

Species	Juglone concentration (M)				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Crimson clover	57a[a]	57a	29b	8c	0c
Hairy vetch	190a	187a	169a	113b	64c
Korean lespedeza	57ab	66a	56ab	42b	8c
Sericea lespedeza	59a	56a	33b	3c	0c
Ginnala maple	102a	85a	89a	41b	1c
Siberian peashrub	77a	69a	27ab	10b	0b
Russian olive	56a	46a	56a	3b	0b
Autumn olive	47a	40a	32a	9b	1b
Amur honeysuckle	70a	44b	42b	7c	1c
White oak	21a	12a	11a	12a	3b
White ash	57a	56a	42b	28c	0d
European black alder	59a	47a	14b	1b	0b
Yellow poplar	67a	76a	71a	24b	2c

[a]Values in each row followed by the same letter are not significantly different at the 5% level.

oak, black alder, and amur honeysuckle) was significantly inhibited by the 10^{-5} M concentration, and that of amur honeysuckle was inhibited by both 10^{-5} M and 10^{-6} M juglone. Only severely inhibited seedlings showed visible chlorosis of leaves and stems; seedlings inhibited by 10^{-6} M and 10^{-5} M juglone showed no visible signs of toxicity. Crown vetch was omitted because of its tendency to form a basal crown rather than elongate.

The pattern and degree of juglone effects on speed of shoot elongation were similar to those on total shoot elongation (data not presented). In all species, 10^{-3} M and 10^{-4} M juglone retarded shoot elongation abruptly at the beginning of the test, while the inhibitory effects (if any) of 10^{-5} M and 10^{-6} M juglone occurred gradually during the tests.

Dry Weight. In several species, plant dry-weight accumulation was more sensitive to juglone than was shoot elongation, i.e., more juglone concentration levels were significantly different from the control (Table 4). Dry weight of white oak roots was not significantly affected by juglone. In nearly all of the remaining species, concentrations of 10^{-4} M and greater significantly inhibited dry weight accumulation. In five species (crimson clover, crown vetch, sericea lespedeza, black alder, and amur honeysuckle) concentrations of 10^{-5} M and greater significantly reduced both shoot and root dry weight accumulation. Dry weight of sericea lespedeza and autumn olive shoots and amur honeysuckle roots was significantly reduced by all concentrations of juglone. In three species (Siberian peashrub, Russian olive, and yellow poplar) shoot dry weight of seedlings grown in 10^{-6} M or 10^{-5} M juglone was higher than in the control, but not significantly so. Juglone had no significant effect on the shoot/root weight ratio (data not shown).

DISCUSSION

Every species tested was found to be sensitive to juglone. Because the species and growth processes responded differently to juglone concentration, some means of assessing overall species sensitivity was needed. Therefore, the summary of species sensitivity to juglone shown in Table 5 is based on the following rationale: (1) the number of concentrations that were significantly inhibitory compared to the control is an indicator of species sensitivity; (2) it is unlikely that 10^{-3} M juglone occurs commonly under natural conditions (see below), so the 10^{-3} M concentration was omitted; (3) species sensitivity during the establishment phase depends on whether they were grown from seed or planted; and (4) following establishment, the principal effects (if any) of juglone will be on plant growth. Thus two rankings of species sensitivity are presented—one based on toxicity to seed germination and radicle elongation and one based on toxicity to seedling shoot elongation and dry weight accumulation. The former ranking applies to establishment from seed, while

TABLE 4. SHOOT AND ROOT DRY WEIGHT (mg) OF 14 SPECIES GROWN IN AERATED NUTRIENT SOLUTIONS AS AFFECTED BY JUGLONE CONCENTRATION

Species	Juglone concentration (M)				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
Shoot dry weight					
Crimson clover	64a[a]	54a	32b	12bc	7c
Crown vetch	163a	166a	28b	10b	6b
Hairy vetch	51a	52a	36a	17b	11b
Korean lespedeza	34a	31a	18b	25ab	3c
Sericea lespedeza	60a	42b	17c	5d	4d
Ginnala maple	108a	180a	177a	70b	8c
Siberian peashrub	185a	229a	100ab	31b	9b
Russian olive	99a	83a	131a	8b	7b
Autumn olive	98a	54b	34bc	6c	4c
Amur honeysuckle	277a	164ab	108bc	24c	9c
White oak	434a	334ab	256ab	206b	212b
White ash	475a	381a	201a	81bc	25c
European black alder	90a	60a	13b	5b	4b
Yellow poplar	317a	376a	341a	88b	5b
Root dry weight					
Crimson clover	18a	20a	9b	4b	4b
Crown vetch	71a	70a	13b	2b	2b
Hairy vetch	21a	23a	19a	9b	7b
Korean lespedeza	7a	8a	5a	6a	2b
Sericea lespedeza	16a	15a	6b	2b	2b
Ginnala maple	29a	19a	21a	5b	3b
Siberian peashrub	74a	64ab	21bc	7c	1c
Russian olive	12ab	13ab	24a	3b	3b
Autumn olive	17a	14a	10ab	2b	2b
Amur honeysuckle	62a	28b	24b	4b	3b
White oak	425	334	310	340	313
White ash	77a	72a	53a	22b	12b
European black alder	31a	23a	4b	2b	2b
Yellow poplar	84a	87a	67ab	19bc	2c

[a]Values in each row followed by the same letter are not significantly different at the 5% level.

TABLE 5. SUMMARY OF NUMBER OF JUGLONE CONCENTRATIONS INHIBITING[a] GROWTH PROCESSES TESTED[b]

Species	Germination		Radicle elongation		Total	Sensitivity ranking	Shoot elongation	Dry weight		Total	Sensitivity ranking
	Blotter	Soil	Blotter	Soil				Shoot	Root		
Crimson clover	0	0	0	0	0	5	2	2	2	6	3
Crown vetch	0	0	0	1	1	4	[c]	2	2	4	–
Hairy vetch	0	0	1	1	2	3	1	1	1	3	6
Korean lespedeza	0	0	3	0	3	2	1	1	0	2	7
Sericea lespedeza	0	1	0	0	1	4	2	3	2	7	2
Ginnala maple	0	0	3	2	5	1	1	1	1	3	6
Siberian peashrub	0	0	0	0	0	5	1	1	2	4	5
Russian olive	0	0	0	0	0	5	1	1	1	3	6
Autumn olive	0	0	1	0	1	4	1	3	1	5	4
Amur honeysuckle	0	0	2	0	2	3	3	2	3	8	1
White oak	0	0	0	0	0	5	0	1	0	1	8
White ash	[c]	[c]	1	0	1	–	2	1	1	4	5
Yellow poplar	[c]	[c]	[c]	[c]	–	–	1	1	1	3	6
European black alder	0	0	0	0	0	5	2	2	2	6	3
Eastern white pine	0	0	0	0	0	5	[c]	[c]	[c]	–	–
Scotch pine	0	0	2	0	2	3	[c]	[c]	[c]	–	–

[a] Differs significantly from control (0 concentration) at the 5% level; 10^{-3} M concentration omitted.
[b] Two rankings of species sensitivity to juglone are presented: one based on toxicity to seed germination and radicle elongation, and one based on toxicity to shoot elongation and dry weight accumulation. Species not included in all of the tests are summarized, but their degree of sensitivity is not ranked with the other species.
[c] Species not included in test.

the second ranking applies to establishment and subsequent growth of planting stock.

When the 10^{-3} M concentration was not considered, few significant effects of juglone on seed germination and radicle elongation remained. These growth processes were less sensitive to juglone in most species than were shoot elongation and dry weight accumulation. Ranking of the species in order of their seed germination and radicle elongation sensitivity to juglone from most sensitive to least sensitive was as follows (Table 5): ginnala maple > Korean lespedeza > hairy vetch = amur honeysuckle = Scotch pine > crown vetch = sericea lespedeza = autumn olive > crimson clover = Siberian peashrub = Russian olive = white oak = European black alder = eastern white pine. Establishing sensitive species that are normally grown from seed (e.g., Korean lespedeza and hairy vetch) should be avoided near existing walnut trees.

Shoot elongation and dry weight accumulation were growth processes most commonly and conspicuously inhibited by juglone. Ranking of the species according to their shoot elongation and dry weight accumulation sensitivity to juglone from most sensitive to least sensitive was as follows (Table 5): amur honeysuckle > sericea lespedeza > crimson clover = European black alder > autumn olive > white oak > hairy vetch = ginnala maple = Russian olive = yellow poplar > Korean lespedeza > white oak. This ranking of species sensitivity would be more applicable than the seed germination–radicle elongation sensitivity ranking to estimating the compatibility of species coestablished with walnut.

In contrast to the growth inhibition by high juglone concentrations, in some instances low juglone concentrations (10^{-6} M and 10^{-5} M) apparently promoted seed germination and/or seedling growth. The responses were due to the exceptional growth of a few individual seedlings. These seedlings were either more resistant to juglone because of their vigor or were more vigorous because of their immunity to juglone. We reported similar stimulatory responses in previous research on juglone effects on the growth of coniferous seedlings (Funk et al., 1979b).

Data from these short-term tests are useful for comparing species sensitivity to juglone and the relative sensitivity of different growth processes. However, biological assays are insufficient as a means of identifying the occurrence of allelopathy and the concentrations of chemicals responsible for allelopathy in the field (Stowe, 1979). Although natural juglone concentrations have not been measured in the field and are therefore unknown, it is unlikely that 10^{-3} M juglone occurs in soil under field conditions. Aqueous solutions of that concentration were difficult to prepare in the laboratory, and some juglone usually precipitated out in hydroponic culture. Perry (1967) found no inhibition of oxygen uptake in bean or tomato plants from 5×10^{-7} M to 10^{-5} M juglone. Between 10^{-5} M and 3×10^{-4} M, the inhibition rose rapidly with the log of juglone concentration. The results of this experiment

generally agree with Perry's findings, although growth of two species (sericea lespedeza and amur honeysuckle) was inhibited by 10^{-6} M juglone. Thus, the allelopathic effects observed in the field are most likely the result of longer-term exposures to moderate juglone concentrations. The experiments also suggest that moderate and low juglone concentrations may inhibit (or stimulate) growth for a period of time before any obvious symptoms of toxicity appear.

Although every species tested was sensitive to juglone under short-term laboratory conditions, the occurrence of allelopathy under field conditions depends on three factors: (1) the sensitivity of associated species to juglone; (2) size and density of walnut trees; and (3) soil and climate conditions that control the disposition of juglone.

Species undoubtedly vary in their sensitivity to black walnut juglone, as evidenced by Brook's extensive surveys of the association frequencies of 218 species with walnut (Brooks, 1951), and the results of the experiments reported here. Brook's species lists include only four of the species that were tested in this study: white oak, white ash, yellow poplar, and white pine. Although Brooks stated that he found no evidence of antagonism between walnut and those species in natural stands, his tables show that white oak and yellow poplar occur less frequently within the crown projection of black walnut. For planted stands, however, the literature contains several reports of phytotoxicity to white pine (Wiant and Ramirez, 1974), Scotch pine (Schreiner, 1949), and other conifers. Pines are often planted with or near walnut and are especially sensitive to juglone (Funk et al., 1979b; Rietveld, 1981). I know of no published reports of phytotoxic effects from walnut on the other species tested in this study.

The occurrence of allelopathy is distinctly different when species are planted near existing large walnut trees than when even-aged mixtures of species, including walnut, are planted. In the former case, the toxic effects may appear within months or a few years, while in the latter case they may take much longer to appear because the walnut trees must grow to sufficient size and density to have a significant chemical effect on the environment. From a survey of 41 mixed, even-aged walnut plantations (Rietveld, 1981), a buildup period of approximately 12–25 years is needed for walnut trees to reach a sufficient size to produce and release enough juglone to have noticeable allelopathic effects. However, it is unknown when and for how long toxicity occurred before symptoms appeared because moderate juglone concentrations may inhibit growth without visible injury.

The principal factor affecting the length of the buildup period appears to be soil characteristics. The speed and completeness of decomposition and immobilization depend on a variety of factors including soil type, moisture and oxygen content, soil reaction, presence of decomposing organisms, concentration of juglone, and juglone's resistance to microbial attack.

Because sterile soil was used in the germination experiments, the reduced allelopathic effects on soil, although variable, can be attributed to fixation of juglone by soil colloids. In a survey of mixed, even-aged walnut plantations, soil drainage was consistently related to the occurrence of allelopathy (Rietveld, 1981). Toxicity occurred earlier on imperfectly drained soils but occurred later or not at all on well-drained soils.

Fisher (1978) found a strong relation between soil moisture and the allelopathic activity of walnut trees. As soil moisture increased, the amount of extractable juglone and allelopathic activity increased. High soil moisture creates anaerobic reducing conditions unfavorable for the chemical and biological oxidation of juglone, and thus allows juglone to accumulate. Although no direct evidence of microbial breakdown of juglone exists, it seems most probable that it occurs (Fisher, 1978). Soil conditions also influence growth rates and vigor, which in turn determine tree size and juglone production by walnut and are related to susceptibility of associated species to juglone toxicity. Thus, it appears that walnut allelopathy is more likely to occur on imperfectly drained soils where chemical and microbial oxidation is restricted and plant vigor is lower. Allelopathy may not occur at all, even in sensitive species, if they are growing vigorously on well-drained soils.

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Announcement

INTERNATIONAL SOCIETY OF PSYCHONEUROENDOCRINOLOGY

XIV International Congress of the International Society of Psychoneuroendocrinology will be held on June 12–16, 1983, New York, NY, USA. Symposia Topics are available from: Dr. Fleur L. Strand, Biology Department, New York University, Washington Square, New York, NY 10003, USA.

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TRAPPING *Dendroctonus brevicomis*[1]: Changes in Attractant Release Rate, Dispersion of Attractant, and Silhouette[2,3]

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Abstract—A sticky trap with 3 m^2 surface area was modified by changes in attractant release rate, vertical dispersion of the attractant, and addition of a tree trunk silhouette to the trap axis. As attractant release rate increased, the number of *Dendroctonus brevicomis* caught at the source of attractant and at 1.5 and 5.2 m above ground on two vertical silhouettes 4.5 m away increased. In one experiment, more beetles were caught at a dispersed source of attractant than at a point source. Fewer beetles were caught at the lower traps on the two outlying silhouettes when a silhouette was at the source, than when no silhouette was at the source. As attractant release rate increased, the catch of a predator, *Temnochila chlorodia*, increased at the source.

Key Words—*Dendroctonus brevicomis*, *Temnochila chlorodia*, Coleoptera, Scolytidae, Trogositidae, western pine beetle, attractant, pheromone, trap, behavior.

[1] Coleoptera: Scolytidae.

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[3] Mention of trade names is for information only and is not an endorsement by the funding or sponsoring agencies.

INTRODUCTION

Bark beetles often attack trees near a source of natural or synthetic attractant (Gara et al., 1965; Knopf and Pitman, 1972; McCambridge, 1967; Rasmussen, 1972; Vité and Pitman, 1970). Frontalin (Kinzer et al., 1969), *exo*-brevicomin (Silverstein et al., 1968), and myrcene (Bedard et al., 1969; Silverstein, 1970) are components of the attractive pheromone of the western pine beetle, *Dendroctonus brevicomis* LeConte (Wood et al., 1976; Bedard et al., 1980). During a field test to evaluate tactics for suppression and survey of *D. brevicomis* using traps baited with racemic *exo*-brevicomin (E), racemic frontalin (F), and myrcene (M), trees near some of the traps were attacked and killed (Bedard and Wood, 1974). A predator, *Temnochila chlorodia* Mannerheim (Coleoptera: Trogositidae), was also trapped. This beetle is attracted by *exo*-brevicomin (Vité and Pitman, 1969; Bedard et al., 1969, 1980).

In a study of the behavior of *D. brevicomis* at and near a source of EFM, only a portion of the attracted beetles were trapped at the source (Tilden et al., 1979). In another study, changes in proportions of trapped beetles generally corresponded to changes in attractant release rate and to the presence of a tree trunk silhouette both at and near the source of attractant (Bedard et al., unpublished observations).

This paper reports a study that attempted to increase the number of *D. brevicomis* trapped at a source of attractant and to decrease the number trapped on nearby vertical silhouettes simulating trees. We hypothesized that changes in attractant release rate, a point or dispersed source of attractant, and the presence or absence of a silhouette at the source are associated with changes in the number and distribution of trapped beetles.

METHODS AND MATERIALS

The study was done during July and August 1972, in the Shasta-Trinity National Forest, about 16 km east of McCloud, Siskiyou County, California. Eight plots were located about 0.8 km apart in an east–west line in 1–2 hectare openings along the edge of a 60- to 80-year-old stand of ponderosa pine, *Pinus ponderosa* Dougl. ex Laws. In each plot two 25.4-cm-diam × 6.1-m Sonotube® cardboard cylinders were erected vertically 9 m apart on a north–south line (Figure 1) to simulate the silhouettes of tree trunks. Two cylindrical hardware cloth (0.95-cm mesh) sticky traps, 38 cm diam × 30.5 cm and coated with Stickem Special®, were hung around each cardboard cylinder 1.5 and 5.2 m above ground. A vane trap (Browne, 1978), its surface reduced by removing one pair of vanes to lessen the number of beetles to count, was placed midway between the two cylinders with the two remaining vanes oriented in an

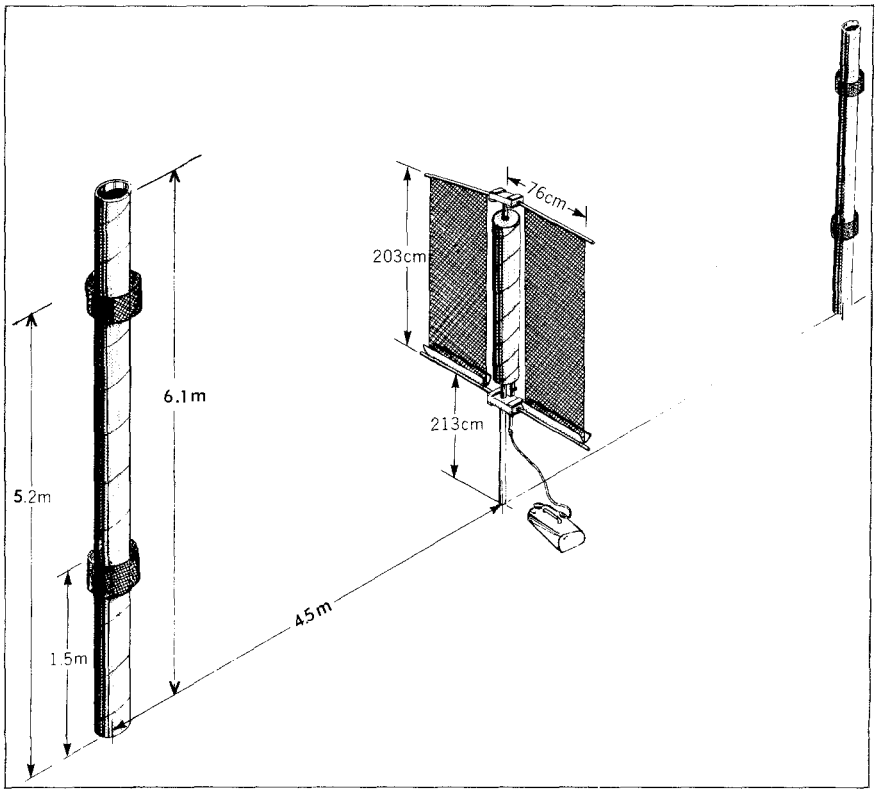

FIG. 1. Plot design for source of attractant at 2-vane trap midway between two cardboard cylinders (McCloud, Siskiyou County, California, July 11–August 11, 1972).

east–west line. Each vane was 76 cm wide × 2 m high. The bottom edge of each vane was 2 m above ground.

Racemic E and F, and natural M were evaporated at the vane trap at rates of 2, 20, and 200 mg/24 hr per compound. M contained 1% Antioxidant 330 (Ethyl Corporation, Baton Rouge, Louisiana) to retard polymerization. E and M were evaporated separately from 3.5-mm ID × 52-mm glass tubes (2 mg/24 hr) or from 9-mm ID × 45-mm 1-dr glass vials (20 and 200 mg/24). F was evaporated from 2.2-mm ID × 62-mm tubes (2 mg/24 hr) or from 5-mm ID × 35-mm 1/2-dr vials (20 and 200 mg/24 hr). For the 2 and 20 mg/24 hr rates, one vial of each compound was placed inside and near the bottom of a 2.7-cm ID × 1.8-m steel electrical conduit attached to the vertical axis of the vane trap. The conduit was sealed at the top and connected at the bottom to a portable air tank (Bedard and Browne, 1969) by 3.2-mm ID polyethylene tubing. For the 200 mg/24 hr rate, 10 vials of each compound evaporating at

20 mg/24 hr were placed inside a 5.1-cm ID × 30.5-cm galvanized pipe connected between the steel conduit and the polyethylene tubing from the air tank. Release rates were checked gravimetrically in the field.

Compounds were released from the conduit through 20 holes (0.4 mm diam), either grouped in two rows of 10 each around the circumference of the conduit near its midpoint (point source) or distributed in a helix along 1.5 m of the conduit (dispersed source). The dispersed source was intended to more closely resemble a portion of a tree under mass attack by *D. brevicomis* than the point source. An air flow of 100 ml/min from the air tank was sufficient to cause release of the compounds from all holes in the dispersed source conduit. The sufficiency of flow was determined by the reaction of moistened litmus paper to ammonia vapor.

To evaluate the effect of a tree trunk silhouette at the source of attractant, a 25.4-cm-diam × 1.5-m cardboard cylinder was mounted on the vane trap's axis. The conduit containing the attractant release devices was mounted on the outside of the silhouette.

Two experiments of eight simultaneously compared treatments each were done, which were all combinations of three factors at two levels each: low or high attractant release rate, point or dispersed source of attractant, and presence or absence of a silhouette at the source. Attractant release rates were 2 and 20 mg/24 hr per compound in the first experiment, and 2 and 200 mg/24 hr per compound in the second experiment. The eight treatments in each experiment were assigned to 64 experimental units (8 plots × 8 days) in a Latin square design, to minimize the effects of variation in trap catch among days and plots, which have been observed in other studies of bark beetle attractants (Wood et al., 1976; Cuthbert and Peacock, 1978; Payne et al., 1978; Tilden et al., 1979; Bedard et al., 1980).

Treatments were put out in mid-afternoon and removed the next morning. *D. brevicomis* and *T. chlorodia* were picked from the traps each morning and stored in labeled vials of solvent (Chevron-325®) for later counting.

Analysis of variance was done on three subsets of the data: (1) catch at the source of attractant, (2) total catch on low and (3) high outlying traps 4.5 m from the source. The analyses were based on an additive linear model with main effects for treatments, days, and plots. The F tests, based on the treatment sums of squares, were tests of the null hypotheses that all treatment means were equal, with the alternative hypotheses that some means were unequal. The days and plots examined in this study were of interest only as representatives of the populations of all possible days and plots and, therefore, might have been treated as random effects in the analyses. Because days and plots, however, were not chosen as random samples from these populations (a necessary assumption for analysis of random effects), they were instead treated as fixed effects. Strictly speaking, this limits the results of

the analyses to the particular days and plots examined. We believe, however, the results are indicative of other times and places. All statistical tests were two-sided tests of size (alpha) 0.05.

The use of F tests in the analysis of variance is usually justified by assuming that the observations come from homoscedastistic normal distributions. We believe this assumption is unrealistic for trap catch data. A distribution-free test of treatment effects can be based on the permutation distribution of the F ratio over all possible Latin squares (Scheffé, 1959). Calculating the permutation distribution is difficult, however, except for small Latin squares (5×5 or smaller). Scheffé (1959, section 9.3) suggests that the critical values of the F distribution are reasonable approximations to the exact critical values of the permutation distribution for Latin squares of size 5×5 or larger. On the basis of this statement by Scheffé, we believe the F tests of treatment effects in this paper are justified. The F ratios based on sums of squares for rows (days) and columns (plots), however, do not have permutation distributions; therefore, no tests were made for these two factors.

For each subset of the data, the treatment sum of squares was partitioned into seven component sums of squares with 1 *df* each: three main effects, three two-factor interactions, and one three-factor interaction. This partitioning allowed the main effects and interactions of the three factors to be examined individually. The test based on the sum of squares for attractant release rate, for example, was a test of the null hypothesis that release rate had no effect on the treatment means.

The analysis of variance was used to examine sources of variation in the differences in catch between low and high outlying traps. For each cell in the Latin square, the difference equaled catch on low traps minus catch on high traps. To determine whether catches at low traps differed from catches at high traps, we used six Wilcoxon signed-rank tests for each experiment. Catches on low and high traps were compared at each level of the three factors: low and high attractant release rate, point and dispersed source of attractant, and presence and absence of a silhouette at the source. Because each level contained half of the cells in the Latin square, each test involved 32 matched pairs.

RESULTS

In all treatments, *D. brevicomis* were caught at all traps (Table 1). The results of the F tests (Table 2) showed significant differences in catch, with attractant release rate being the largest source of variation. In both experiments, more beetles were caught at the source of attractant and at low and high outlying traps with the high release rate than with the low release rate (Table 1).

In the first experiment, the number of beetles caught at a point source

TABLE 1. CATCH OF *Dendroctonus brevicomis* AT TWO HEIGHTS ON TWO CARDBOARD CYLINDERS[a] 9 m APART AND AT A SOURCE OF ATTRACTANT[b] MIDWAY BETWEEN CYLINDERS (MCCLOUD, SISKIYOU COUNTY, CALIFORNIA)

Attractant rate	Attractant source[c]	Silhouette[d]	Experiment 1[e]			Experiment 2[f]		
				Outlying cylinders			Outlying cylinders	
			Trap at source	Low traps	High traps	Trap at source	Low traps	High traps
Low	Point	No	20.9 (17.3)[g]	2.3 (1.6)	1.6 (2.3)	36.5 (32.5)	4.9 (4.3)	3.0 (2.7)
Low	Dispersed	No	25.9 (27.2)	1.6 (1.8)	1.1 (1.8)	50.9 (52.4)	3.0 (6.1)	1.5 (3.1)
Low	Point	Yes	34.8 (24.8)	0.6 (0.7)	1.3 (1.5)	69.0 (59.7)	2.4 (2.1)	1.4 (1.4)
Low	Dispersed	Yes	42.6 (43.9)	1.0 (1.1)	0.6 (0.9)	98.4 (80.7)	4.1 (4.6)	1.8 (2.3)
High	Point	No	87.0 (71.8)	4.6 (2.8)	8.8 (4.8)	243.1 (289.3)	15.1 (10.5)	31.5 (23.1)
High	Dispersed	No	111.6 (91.7)	3.9 (3.6)	6.5 (5.0)	147.1 (154.8)	10.5 (9.0)	24.6 (25.7)
High	Point	Yes	96.8 (56.6)	2.0 (1.6)	7.3 (6.8)	175.6 (144.4)	8.0 (5.4)	18.8 (13.9)
High	Dispersed	Yes	134.5 (96.7)	3.5 (2.3)	5.5 (4.4)	187.1 (111.1)	8.1 (6.4)	31.0 (26.3)

[a] 25.4 cm diam × 6.1 m, each with 38-cm-diam × 30.5-cm cylindrical traps at 1.5 and 5.2 m above ground.
[b] Racemic *exo*-brevicomin and frontalin and natural myrcene each released at 2 mg/24 hr (low rate in experiments 1 and 2) and at 20 mg/24 hr (high rate in experiment 1) or 200 mg/24 hr (high rate in experiment 2). Trap at source was two vanes, each 76 cm × 2 m. Bottom of vanes 2 m above ground.
[c] Attractant released from 20 holes (0.4 mm diam) at midpoint (point source) or distributed in a helix along 1.5 m (dispersed source) of 2.7-cm ID × 1.8-m steel conduit.
[d] Cardboard cylinder 25.4 cm diam × 1.5 m on axis of vane trap.
[e] July 11–14, 17–20, 1972.
[f] July 31–August 1, 3–4, 7, 9–11, 1972.
[g] Daily mean (standard deviation of observations), $N = 8$. For outlying cylinders, each mean is calculated from total of two traps.

TABLE 2. F RATIOS FROM ANALYSES OF VARIANCE OF *Dendroctonus brevicomis* CAUGHT AT TWO HEIGHTS ON TWO CARDBOARD CYLINDERS[a] 9 m APART AND AT A SOURCE OF ATTRACTANT[b] MIDWAY BETWEEN CYLINDERS (MCCLOUD, SISKIYOU COUNTY, CALIFORNIA)

		Experiment 1[c]				Experiment 2[d]			
Factors	df	Trap at source	Low outlying traps	High outlying traps	Difference[e]	Trap at source	Low outlying traps	High outlying traps	Difference[e]
Treatments[f]	7	11.45*[g]	3.59*	9.72*	3.11*	3.53*	3.73*	6.38*	4.04*
Rows (days)	7	4.93	1.23	2.57	2.95	1.02	0.92	1.22	1.29
Columns (plots)	7	11.60	1.54	5.78	4.75	5.56	2.14	1.72	1.26
Residuals	42								
Partition of treatment sums of squares									
A (rate)	1	70.72*	15.95*	62.42*	17.17*	20.21*	18.86*	40.99*	25.11*
B (source)	1	4.28*	0.06	3.00	2.46	0.12	0.50	0.11	0.47
C (silhouette)	1	3.02	6.08*	1.30	0.27	0.26	2.96	0.19	0.10
$A \times B$	1	1.85	0.22	0.94	1.17	1.32	0.30	0.23	0.60
$A \times C$	1	<0.01	0.12	0.30	0.06	0.88	1.38	0.06	0.07
$B \times C$	1	0.20	2.33	0.02	0.64	1.23	2.10	1.85	0.68
$A \times B \times C$	1	0.08	0.34	0.04	0.03	0.67	0.04	1.24	1.27

[a] 25.4 cm diam × 6.1 m, each with 38-cm-diam × 30.5-cm cylindrical traps at 1.5 and 5.2 m above ground.
[b] Racemic *exo*-brevicomin and frontalin and natural myrcene. Trap at source was two vanes, each 76 cm × 2 m. Bottom of vanes 2 m above ground.
[c] July 11–14, 17–20, 1972.
[d] July 31–August, 1, 3–4, 9–11, 1972.
[e] Catch on low traps minus catch on high traps.
[f] Three factors with two levels each: (a) attractant release rates of 2 or 20 mg/24 hr per compound in experiment 1, and 2 or 200 mg/24 hr per compound in experiment 2; (b) attractant source, with compounds released from 20 holes (0.4 mm diam) at midpoint (point source) or distributed in a helix along 1.5 m (dispersed source) of 2.7-cm ID × 1.8 m conduit; (c) presence or absence of cardboard cylinder 25.4 cm diam × 1.5 m on axis of trap at source.
[g] F ratios for treatment sums of squares indicated by (*) are significant at alpha = 0.05. Critical values of F are 2.23 (7 and 42 *df*) and 4.07 (1 and 42 *df*). Row or column F ratios were not tested.

differed significantly from the number caught at a dispersed source of attractant (Table 2). More beetles were caught at a dispersed source than at a point source (Table 1). Although not statistically significant, a similar trend was observed in the second experiment in three of four comparisons of dispersed vs. point source (Table 1). A point or dispersed source of attractant did not significantly affect the numbers of beetles caught at either low or high outlying traps.

In the first experiment, the number of beetles caught at low outlying traps differed significantly when presence and absence of a silhouette at the source of attractant were compared (Table 2). Fewer beetles were trapped when a silhouette was present than when it was absent (Table 1). A similar trend was observed at the high outlying traps in the first experiment, but it was not statistically significant. No such trends were apparent in the second experiment. More beetles were caught at the source when a silhouette was present than when absent, at both release rates in the first experiment and at the low release rate in the second experiment (Table 1), but these differences were not statistically significant (Table 2).

No significant interactions were indicated among attractant release rate, point or dispersed source of attractant, and presence or absence of a silhouette at the source (Table 2).

In both experiments, attractant release rate significantly affected the differences in catch between low and high outlying traps (Table 2). The Wilcoxon tests, which compared catches on low and high traps, were also significant only for comparisons within each attractant release rate. In the first experiment, catches on low and high traps differed at the high release rate ($P = 0.007$). Low traps caught fewer beetles than high traps (Table 1). In the second experiment, catches on low and high traps differed at the low ($P = 0.002$) and at the high ($P = 0.001$) release rates. At the low rate, low traps caught more beetles than high traps, but at the high rate, low traps caught fewer beetles than high traps (Table 1).

T. chlorodia were also trapped at the source of attractant and at low and high outlying traps, but the individual trap catches were too few to analyze statistically. In both experiments more *T. chlorodia* were trapped with the high rate than with the low (Table 3).

DISCUSSION

Large variations in numbers of beetles trapped resulted from day and plot effects. Although the Latin square design accounts for these sources of variation, it does not account for variations resulting from any interactions between day, plot, and treatment. Such interactions inflate the residual mean square, thereby reducing the power of the F tests (Scheffé, 1959; sections 5.1 and 9.2), and they may have made it difficult to detect variations resulting

TABLE 3. CATCH OF *Temochila chlorodia* ON TWO CARDBOARD CYLINDERS[a] 9 m APART AND AT A SOURCE OF ATTRACTANT[b] MIDWAY BETWEEN CYLINDERS (MCCLOUD, SISKIYOU COUNTY, CALIFORNIA)

	Experiment 1[c]		Experiment 2[d]	
Attractant rate	Trap at source	Traps on outlying cylinders	Trap at source	Traps on outlying cylinders
Low	40(0–13)[e]	3(0–1)	23(0–3)	10(0–2)
High	251(0–31)	17(0–3)	441(1–51)	40(0–9)

[a] 25.4 cm diam × 6.1 m, each with a 38-cm diam × 30.5-cm cylindrical trap at 1.5 and 5.2 m above ground.
[b] Racemic *exo*-brevicomin and frontalin and natural myrcene each released at 2 mg/24 hr (low rate in experiments 1 and 2) and at 20 mg/24 hr (high rate in experiment 1) or 200 mg/24 hr (high rate in experiment 2). Trap at source was two vanes, each 76 cm × 2 m. Bottom of vanes 2 m above ground.
[c] July 11–14, 17–20, 1972.
[d] July 31–August 1, 3–4, 7, 9–11, 1972.
[e] Total (range), $N = 32$. For outlying cylinders, each number is total of four traps (one trap × two heights × two cylinders).

from a point or dispersed source of attractant and the presence or absence of a silhouette at the source. In only two instances did these modifications have a statistically significant effect on numbers of *D. brevicomis* trapped, although the trends in the other data were usually consistent with these differences.

A tree trunch silhouette is an important visual stimulus to the behavior of *D. brevicomis* responding to a source of attractant (Bedard et al., unpublished observations). In most of the comparisons in our study, the presence of a 1.5-m-high silhouette was associated with higher catches at the source of attractant, although this effect was not statistically significant. A silhouette taller than 1.5 m at the source, as well as a more dispersed source of attractant, might have produced more detectable effects—both at the source and at the cylinders 4.5 m away.

Tenfold (experiment 1) and 100-fold (experiment 2) increases in attractant release rate were associated with ca. threefold increases in catch both at the source and at low outlying traps (Table 1). At the high outlying traps a 10-fold increase in release rate was associated with ca. sixfold increases in catch, and a 100-fold increase in release rate was associated with ca. 14-fold increases in catch. These data are consistent with the results of Coster and Gara (1968) and Johnson and Gara (1978) and indicate that the risk of trees being attacked close to a source of attractant may increase as the attractant release rate increases. Daily variation in catch on the cylinders (Table 1) may reflect fluctuations in attractant concentration at or near the cylinders caused

by variable wind patterns, and fluctuations in the number of beetles available to respond to these chemical and visual stimuli.

The apparent response of *T. chlorodia* to different attractant release rates is, to our knowledge, the first account of this aspect of its behavior. Vité and Williamson (1970) reported an increase in the catch of *Thanasimus dubius* (F.) (Coleoptera: Cleridae), a predator of *D. frontalis*, when the release rate of frontalin was increased 10-fold.

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MEASUREMENT OF RELEASE RATES OF GOSSYPLURE FROM CONTROLLED RELEASE FORMULATIONS BY MINI-AIRFLOW METHOD

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Abstract—The use of the mini-airflow apparatus for the measurement of gossyplure release rates is described. The method involves the passage of air over controlled-release dispensers containing radiolabeled pheromone, through a coarse frit, and entrapment of the gossyplure on glass beads. Desorption of the beads with solvent is followed by quantification by liquid scintillation counting. The results of release rate measurements from hollow fibers, red rubber septa, and red rubber wicks are discussed.

Key Words—Gossyplure, release rates, formulations, controlled release, hollow fibers, septa, wicks.

INTRODUCTION

The development and field testing of pheromone-based insect pest control products has advanced rapidly in the recent past. Such strategies involve the use of controlled release formulations to dispense the pheromone. In several areas, the development of a technology to evaluate the pheromone formulations has lagged behind the design and development of the formulations themselves. This has especially been the case where the measurement of the release rates from controlled release devices has been concerned. Indeed, as noted by Roelofs (1979) many field tests had, in the past, been conducted without any knowledge whatsoever of the release rate of the pheromone from the formulation used. While release rates measured under the controlled conditions of the laboratory will vary from the actual release rates under field conditions, laboratory data obtained from different substrates at different

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conditions and wind speeds assist greatly in the design and evaluation of formulations.

In particular, a reliable, reproducible method of measuring the effluent from the controlled release devices was needed. An earlier paper by Weatherston et al. (1981) described the various methods currently in use for the measurement of the release rates and discussed some of the problems associated with these methods and measurements. One problem which arises frequently (Bierl and DeVilbiss, 1975; Rothschild, 1978) is lack of agreement when rates are measured by different methods. In the case of measurements by the airflow methods, one manifestation of this problem was in inability to achieve a mass balance. In the cited paper, Weatherston and coworkers introduced an apparatus and a technique which has been used to measure the release rate of [*acetyl*-1-^{14}C]gossyplure from hollow fiber formulations and demonstrated that a mass balance could be achieved with this method. This paper reports on further measurements of the release rate of [*acetyl*-1-^{14}C]gossyplure from hollow fiber formulations and on the extension of the method to include other types of release devices.

METHODS AND MATERIALS

Liquid scintillation counting was done either in a Nuclear Chicago Isocap 300 scintillation counter, at an efficiency of 80%, or a Tracor Analytic Mark III model 6881 scintillation counter, at an efficiency of 97%, except as noted. Liquid scintillation counting (LSC) cocktail was prepared either by mixing PPO, 2 g, and POPOP, 50 mg, in liquid-scintillation-grade toluene, 500 ml, or by mixing 1 Gal-Pak of Omnifluor® with 1 gallon liquid-scintillation-grade toluene. The PPO was obtained from Amersham/Searle, Oakville, Ontario; the POPOP from Sigma Chemical Company, St. Louis, Missouri; and Omnifluor from New England Nuclear, Boston, Massachusetts.

Gossyplure [the 1:1 mixture of (*Z,Z*)-and (*E,Z*)-7,11-hexadecadien-1-yl acetate] was purchased from Albany International Chemicals Division (formerly Chemical Samples Company), Columbus, Ohio. Gas chromotography was performed on a Bendix model 2500 equipped with an FID, at an oven temperature of 180° C and N_2 flow rate of 40 ml/min through a glass 1.8-m × 4-mm ID column packed with 3% OV-17 on 80/100 WNP Chromosorb.

Molecular sieves were Linde 13X pellets purchased from Ventron Corporation, Danvers, Massachusetts, and Amberlite® XAD-4 was purchased from Mallinckrodt Chemical Works, St. Louis, Missouri. Flowmeters equipped with a differential regulator were purchased from Kontes, Vineland, New Jersey. Glass beads, 1.00–1.05 mm diameter, were manufactured by B.

Braun, Melsungen, Federal Republic of Germany. Hexamethyldisilazane was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, and was used as received. Red rubber septa, catalog number 8753-D22, were purchased from A.H. Thomas Company, Philadelphia, Pennsylvania, and the red rubber wicks were supplied by Dr. Roger T. Huber, University of Arizona, Tuscon, Arizona.

[*Acetyl-1-*^{14}C] *gossyplure*. (*Z*,*Z*) and (*E*, *Z*)-7,11-hexadecadien-1-ol, a 1:1 mixture obtained by hydrolysis of gossyplure with methanolic NaOH, were dissolved in dry pyridine (1 ml) and treated with [1-^{14}C]acetic anhydride (50 μCi, 435 mg, 3.89 mmol). After 18 hr in a stoppered tube at room temperature, additional acetic anhydride (500 mg, 4.46 mmol) was added and the mixture allowed to remain at room temperature for a further 48 hr. Pesticide-grade hexane (20 ml) was added and the solution washed sequentially with 2 N H_2SO_4, water and saturated $NaHCO_3$. The hexane was dried ($MgSO_4$), concentrated under a N_2 stream to about 10 ml, and filtered. The filter cake was washed with hexane, and the combined filtrate and washings evaporated to an oil under aspirator vacuum. The oil was distilled to yield the product: 780 mg (66%), bp 125–127°/0.1 mm, homogenous to GLC (99%) with a retention time (3 min 25 sec) identical to that of an authentic sample, and a specific activity of 0.02 μCi/mg.

General Mini-Airflow Procedure. The mini-airflow apparatus (see Weatherston et al., 1981) was silanized with hexamethyldisilazane. The collection chamber was filled with a known weight (ca. 8 g) of unsilanized glass beads which were held in place with a plug of silanized glass wool. The formulation to be measured was placed in the sample chamber and a prefilter consisting of a mixture of molecular sieves and XAD-4 resin in a jointed glass tube was placed in front of the sample chamber. The Luer joint was attached to a vacuum pump through a flowmeter, and air at ambient temperature was drawn through the apparatus at 1.0 l/min for 24 hr.

After 24 hr the apparatus was detached from the vacuum line and disassembled. The formulation was removed from the apparatus and placed in the cap of a scintillation vial. The apparatus itself was inverted over the vial and a syringe containing solvent was attached to the Luer joint. The trapped pheromone was desorbed from the beads with three washes of 7 ml each, except as noted. Each wash was collected in a separate scintillation vial and LSC cocktail (10 ml) was added. The apparatus was flushed with nitrogen in both directions, the formulation replaced in the sample chamber, and the mini-airflow apparatus reassembled for further measurements. Measurements were continued for the number of days specified.

After the final day of measurements the formulation was extracted by soaking in either LSC cocktail alone or a mixture of LSC cocktail (10 ml) and hexane (7 ml) for 48 hr to remove the residual pheromone. The beads and glass

wool were removed from the apparatus, and each was separately added to LSC cocktail to determine what, if any, material remained on these parts of the apparatus. The airflow unit itself was also washed again and these washes examined by liquid scintillation counting as well.

Hollow-Fiber Formulations. A group of nine hollow fibers filled with [*acetyl*-1-^{14}C]gossyplure was selected at random. To ascertain the initial loading, six of these fibers were individually extracted by soaking in the LSC cocktail–hexane mixture. Each of the remaining fibers were individually placed in a mini-airflow apparatus and the release rate measured as described at 22 ± 1° C. The beads were washed with hexane daily and the residual [*acetyl*-1-^{14}C]gossyplure was extracted from the fibers at the end of 13 days in the LSC cocktail–hexane mixture.

Red Rubber Septa Formulations. Septa were loaded with a solution (135 μl, 7.5 μg/μl) of [*acetyl*-1-^{14}C]gossyplure in hexane. When the hexane had evaporated, three of the septa were individually extracted in LSC cocktail to determine initial loading. Another three septa were individually placed in mini-airflow units and the release rates measured at 26 ± 1° C for 21 days except as noted. The airflow units were routinely desorbed with two washes (7 ml each) of toluene and the residual pheromone in the septa extracted in LSC cocktail alone.

Red Rubber Wick Dispensers. The sections of red tubing were supplied by and identical to those used by Huber and Hoffman (1979). They varied in both length and weight. A solution (22 μl, 864 μg/μl) of [*acetyl*-1-^{14}C]gossyplure in pesticide-grade hexane was poured into a Petri dish lid containing 17 of these sections cut side down. After 2 hr at 23° C, the hexane had completely evaporated and the lures were transferred to sealed pouches for storage except that four were selected at random for determination of the initial loading. The Petri dish was washed with hexane and any [*acetyl*-1-^{14}C]gossyplure remaining was recovered.

To determine whether any [*acetyl*-1-^{14}C]gossyplure had been lost by evaporation, a blank experiment was conducted in which the same volume of the [*acetyl*-1-^{14}C]gossyplure solution was allowed to stand at room temperature in a Petri dish lid for 4 hr. The lid was then washed with hexane to recover the [*acetyl*-1-^{14}C]gossyplure.

For the wicks selected to determine the loading, in two cases the wicks were extracted directly into LSC cocktail (10 ml) by soaking for 48 hr and in the other two cases the wicks were extracted by soaking in hexane (2 ml) for 24 hr and then again in fresh hexane (2 ml) for 48 hr twice. Each hexane extract was separately added to LSC cocktail (10 ml).

An additional three wicks were randomly selected and the release rates measured at 22 ± 1° C for 20 days as described. Hexane was used to wash the apparatus daily and the residual activity was extracted from the wicks in LSC cocktail alone.

TABLE 1. RECOVERY OF [*acetyl*-1-^{14}C]GOSSYPLURE FROM HOLLOW-FIBER FORMULATIONS

	Amount released (μg)	Residue in fiber (μg)	Total recovered (μg)	Recovered (%)
Fiber 1	47.9	185.1	233.0	104
Fiber 2	48.9	166.1	215.0	96
Fiber 3	60.9	151.0	211.9	94

RESULTS AND DISCUSSION

Hollow-Fiber Formulations. The mean initial loading of the six fibers assayed was 224.8 μg/fiber (±14%) of [*acetyl*-1-^{14}C]gossyplure. The total recovery and percent recovery are shown in Table 1. The mean recovery was 98% (±14%).

The release profiles of the individual fibers measured by mini-airflow are shown in Figure 1 as plots of total days elapsed versus cumulative amount of [*acetyl*-1-^{14}C]gossyplure released. The mean release rate represented by the fibers shown in Figure 1 was 3.80 μg/day (±14%). The release rates were

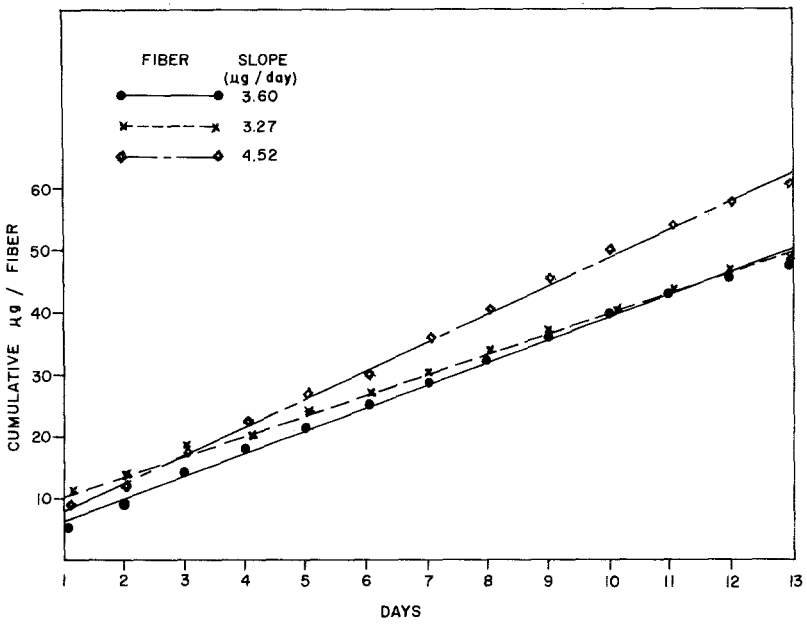

FIG. 1. Release rates of [*acetyl*-1-^{14}C]gossyplure from hollow fibers.

calculated by linear regression for each fiber, and in all cases the correlation coefficient was equal to or greater than 0.997.

The small standard deviations and the high percent recovery both indicate that the use of the mini-airflow method is a reliable, reproducible method for measuring release rates.

Red Rubber Septa. To test the hypothesis that the method was applicable to release rate measurements in general, the release of [*acetyl*-1-^{14}C]-gossyplure was measured from two additional release devices. Since rubber septa have been used as controlled release device in attractancy tests and monitoring traps for several years, septa were selected for release rate measurements.

Butler and McDonough (1979), in their extensive study of the evaporation rates of pheromones from natural rubber septa, used dichloromethane as the solvent, citing an earlier paper by Maitlen et al. (1976) which indicated that in the case of (*E,E*)-8,10-dodecadien-1-ol, dichloromethane was the preferred solvent over hexane, chloroform, and dimethylformamide. Maitlen et al. (1976) speculated that solvent effect differences in the evaporation rate from septa could be due to the ability of a solvent to penetrate the rubber. The present authors believe it is more likely that the solution of a pheromone in a solvent will contribute to the penetration of the pheromone into the septa. In the present study the septa were loaded with an hexane solution of radioactive

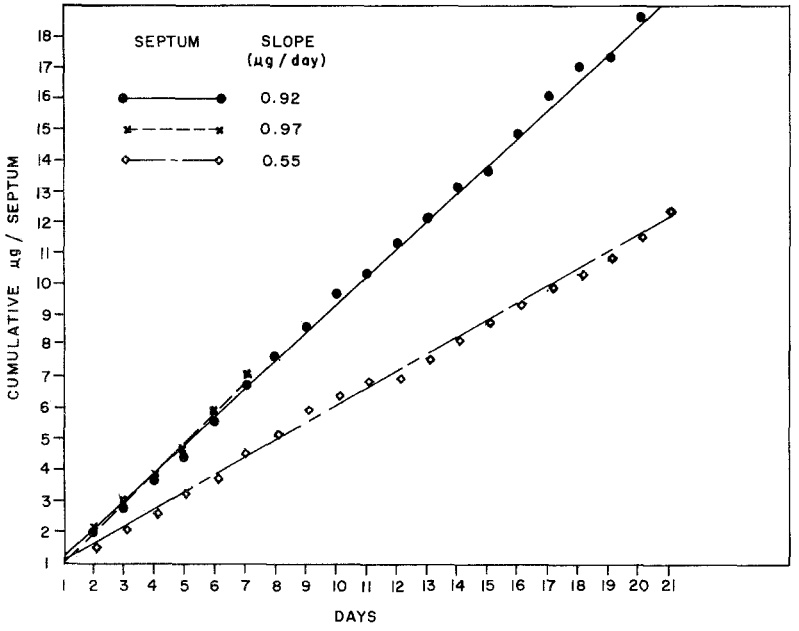

FIG. 2. Release rates of [*acetyl*-1-^{14}C]gossyplure from rubber septa.

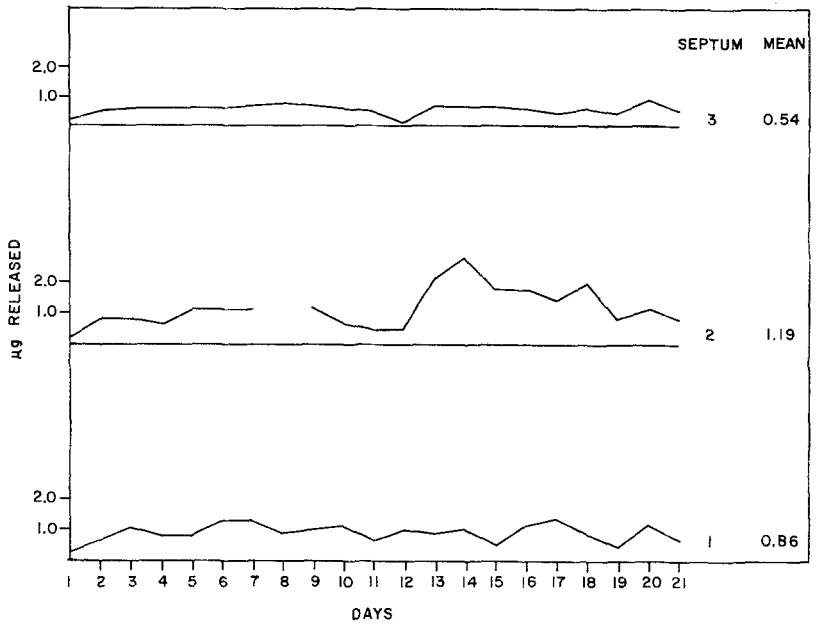

FIG. 3. Daily release of [*acetyl*-1-^{14}C]gossyplure from rubber septa.

gossyplure. The release profiles of the red rubber septa are shown in Figure 2 as plots of total days elapsed versus cumulative amount released. It should be noted that the plot for one septum terminates at day 7. The mini-airflow unit in which this release rate was being measured was broken on day 8, resulting in the loss of that sample. A new unit was assembled and the release rate measurements continued for the remainder of the experiment, but the cumulative total was not calculated for that septum beyond day 7. The daily release for all three septa is shown in Figure 3 where the missing sample is indicated as a discontinuity in the plot. The release rates calculated by both linear regression and as the mean of the daily amount released is shown in Table 2. The difference in the day 1–7 and 9–21 values for the second septum are due to the large releases which occurred from days 13–18 as shown in Figure 3. When the mean value for the 19 days on which pheromone was collected is calculated, it is found to be 1.9 μg/day (±53%).

The half-life of gossyplure in rubber septa has been reported by Flint et al. (1978) to be 159 days. Using this value and the equation used by Butler and McDonough (1979), the anticipated release rate of gossyplure from the septa was calculated to be 4.94 μg/day. That the mean measured rate was less than 20% of this may be due to a variety of differences between the two experiments including factors such as the type of septum used, the application solvent, the initial loading, and wind speed.

TABLE 2. RELEASE RATE OF [*acetyl*-1-^{14}C]GOSSYPLURE FROM RUBBER SEPTA

Septum	Days		μg/day			
	From	To	Mean	CV (%)[a]	Slope[b]	R[c]
1	1	21	0.87	±33	0.92	0.999
2[d]	1	7	0.87	±37	0.97	0.996
	9	21	1.36	±51	1.39	0.994
3	1	21	0.53	±32	0.55	0.999

[a]Coefficient of variance.
[b]Calculated by linear regression of days elapsed versus cumulative amount released.
[c]Correlation coefficient.
[d]Day 8 sample lost.

Both Flint et al. (1978) and Butler and McDonough (1979) indicate that the half-lives observed for field-aged septa were considerably less than those observed in laboratory studies and attribute the shorter life of field-aged septa to factors such as temperature and wind speed. The wind speed through the mini-airflow apparatus is less than 0.25 mph, well under what could be expected in the field. These experiments did not include any variation in the speed of the air drawn through the mini-airflow apparatus, but such experiments are presently in progress.

The expected loading for the septa was 1012.5 μg/septum. The mean initial loading was, in fact, found to be 1133.3 μg/septum (±7%) at a counting efficiency of 32%. The low counting efficiency was due to the significant amount of color which was also extracted from the septa.

The total recovery was also calculated for these septa. The recoveries are given in Table 3. Even though the sample for one day is missing from one septum, the mean recovery of pheromone was 98% (±3) for all septa.

Red Rubber Wick Dispensers. The second release device chosen to test the applicability of the mini-airflow method was red rubber wick dispensers.

TABLE 3. AMOUNT OF [*Acetyl*-1-^{14}C]GOSSYPLURE RECOVERED FROM RUBBER SEPTA

	Amount recovered (μg)		
Septum number	1	2	3
Amount released	18.1	23.8	11.2
Recovered from septum	1049.4	1105.4	1121.9
Recovered from apparatus	0.3	0.5	0.5
Total	1067.8	1129.7	1133.6
% Recovered	94.2	99.7	100.0

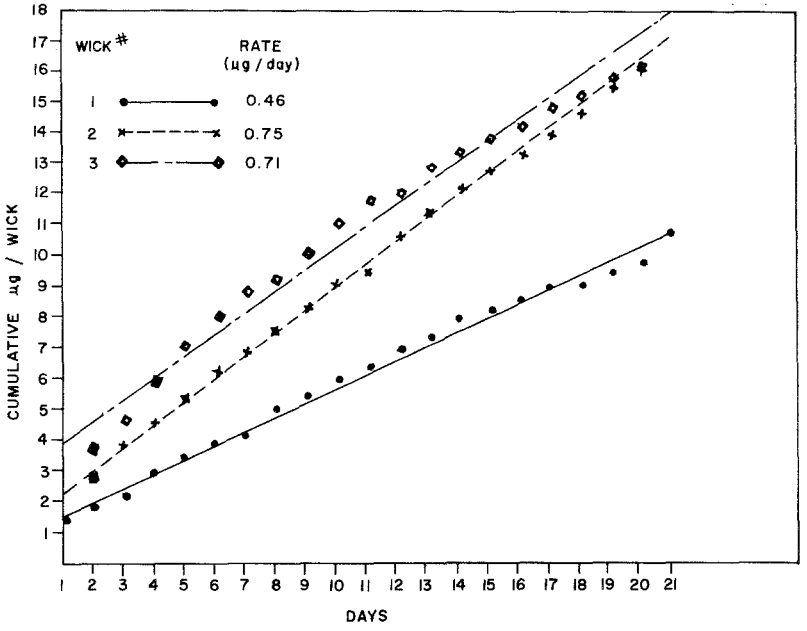

FIG. 4. Release rates of [*acetyl*-1-^{14}C]gossyplure from rubber wicks.

These dispensers described by Huber and Hoffman (1979) are used commercially in a mass trapping program in Arizona. The protocol for loading these wicks was obtained from Dr. R. Huber (person communication) and followed implicitly. The release profiles of the red rubber wick formulations are shown in Figure 4. The correlation coefficients of these plots are 0.994, 0.997 and 0.983 for wicks number 1, 2 and 3 respectively.

The recovery of [*acetyl*-1-^{14}C]gossyplure from the blank experiment was 97%; 20% of the initial amount of [*acetyl*-1-^{14}C]gossyplure was recovered from the Petri dish after loading the wicks. The weights of 34 rubber wicks varied from 359.3 mg to 580. 3 mg with a mean weight of 435.1 mg (±13%).

TABLE 4. RECOVERY OF [*Acetyl*-1-^{14}C]GOSSYPLURE FROM RED RUBBER WICKS AFTER MEASUREMENT OF RELEASE RATES

	Amount recovered (µg)		
Lure number	1	2	3
Amount release	9.8	16.2	16.3
Recovered from lure	484.5	1166.2	910.5
Recovered from apparatus	0.1	0.1	0.1
Total recovered	494.4	1182.5	926.9

The amounts recovered from the four wicks extracted immediately after loading were 1641.2 and 680.8 μg in hexane and 703.4 and 859.3 μg in LSC. Considering the method of loading and the lack of uniformity in the size of these wicks, this large variation is not surprising. However, the wide range of initial loadings made calculation of percent recovery less meaningful for these lures than for the other types of lures examined. Nevertheless, the data in Table 4 indicate that the recoveries did not fall within an acceptable range.

CONCLUSIONS

Use of the mini-airflow apparatus appears to have eliminated many of the problems previously described by Weatherston et al. (1981) as being associated with measurements made by airflow methods. Miniaturization of the apparatus has resulted in the ability to obtain a quantitative recovery of the pheromone released on a daily basis and has allowed the attainment of a mass balance for the entire measurement. Since the loading in the septa and rubber wicks is much greater than in the fibers, and since the amount released from the septa and wicks is small by comparison to the initial loading, the test of mass balance may not be as valid for these devices as it is for hollow fibers. However, the value of the methodology in these cases is in being able to determine the release rate when the total amount released is extremely small by comparison to the initial loading. The mini-airflow method eliminates the problems inherent in methods which attempt to measure small decreases from large quantities.

The versatility of the apparatus has been demonstrated through the measurement of release rates from a small, but representative, sample of controlled release devices. The data presented indicate that the mini-airflow apparatus provides an accurate, reproducible method of measuring release rates from a variety of controlled release formulations.

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SEX PHEROMONE COMPONENTS OF *Euxoa drewseni*[1]: Chemical Identification, Electrophysiological Evaluation, and Field Attractancy Tests

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Abstract—Eleven compounds structurally similar to known lepidopterous pheromone components were identified in the extract from 18 calling female moths of *Euxoa drewseni* (Staudinger). The identifications were done by gas chromatography–mass spectrometry and high-resolution gas chromatography with flame ionization and electroantennographic detectors simultaneously. Detector antennae were from five species of moths. In the field, male moths were specifically attracted to a three-component blend of dodecyl, (*Z*)-5-dodecenyl, and (*Z*)-7-tetradecenyl acetates in a ratio of 2:6:1. This blend at 1000 μg/rubber septum dispenser is recommended as a trap bait for monitoring purposes. Low concentrations of (*Z*)-7-dodecenyl acetate or (*Z*)-7-tetradecenol inhibited the attraction of moths to the three-component blend. (*Z*)-7-Pentadecenyl acetate functioned as a parapheromone in place of (*Z*)-7-tetradecenyl acetate in the pheromone blend, and they appear to react via the same antennal receptor.

Key Words—*Euxoa drewseni*, *Chorizagrotis thanatologia*, Lepidoptera, Noctuidae, sex pheromone, attractant inhibitor, parapheromone, (*Z*)-5-dodecenyl acetate, (*Z*)-7-dodecenyl acetate, (*Z*)-7-tetradecenyl acetate, (*Z*)-7-pentadecenyl acetate.

INTRODUCTION

Euxoa drewseni (Staudinger) occurs throughout the southern parts of Canada and the northern United States and has been reported in Greenland and Alaska. There are several color phases of the moths, but dark red and

[1] Lepidoptera: Noctuidae.
[2] Author: For Agriculture Canada, Government of Canada.

blackish-brown forms predominate. The dark red color phase is very similar to the common red color phase of the redbacked cutworm (RBC), *Euxoa ochrogaster* (Guenée), and examination of their genitalia is required to distinguish these species (Hardwick, 1970). Since the moths of these species are also about the same size, they may be easily misidentified, especially in light trap catches. *E. drewseni* is synonymous with *Chorizagrotis thanatologia* Dyar (Hardwick, 1970) which has been reported in mixed infestations with RBC on oats and flax in the prairie provinces (Beirne, 1971). Since *E. drewseni* both as larvae and as moths could be mistaken for RBC, it seems that their abundance and, to a lesser extent, their distribution may not be well documented.

E. drewseni male moths were consistently attracted to some of the chemical blends that were included in a field attractancy test for RBC in 1974 (Struble and Swailes, 1978). These field data suggested that similar chemicals may be involved with the sex pheromone of both species. This report describes the identification of *E. drewseni* pheromone components and the development of a species-specific pheromone blend for the attraction of *E. drewseni* male moths. This will be useful for determining their abundance and distribution, and for studying the behavior of the moths.

METHODS AND MATERIALS

Pheromone was recovered from 13- to 18-day old females (kept at 22 ± 2° C, 60% relative humidity, 16L:8D) that exhibited maximum calling behavior during a 1-hr simulated twilight (21 to 8 lux) before the scotophase. Their everted ovipositors were removed with fine forceps, adhering abdominal tissue was removed, the ovipositor was washed with ca. 100 μl of *n*-hexane (B.D.H. OmniSolv, glass distilled), and then the ovipositor was soaked in *n*-hexane. Accumulated washes and tip extracts from 18 females were separately filtered and without further clean-up were reduced to an appropriate volume of 0.5 female equivalents (FE)/μl under N_2.

Combined pheromone washes and tip extracts were analyzed by gas chromatography (GC) using a flame ionization detector (FID) and an electroantennographic detector (EAD) simultaneously. The EAD was similar to that described by Arn et al. (1975). It was built on a Varian 2700 chromatograph that was converted here for use with glass capillary columns, and the column effluent was split ca. 3:1 between the FID and EAD. Glass capillary columns (28 m, 0.25 mm ID) coated with Carbowax 20 M TPA (Varian) with a film thickness of 0.23 μm, or SP-2340 (Supelco) with a film thickness of 0.07 μm were used for these analyses. All glass (borosilicate) capillary columns were treated here by the method of Grob and Grob (1976) and coated by the dynamic method. Helium carrier gas linear flow velocity

was 30 cm/sec for all analyses. A typical temperature program for FID-EAD analyses was splitless injection for 0.3 min at 50° C, 30° /min to 110° C, hold for 2 min, 4° /min to 205° C. The EAD antennae were from laboratory-reared 7- to 28-day old *E. drewseni* male moths and from field collected *E. drewseni*, *E. auxiliaris* (Grote), *E. messoria* (Harris), *Autographa californica* (Speyer), and RBC.

GC (FID) analyses were also done on a Hewlett Packard 5830A with a capillary inlet, and an SP-2340 glass capillary column (78 m, 0.25 mm ID, film thickness 0.06 μm). A typical temperature program was splitless injection for 0.6 min at 50°C, 20°/min to 110°C, and 0.5°/min to 180°C. Various temperature program rates were used to completely resolve the pheromone components, e.g., baseline resolution of (*Z*)-5- and (*Z*)-7-tetradecenyl acetates (abbrev. *Z*5- and *Z*7-14:Ac).

GC-MS chemical ionization (CI) analyses were done with a Hewlett Packard 5985B using glass capillary columns, e.g., 15 m, 0.32 mm ID, coated with Carbowax 20 M TPA, with a film thickness of 0.24 μm. Helium carrier gas linear flow velocity was 30 cm/sec and typical temperature program was splitless injection for 1.0 min at 50° C, 30° /min to 95° C, and 4° /min to 200° C. The temperature of the Pt/Ir transfer line from the GC column to the MS ion source was 225°C, and the ion source was 180°C. Isobutane reactant gas pressure was 0.5 torr in the ion source. Mass range was 130–350 amu/0.82 sec.

Synthetic chemicals were >99% chemically pure and >99.8% isomerically pure. Their sources and and purification, red rubber septa dispensers, field attractancy test procedures, and traps were as previously described (Struble, 1981a). Traps were spaced 20–30 m apart along the edge of wheat fields near Lethbridge, Alberta, and moth catches were recorded at least three times a week. Identification of the moths was confirmed here by examining the genitalia of representative specimens from each replicated experiment (Hardwick, 1970). Analyses of variance were done on the data, and treatment means were compared by regression analysis or Tukey's test (Steel and Torrie, 1960). In all instances, in the following section, mean trap catches followed by the same letter did not differ, $P > 0.05$, by Tukey's test.

RESULTS AND DISCUSSION

Female Calling. Female moths were at least 13 days old before they exhibited a calling behavior which consisted of extending their ovipositor while in a resting position (Struble et al., 1980b). They called for about 1 hr during a simulated twilight period. These observations suggest that the females would call shortly after sunset under natural conditions and that the moths would be about 2 weeks old before mating.

GC-MS and GC Analyses. A total ion current CI mass chromatogram of

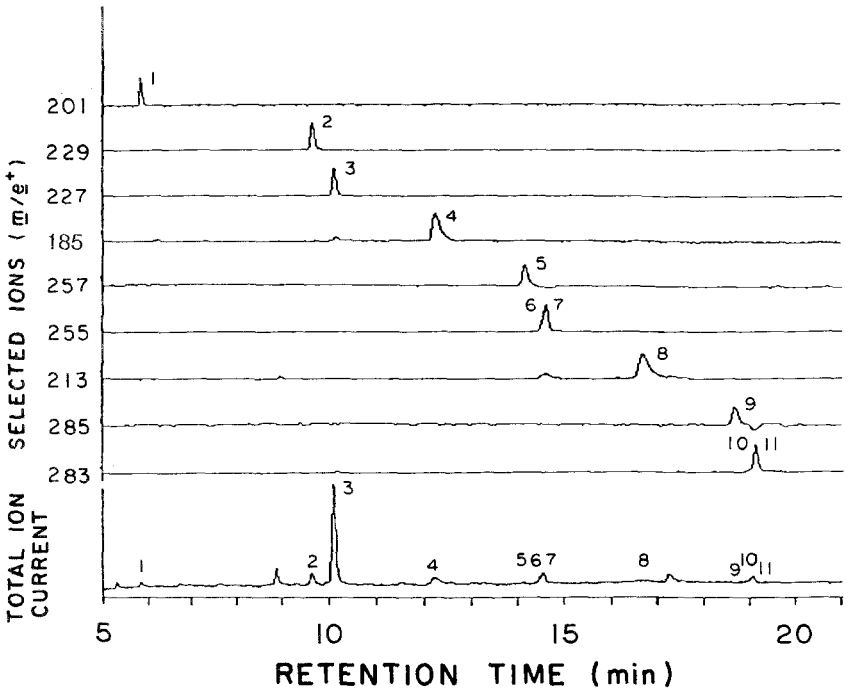

FIG. 1. Mass chromatograms with chemical ionization (isobutane) showing the total ion current and selected ions for an *E. drewseni* abdomen tip extract (3 FE injected on a Carbowax 20 M TPA column). The components were: 1, 10:Ac; 2, 12:Ac; 3, Z5-12:Ac; 4, Z5-12:OH; 5, 14:Ac; 6, Z5-14:Ac; 7, Z7-14:Ac; 8, Z7-14:OH; 9, 16:Ac; 10, Z7-16:Ac; 11, Z9-16:Ac.

female abdomen tip extract is illustrated in Figure 1. The abdomen tip wash contained identical components but at a lower concentration. Mass chromatograms based on selected ions, (M + 1) for C_{10} to C_{18} saturated, mono-, and diunsaturated acetates, alcohols, and aldehydes (Struble, 1982), were reconstructed with the data system. The detectable selected ions of diagnostic value are included in Figure 1; complete mass spectra were obtained for these components. C_{10} to C_{18} aldehydes and diunsaturated alcohols and acetates were not detected in the pheromone extracts.

The structural assignments of components 1 to 11 were based on their CI differential mass spectra in comparison to those of synthetic chemicals obtained under identical GC-MS conditions. The isomer assignments were based on retention times and cochromatography of these components with synthetic chemicals on high-resolution capillary GC columns of Carbowax 20 M TPA and SP-2340 under different temperature program conditions. Most of the positional and geometrical isomers of monounsaturated C_{10} to C_{16}

acetates and alcohols with the double bond beyond carbon number 4 could be resolved on these liquid phases (Heath et al., 1980). Isomer assignments were also confirmed by GC-EAD and field attractancy data which are discussed later in this section. The suggested structural assignments of pheromone components 1 to 11; their relative quantity; and m/e^+ (relative intensity) of the major ions, M + 1 and (M + 1) − 60 for acetates and M + 1, M − 1, (M + 1) − 18 and (M − 1) − 18 for alcohols were:

1.	10:Ac	0.3	201(100), 141(7)
2.	12:Ac	11	229(100), 169(9)
3.	*Z*5–12:Ac	100	227(100), 167(51)
4.	*Z*5–12:OH	20	185(100), 183(10), 167(4), 165(6)
5.	14:Ac	2	257(100), 197(20)
6.	*Z*5–14:Ac	4	255(100), 195(50)
7.	*Z*7–14:Ac	18	255(100), 195(33)
8.	Z7–14:OH	5	213(100), 211(18), 195(8), 193(5)
9.	16:Ac	0.8	285(100), 225(2.4)
10.	*Z*7–16:Ac	0.8	283(100), 223(48), (background ions present)
11.	*Z*9–16:Ac	6	283(100), 223(63) (background ions present)

Ions (M + 39) and (M + 57), which are common with isobutane as reactant gas, were detectable in all of these spectra, but their relative intensities were generally low (<3) (Struble, 1982). The abdomen tip extract contained an average of 14 ng of *Z*5–12:Ac/FE.

FID-EAD Analyses. An FID-EAD chromatogram of the abdomen tip extract is illustrated in Figure 2. *E. drewseni* male antennae gave consistent responses to pheromone components 2, 3, 6, 7, and 8. Responses of the same intensity were recorded to similar quantities of the corresponding synthetic chemicals, which helped confirm the structural assignments of the pheromone components.

The assignments of components 3, 6, and 7 in the pheromone extract were also confirmed by a special EAD technique in which the detector antennae were from male moths of other species that were known to have specific responses to these components.

RBC male antennae were known to respond specifically to *Z*5–10:Ac and *Z*5–12:Ac (Struble et al., 1980a) and, as detector antenna for the *E. drewseni* extract, strongly responded (0.7 mV) only to pheromone component 3. This was consistent with the structural assignment of *Z*5–12:Ac. The lack of any other RBC antennal responses indicated that *Z*5–10:Ac was not present in the *E. drewseni* pheromone extract.

A. californica male antennae were known to respond specifically to *Z*7–12:Ac and *Z*7–14:Ac (Struble, unpublished results; Steck et al., 1979) and injections of 1 ng of each compound under the GC-EAD conditions outlined in Figure 2 gave antennal responses of 0.8 and 0.14 mV, respectively.

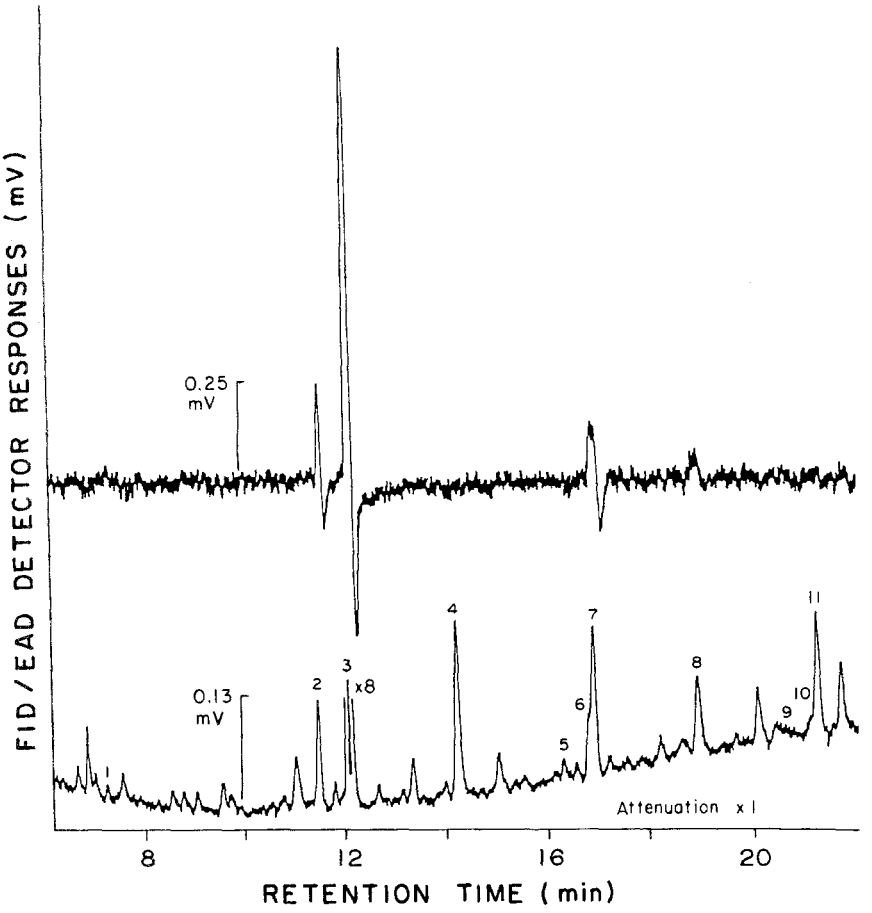

FIG. 2. Gas chromatogram with FID and EAD responses to *E. drewseni* abdomen tip extract (0.5 FE injected on a Carbowax 20 M TPA column) with *E. drewseni* as detector antenna. The FID and EAD responses were recorded simultaneously.

A. californica as detector antenna for the *E. drewseni* pheromone extract gave responses of 0.1 and 0.13 mV at retention times that corresponded to *Z*7–12:Ac and component 7 (Figure 2). This was consistent with the structural assignment of *Z*7–14:Ac for *E. drewseni* pheromone component 7, and it suggested the presence of a trace amount, ca. <10 pg/FE, of *Z*7–12:Ac. *Z*7–12:Ac was not detectable by GC-FID or GC-MS.

E. auxiliaris male antennae were known to respond specifically to *Z*5–14:Ac (Struble, unpublished results) and as detector antenna gave a distinct response (0.2 mV) to the *E. drewseni* pheromone component 6. This was in

agreement with the structural assignment of Z5-14:Ac. Under similar conditions *E. messoria* male antenna, which were known to respond to Z11-16:Ac (Struble, unpublished results; Struble et al., 1977), gave weak responses of 0.1 mV to the *E. drewseni* extract. This suggested that a trace amount, ca. <10 pg/FE, of Z11-16:Ac may be present in the *E. drewseni* pheromone, although it was not detectable by GC-FID or GC-MS.

Field Attractancy Tests. E. drewseni males were consistently attracted to blends of Z5-12:Ac and Z7-14:Ac in systematic field screening attractancy tests of binary mixtures that were tested for RBC (Struble and Swailes, 1978). Consequently, several attractancy tests were done for *E. drewseni* before the pheromone components were identified, and the results are in good agreement.

The ratio of Z5-12:Ac and Z7-14:Ac that attracted the most *E. drewseni* males was determined by testing (4 replications, 200 μg of attractant/dispenser, August 19-29, 1975) the following ratios of these components, and the mean catches of moths (in parentheses) were: 100:0 (0.5), 10:1 (32), 6:1 (35), 3:1 (25), 1:1 (12), 1:3 (4), 1:6 (2), and 0:100 (0). Regression analysis indicated the catches increased linearly ($P < 0.01$) as the quantity of Z7-14:Ac increased, with maximum catches at a ratio of 6:1. The ratio of these components in the pheromone extract was about 5.5:1.

Blends of Z5-12:Ac and Z7-14:Ac in a ratio of 6:1 were tested with several other compounds as third components to determine whether any of them had synergistic or inhibitory effects on the attraction of males (Table 1). 12:Ac at ratios of 1 or 3 improved ($P < 0.05$) the catches and, since it was present in the pheromone extract, it can be considered as an essential pheromone component. Several other ratios of these three components were

TABLE 1. MEAN *E. drewseni* MALES CAPTURED WITH COMBINATIONS OF Z5-12:AC AND Z7-14:AC AT A 6:1 RATIO AND 200 μg/DISPENSER WITH VARIOUS RATIOS OF OTHER COMPONENTS (4 REPLICATIONS, AUGUST 6-17, 1976)

Component added	Ratio	Mean[a] males captured
None	—	44 b
12:Ac	1	80 a
12:Ac	3	71 a
E5-12:Ac	1	45 b
Z5-12:OH	2	37 b
Z7-12:Ac	1	1 c
Z7-14:OH	1	1 c

[a]Means followed by the same letter do not differ ($P > 0.05$) by Tukey's test.

tested, but blends of 12:Ac, Z5-12:Ac, and Z7-14:Ac at ratios of 1:6:1 or 2:6:1 attracted the most moths.

E5-12:Ac or Z5-12:OH had no effect on the attraction of males (Table 1). Since *E. drewseni* male antennae did not respond to Z5-12:OH (Figure 2), the alcohol in the pheromone extract may be a precursor of Z5-12:Ac. E5-12:Ac was not detectable in the pheromone. Other compounds that had no effect on the attraction of males when tested as third components, but were not listed in Table 1, were: 12:OH, 14:Ac, 14:OH, E7-14:Ac, 16:Ac, E7-, and Z9-16:Ac. Z7-12:Ac and Z7-14:OH both had strong ($P < 0.01$) inhibitory effects on the attraction of males at the concentrations used in these early field tests (Table 1).

The pheromone extract contained Z5-14:Ac, and GC-EAD data with *A. californica* and *E. messoria* as detector antennae indicated that trace amounts (ca. <10 pg/FE) of Z7-12:Ac and Z11-16:Ac may also be present. The effects of very low concentrations of these compounds on the attraction of males were determined in the following tests.

A blend of 12:Ac, Z5-12:Ac, and Z7-14:Ac in a μg ratio of 120:600:140 captured a mean of 58a males (4 replications, July 31-September 2, 1981). [Means followed by the same letters do not differ ($\rho > 0.05$) by Turkey's test.] Addition of 3 μg of Z7-12:Ac, or 60 μg of Z5-14:Ac, or 6 μg of Z11-16:Ac as fourth components resulted in mean catches of 18c, 47a, and 63a, respectively. A blend of 12:Ac, Z5-12:Ac, Z7-14:Ac, Z5-14:Ac, and Z11-16:Ac in a μg ratio of 120:600:140:60:6 captured a mean of 48a moths, and addition of 3 μg of Z7-12:Ac to this blend resulted in the capture of 10c moths. In each instance the addition of only 3 μg of Z7-12:Ac reduced ($P < 0.05$) the catches of moths, which confirmed its potent inhibitory effect. On the other hand, Z5-14:Ac and Z11-16:Ac at the concentrations tested had no significant effect on the number of males captured. These components were tested again at even lower concentrations to determine whether any synergistic effects could be established.

A test was done (4 replications, August 13-September 3, 1981) using a blend of 12:Ac, Z5-12:Ac, and Z7-14:Ac in a μg ratio of 200:600:100 and a mean of 39 moths/trap were captured. Addition of Z5-14:Ac at 1, 10, and 25 μg; or Z11-16:Ac at 1, 6, and 12 μg as fourth components did not ($P > 0.05$) affect the captures of moths. Addition of both Z5-14:Ac and Z11-16:Ac also had no effect on the catches of moths.

The three-component blend is an excellent trap bait for monitoring the population densities of *E. drewseni* males, and it was important to establish the quantity/dispenser that functioned the best over an average flight period (August 1-September 15). Blends of 12:Ac, Z5-12:Ac, and Z7-14:Ac in a ratio of 2:6:1 at 50, 100, 500, and 1000 μg/dispenser captured (4 replications, August 19-September 9, 1981) a mean of 18c, 20bc, 32ab, and 35a moths, respectively. The 500 and 1000 μg/dispenser functioned equally well for a

3-week period, but the data showed a relative decrease ($P > 0.05$) in the catches to the 500-μg treatment during the last week of the test. Therefore, this three-component blend at 1000 μg/dispenser is recommended for monitoring or survey purposes.

The three-component blends of 12:Ac, Z5–12:Ac, and Z7–14:Ac were essentially species-specific for *E. drewseni*. About 9500 males were captured in these tests (1974–1977, and 1981), and even though many other species of Lepidoptera were known to be in the test area, they were not attracted. Two unbaited traps did not capture any moths. The other pheromone-like compounds identified in the *E. drewseni* extract that had no effect on trap catches may be involved with some other aspect of their mating behavior. This was not investigated.

E. drewseni, *E. auxiliaris*, and RBC occur sympatrically in southern Alberta and presumably in other areas of Canada and the United States. There is considerable overlap of their seasonal flight periods which are from late July to October. It is likely that these species maintain reproductive isolation, at least in part, by their pheromones. Of the three species, *E. drewseni* and *E. auxiliaris* are the most closely related as both are in the small subgenus *Chorizagrotis* which is represented in North America by six species (Hardwick, 1970). The primary pheromone component of *E. drewseni*, Z5–12:Ac, is a potent attractant inhibitor for *E. auxiliaris* (Struble, 1981b). The RBC, although in a different and much larger subgenus (Lafontaine, 1982), *Euxoa*, also has Z5–12:Ac as its primary component, but Z7–12:Ac, an essential secondary component of the RBC pheromone (Struble, 1981a), is a potent inhibitor for *E. drewseni*.

Pheromone Receptors and Parapheromones. E. drewseni male antennal responses were recorded for most saturated and monounsaturated C_{10} to C_{16} acetates and alcohols where the double bond was beyond carbon number 4. The strongest responses were to saturated, Z5 and Z7 acetates (Figure 3), which reached a maximum with the C_{12} compounds and decreased as the carbon chain length changed to C_{10} and C_{16}. Similar results have been reported for single receptor recordings of other noctuids (Priesner, 1979). Although single receptor recordings were not done for *E. drewseni*, it is assumed that the main receptor type was for Z5–12:Ac, the primary pheromone component. The other Z5 acetates presumably react on the receptor for Z5–12:Ac and the decrease in their relative EAD responses with changes in the carbon chain length is an indication of a decrease in their "fit" on the receptor.

Z7–12:Ac gave the strongest responses of the Z7 acetates, and it was found to be an attractant inhibitor. There presumably is a specific receptor for Z7–12:Ac that may be an evolutionary "hang over" (Priesner, 1979) to provide some interspecific responses such as reproductive isolation. There is likely a specific receptor for Z7–14:Ac, an essential pheromone component,

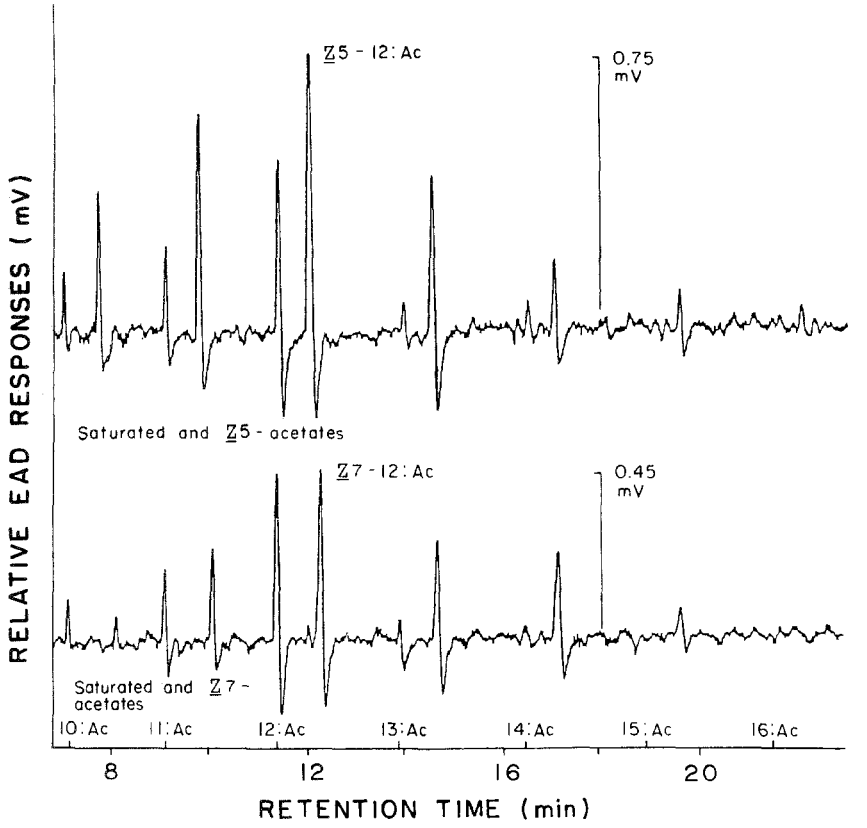

FIG. 3. EAD chromatograms with *E. drewseni* male moth relative antennal responses to 1 ng (injected) of C_{10} to C_{16} saturated, Z5-, and Z7-monounsaturated acetates on a Carbowax 20 M TPA column.

and the responses recorded to Z7-13: Ac and Z7-15: Ac may be from the receptors for Z7-12: Ac and Z7-14: Ac.

Since *E. drewseni* male antennae responded to Z7-13: Ac and Z7-15: Ac (Figure 3), the attractancy of these compounds was determined by including them in the three-component blend in place of Z7-14: Ac. Three blends consisting of 12: Ac, Z5-12: Ac, and Z7-14: Ac; 12: Ac, Z5-12: Ac, and Z7-13: Ac; and 12: Ac, Z5-12: Ac, and Z7-15: Ac (at ratios of 1: 6: 1 and 200 μg/dispenser, 4 replications, August 17-27, 1976) captured a mean of 17a, 3b, and 8b moths, respectively. Since blends containing Z7-13: Ac and Z7-15: Ac did result in the capture of some moths, these parapheromone components must accomplish some "fit" on the receptor for Z7-14: Ac. The Z7-13: Ac blend attracted the smallest number of moths, and this may indicate that it also had some reaction at the receptor for Z7-12: Ac, an

attractant inhibitor. Odd-numbered carbon compunds have been reported as parapheromones for other lepidopterans (Voerman et al., 1975; Cardé and Roelofs, 1977; Struble, 1981a), and these may also function via their corresponding pheromone receptors.

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SOLDIER DEFENSE SECRETIONS OF THE MALAYSIAN TERMITE, *Hospitalitermes umbrinus* (ISOPTERA, NASUTITERMITINAE)

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Abstract—Trinervita-1(15),8(19)-dien-2β,3α-diol and the new trinervita-1(15),8(19)-dien-2β,3α-diol 2-O-acetate constitute the major diterpene constituents of the soldier defense secretions of the Nasutitermite *Hospitalitermes umbrinus* of Malaysian region. Studies on the intraspecific variations on sympatric and allopatric populations indicate two distinct patterns of chemical composition.

Key Words—Termite, *Hospitalitermes umbrinus*, Isoptera, Nasutitermitinae, defense secretions, terpenes, trinervitenes.

INTRODUCTION

Soldier defense secretions of the highly specialized genera of the termite subfamily Nasutitermitinae have recently generated considerable interest and have been reviewed by Prestwich (1979a). Comprising mostly monoterpenoid and diterpenoid compounds, these secretions are produced in the enlarged frontal glands and ejected via the conical, nozzle-shaped projections of the soldier heads. Besides the identification of the various chemical components and structures which has led to the discovery of a number of new natural products with some novel bi-, tri-, and tetracyclic diterpenoid structures, such studies have also provided for a better understanding of the use of such secretions in defense of the colony (Moore, 1964, 1968; Eisner, 1976). Investigations of interspecific and intraspecific variations have currently

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provided evidence that the differences in chemical composition can be relied upon as chemotaxonomic characters for generic and species separation and hence could contribute towards unraveling some of the current problems centered around termite phylogenetic relationships (Prestwich and Collins, 1981).

Many of the reported studies have been based on Ethophian and Neotropical termite material. However, over the last two years, we have initiated studies towards the documentation and identification of the chemical secretion of the Indomalayan nasute genera and species, especially those found in peninsular Malaysia. Some aspects of these studies have already been reported (Prestwich et al., 1981a,b; Goh et al., 1982).

This paper reports on the chemical composition and some preliminary investigations of intraspecific variations in the soldier defense secretions of *Hospitalitermes umbrinus*, an ebony black species that is common to the lowland and hill dipterocarp forest in peninsular Malaysia and habitually builds nests of a black carton material at the bases of living trees and in tree stumps.

These termites have a specialized dietary preference for lichen that grows on the bark of trees. However, their food often includes a mixture of bark scrapings, fungal hyphae, fungal spores, and algae. In foraging for food, these termites emerge from their nests, usually in the early hours of the morning, and form long processionary trails, of up to 5 termites abreast, that traverse fallen branches, logs, and often up along the trunks of trees to the canopy. The food collected is worked into small round balls, slightly larger than the termite heads, which are then carried back along the foraging column to the nest by the workers. Hence, this form of open foraging behavior would demand an efficient defense strategy. In this respect, the soldiers play an important role by positioning themselves on the flanks of the column and they readily attack by "squirting" a stream of frontal gland secretions at any would-be predators, such as ants, that attempt to break into their ranks.

METHODS AND MATERIALS

Termite Material. The soldier termites' materials used for the present studies were all collected within closed canopy dipterocarp forests from four localities as follows: (1) Sungei Menyala forest reserve (2°29′ N, 101°55′ E; 300 m above sea level)—a lowland dipterocarp forest in the state of Negeri Sembilan; (2) Pasoh forest reserve (2°58′ N; 101°55′ E; 100 m above sea level)—a lowland dipterocarp forest which has been the site for intensive ecological studies under the auspices of the International Biological Programme; (3) Gombak forest reserve (3°20′ N, 101°46′ E; 500 m above sea level)—a hill dipterocarp forest in the state of Selangor; and (4) Fraser's Hill

forest reserve (3°43′ N, 101°44′ E; 1200 m above sea level)—a upper hill dipterocarp forest boardering on the oak montane forest type.

The termites were collected with minimum disturbance in plastic bags and chilled to −10° C after which they were decapitated and the heads crushed and extracted by hexane or carbon disulfide.

Analytical Methods. Gas chromatography (GC) was performed on a Tracor 560 instrument fitted with a flame ionization detector. Gas chromatography–mass spectrometry was performed on a Pye 104 gas chromatograph interphased to a double-beam Kratos MS30 mass spectrometer by a membrane separator. Mass spectra were obtained at 70 eV with the source and interphase temperatures at 150° and 210°C, respectively, and data were accumulated by a Kratos DS50 data system. Two glass columns, viz. 5 ft × 3 mm 3% OV-17 and 6 ft × 3 mm 3% OV-1 on 100/120 Gas Chrom Q, were used to analyze the terpenoids. A 10-ft × $\frac{1}{8}$-in. stainless-steel column packed with 10% Carbowax 20 M on 120/140 Gas Chrom Q was also used to analyze monoterpenes. Retention times of diterpenes were made relative to *n*-alkane standards and quantitation was made relative to C_{14} or C_{20} *n*-alkanes. Results are given in Tables 1–4.

Isolation of individual compounds from the crude secretion (ca. 80 mg) was aided by a Waters 440 HPLC instrument fitted with a 25-cm × $\frac{1}{4}$-in. Ultrasphere silica gel column. Elution of the diterpenes was by a gradient of 2–10% ethyl acetate in petroleum ether (bp 60–80° C). Isolated samples were checked by GC or TLC on 5 × 10-cm 0.25-mm silica gel 60 plates. Proton and carbon NMR spectra were recorded on a Jeol JNM-FX100 spectrometer.

2,3-Dihydroxy-1(15),8(19)-trinervitadiene (VII) and its 2-*O*-monoacetate

TABLE 1. VARIATION IN MONOTERPENE COMPOSITION (%)

	Colonies[a]					
Compound	I	IIA[b]	IIB[b]	IIIA[b]	IIIB[b]	IV
α-Pinene	68	80–85	61–64	64–65	69–73	60
Camphene	t[c]	t	2–3	t	0–3	3
β-Pinene	30	12–13	27–33	24–28	22–28	30
Carene	t	t	t	0–3	t	t
α-Terpinene		t		t		
Limonene	2	2–6	2–6	2–5	2–4	6
Terpinolene		0–1	0–1	0–1	0–1	1

[a]I, Sungei Menyala Forest Reserve, Negeri Sembilan; IIA, B, Gombak Forest Reserve, Selangor; IIIA, B, Pasoh Forest Reserve, Kuala Pilah; IV, Fraser's Hill, Pahang.
[b]Range for three colonies.
[c]t = trace (less than 1%).

TABLE 2. VARIATION OF DITERPENE COMPOSITION (%) IN *Hospitalitermes umbrinus*

Compound[a]	Colony[b]					
	I[c]	IIA[d]	IIB[c,d]	IIIA[d]	IIIB[c,d]	IV[c]
Ripperten-3α-ol(I)		0–1		0–2		
Trinervita-1(15),8(19)-dien-9β-ol(V)		0–2		2–6		
Trinervita-1(15),8(9)-dien-2β-ol(II)		0–1		1–4		
2-oxotrinervita-1(15),8(19)-dien-3α-ol(IV)	4	0–3	3–6	6–7	7–8	7
Trinervita-1(15),8(19)-dien-2β,3α-diol 2-*O*-acetate(VI)[e]	26	16–28	28–38	25–30	17–18	27
Trinervita-1(15),8(19)-dien-2β,3α-diol (VII)	70	69–72	47–59	37–47	74–75	66
Trinervita-1(15),8(9)-dien-2β,3α-diol(III)		0–1		0–1		
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 9-*O*-acetate(IX)		0–13		4–17		
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 2,3-*O*-diacetate(X)		0–1		0–1		
13-oxotrinervita-1(15),8(19)-dien-2β,3α-diol 2,3-*O*-diacetate(XI)		0–1		0–1		
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 2,3,9-*O*-triacetate(XII)		0–1		0–2		

[a]Listed in increasing order of retention times on 5-ft 3% OV-17 column.
[b]I—Sungei Menyala Forest Reserve, Negeri Sembilan; IIA&B—Gombak Forest Research, Selganor; IIIA&B—Pasoh Forest Reserve, Negeri Sembilan; IV—Fraser's Hill, Pahang.
[c]Only small amounts of diterpene derivatives; some unknown mixed acetate-propionate esters of a tetrahydroxy diterpene are also present.
[d]Range for 3 colonies.
[e]Decomposes in GC.

derivative VI were isolated as major components. The former VII had [^{1}H]NMR, [^{13}C]NMR, and mass spectra in agreement with that reported previously (Vrkoc, 1978).

The 2-*O*-acetate VI was obtained as fine feather-shaped crystals of mp 152–153°C. [^{1}H]NMR ($CDCl_3$) resonance at δ 5.37 (1H, br d, 9, H-2), 4.95, 4.79 (2H, two br s, H-19), 3.98 (1H, d, 9, H-3), 3.14 (1H, dt, 11, 9, H-7), 2.40 (1H, br d, 11, H-16), 2.19 (3H, s, OAc), 1.78 (3H, s, H-17), 1.06 (3H, s, H-18), 0.93 (3H, d, 6.6, H-20). IR ν_{max} (CCl_4) 1735 (C=O), 3500 (O—H), 1635, and 905 cm^{-1}(C=CH_2). The [^{13}C]NMR ($CDCl_3$) spectrum showed two quaternary olefic carbons at 112.2 and 152.5 ppm and a single C=O carbon at 182.9 ppm. EI-mass spectrum *m/e* 328 (5, $M^+ - H_2O$), 286 (35, $M^+ - AcOH$), 175 (15, $C_{12}H_{15}O^+$), 149 (5, $C_{10}H_{13}O^+$), 135 (70, $C_9H_{11}O^+$), 123 (10, $C_9H_{15}^+$), 119 (15, $C_9H_{11}^+$), 109 (5, $C_8H_{13}^+$), 105 (10, $C_8H_9^+$), 93 (20, $C_7H_9^+$), 91 (30, $C_7H_7^+$), 81 (25, $C_6H_9^+$), 77 (25, $C_6H_5^+$), 69 (15, $C_5H_9^+$), 67 (15, $C_5H_7^+$), 55 (45, $C_4H_7^+$), 53 (20, $C_4H_5^+$), 43 (100%, CH_3CO^+), and 41 (75, $C_3H_5^+$). Calculated for

TABLE 3. CHROMATOGRAPHIC PROPERTIES AND MAJOR MASS SPECTRAL FRAGMENTS OF DITERPENES

Compound	Mol wt	R_f (color)[a]	OV-1[b]	OV-17[b]	(m/e)[c]
I	288	0.48 (sky blue)	21.8	24.1	270, 255, 163, 159, *41*
V	288	0.58 (wistaria violet)	22.0	24.3	270, 255, 175, *136*, 135,41
II	288	0.54 (violet)	22.3	24.7	270, 255, 175, 161, 135, *41*
IV	302	0.61 (lilac)	22.8	25.5	287, 274, 259, 231, *151*, 41
VI	346	0.36 (pink)	23.5[d]	26.2[d]	328, 286, 175, 135, 81, *43*, 41
VII	304	0.23 (pink)	23.5	26.2	286, 251, 175, 151, 138, 135, *41*
III	304	0.16 (greyish blue)	24.0	26.7	286, 251, 175, 161, 151, 135, *41*
IX	362	0.14 (deep purple)	24.5	27.4	302, 284, 256, 151, 135, *43*
X	404	0.42 (greenish blue)	27.1	28.8	386, 344, 284, 159, 135, *43*
XI	402	0.56 (violet)	27.1	29.0	342, 282, 267, 254, 239, *43*
XII	446	0.52 (orange)	~29	31.0	344, 284, 159, 135, *43*

[a] 5% ethyl acetate–dichloromethane (v/v), TLC on silica gel G (stained by vanillin).
[b] Relative retention times; 6 ft × 3 mm 3% OV-1 on 100/120 Gas Chrom Q, 5 ft × 3 mm 3% OV-17 on 100/120 Gas Chrom Q.
[c] Major mass fragments with base peaks in italics.
[d] Compound decomposed in the GC column.

TABLE 4. RELATIVE AMOUNTS OF DEFENSIVE SECRETION OF *Hospitalitermes umbrinus*

Colony	Termite location	Monoterpenes (μg)	Diterpenes (μg)	Diterpenes–Monoterpenes	Secretion (%)[a]
I	Sungei Menyala Forest Reserve	16.0	0.5	0.03	0.8
IIA	Gombak Forest Reserve	7.6	23	3.0	1.3
IIB[b]	Gombak Forest Reserve	10.6–15.8	0.5–0.52	0.03–0.05	0.8
IIIA[b]	Pasoh Forest Reserve	6.3–7.6	15.5–23.0	2.5–3.0	1.2
IIIB[b]	Pasoh Forest Reserve	6.3–7.6	0.2–0.3	0.03–0.05	0.5
IV	Fraser's Hill	16.2	0.5	0.03	0.4

[a]Percent of body weight of fresh termite soldier.
[b]Range for three colonies.

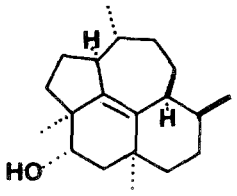
$C_{22}H_{32}O_2$ ($M^+ - H_2O$) and $C_{20}H_{30}O_1$ ($M^+ - 60$): 328.244 and 286.229 respectively. Found: 328.249 and 286.230, respectively. Silation of VI (1 mg, 20 μl HMDS, 20 μl TMCS, and 60 μl pyridine, 100°C, 8 hr) gave the silyl derivative XIII which had mass spectrum m/e 418 (1, M^+), 358 (15, $M^+ - AcOH$), 343 (5, $M^+ - AcOH - CH_3$), 196 (13, $Me_3Si^+OCH{=}CMeCH_2CH_2CH{=}C{=}CH_2$), 143 (9, $C_7H_{15}OSi^+$), 133 (5, $C_{10}H_{13}^+$), 119 (6, $C_9H_{11}^+$), 105 (5, $C_8H_9^+$), 91 (6, $C_7H_7^+$), 73 (100%, Me_3Si^+), 55 (10, $C_4H_7^+$), and 41 (11, $C_3H_5^+$). Calculated for $C_{25}H_{42}O_3Si$: 418.290. Found: 418.281.

Monohydroxyditerpenes (I–V) were identified by their [^{1}H]NMR and mass spectra as well as by comparison of their retention times with those previously identified (Prestwich, 1979c). Di- and tri-acetoxyditerpene derivatives (X–XII) were identified by their mass spectra and compared to those reported (Vrkoc, 1978).

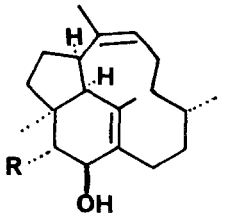
RESULTS AND DISCUSSION

Hospitalitermes umbrinus colonies were collected in Malaysian jungles over a fairly widespread region near Kuala Lumpur, Malaysia. The chemical secretions were analyzed for monoterpenes and diterpenes, and the results are summarized in Tables 1 and 2. Pinenes (usually α-pinene) constituted the major monoterpenes, but limonene was also present as a minor component. Traces of camphene, carene, α-terpinene, and terpinolene were also found. Volatile monoterpenes have been demonstrated to have irritant and repellent properties (Eisner, 1976) or even mild toxic effects (Howse, 1975) on predators. It has also been suggested that they serve as alarm chemicals whereby soldiers alert one another to converge at the site of danger (Eisner, 1976). *H. umbrinus* soldiers were observed to be agitated by vapors of pinenes, limonene, or myrcene.

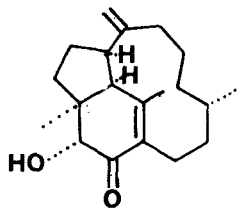
Diterpenes were also found to be an important part of the defensive secretion. Most of the diterpenoids identified were trinervitene derivatives (II–XII), except for a small amount of rippertenol (I) detected in three of the colonies (Figure 1 and Table 2). Rippertenol, a methyl-shifted derivative in the biogenetic cyclization of a trinervitene structure, has also been detected in *N. rippertii (Prestwich, 1979b), Grallatotermes* (Prestwich, 1979c), and *Longipeditermes* (Goh et al., unpublished). Defense secretions based predominantly on trinervitanes are also found in two other constricted head genera, *Grallatotermes* and *Lacessititermes*, both of which are closely related to *Hospitalitermes*. However, in *Grallatotermes africanus*, a greater amount of the methyl-shifted rippertenol was found. The secretions of *Hospitalitermes umbrinus* were observed having GC profiles that were distinguishable from those of *Lacessititermes* (Goh et al., unpublished) which is a genus that is morphologically very similar. Trinervitene monoalcohols II, IV, and V have



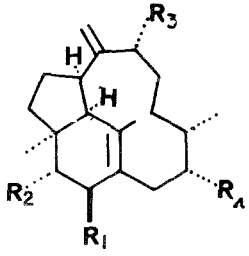
I



II R=H
III R=OH



IV



V R_1=H, R_2=H, R_3=OH, R_4=H
VI R_1=OAc, R_2=OH, R_3=H, R_4=H
VII R_1=OH, R_2=OH, R_3=H, R_4=H
VIII R_1=OH, R_2=OAc, R_3=H, R_4=H
IX R_1=OH, R_2=OH, R_3=OAc, R_4=H
X R_1=OAc, R_2=OAc, R_3=OH, R_4=H
XI R_1=OAc, R_2=OAc, R_3=H, R_4=O
XII R_1=OAc, R_2=OAc, R_3=OAc, R_4=H
XIII R_1=OAc, R_2=OSi(Me)$_3$, R_3=H, R_4=H

FIG. 1.

been observed before in *Trinervitermes* (Prestwich, 1978) as have the compounds III and VII. The diol monoacetate VI, which was secreted in relatively large quantities in *H. umbrinus*, was identified on the basis of its proton NMR and mass spectral data. The C-2 proton appeared as a broad 9-Hz doublet at δ 5.37 and hydrolysis of VI by methanolic sodium hydroxide gave the known 2β,3α-dihydroxy-1(15),8(19)-trinervitadiene (VII). The EI-mass spectrum of VI did not give the molecular ion but gave the M-AcOH ion. However, the trimethylsilyl derivative XIII gave the correct molecular ion and an expected fragmentation pattern, including a retro Diels-Alder fragment $[Me_3SiOCH{=}CMeCH_2CH_2CH{=}C{=}CH_2]^+$ at *m/e* 196. The other isomer of VI, the 3-*O*-acetate VIII, was isolated by Vrkoc (1978) from *N. costalis*. Trinervitene diacetate derivatives IX–XII appeared as minor components and were detected by GCMS.

The compositions of the diterpenoid fractions from the various colonies collected were analyzed by TLC, GC, and GC-MS, and the results are

summarized in Tables 1-4. Diol VII and the corresponding monoacetate VI represent the major diterpene components of the defensive secretion and the composition appears to be fairly consistent for all the colonies. Diol VII appears to be a fairly common, gluey component found in soldier secretions of the Nasutitermitinae. Diterpenes are considered to constitute a sticky base to retard the evaporation of the monoterpenes and, in addition, they function to enmesh the assailant. They could also serve as an irritant glue and induce scratching and other cleaning reflexes almost as soon as the secretion has been sprayed. However, the toxicity of diterpenes is still in doubt (Eisner, 1976).

Surprisingly, it was observed that the relative proportions of diterpenes and monoterpenes varied considerably between the different colonies studied. As shown in Table 4 the diterpenediol–monoterpene ratios obtained for the different colonies appear to fall in two broad classes being either small (ca. 0.03–0.05) or "normal" (ca. 2.5–3.0). The termite samples were collected from a fairly wide area, and allopatric or sympatric populations also showed this same dichotomy (Table 4). We do not as yet know the reason or reasons for this result, but there are many possible explanations. One possibility is that the ratio reflects the condition or state of health of a colony, and the termites are using terpenoids with maximum effectiveness. Secretions with low diterpenediol content (i.e., relatively larger content of monoterpene) would mean that the secretion would be very volatile, as there is little diterpene solute to hinder evaporation of the pinenes. Thus alarm would be triggered instantly. Preliminary observations also bear out this point in that colonies of the termites that secrete low diterpene content tend to scramble and flee to the safety of the nests after the soldier termites begin spraying. On the other hand, termite colonies with secretions containing more diterpenes have a greater tendency to "stand and fight" after being provoked to spray.

Alternatively, it is possible that colonies with a reduced soldier-to-worker ratio would have a lesser tendency to engage in combat and therefore would secrete less diterpene while relying upon the volatile monoterpenes for alarm. Yet another possibility could be that the mono-to-diterpene ratio functions in a pheromonal role to control changes in the caste ratio in the colony. It is hoped that some of these questions will be answered by further investigations currently being pursued.

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ATTRACTANCY TO *Oryzaephilus surinamensis* (L.), SAW-TOOTHED GRAIN BEETLE, OF EXTRACTS OF CAROBS, SOME TRIGLYCERIDES, AND RELATED COMPOUNDS

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Abstract—A laboratory method has been devised for testing the attractancy of vapors to adult *Oryzaephilus surinamensis* (L.). Of four solvent extracts prepared from pods of the carob tree [*Ceratonia siliqua* (L.)], all attracted *O. surinamensis*, but the least polar extract was the most active. Subdivision of extracts yielded a series of fractions, the most attractive of which contained a mixture of triglycerides with three or more double bonds per molecule. Twelve authentic triglycerides, either synthesized or purchased, elicited responses ranging from high to very low attractancy in a way which appeared to be related to the number, position, and geometrical shape of the double bonds in the molecules. By studying the response of the insects to five glyceride acids, three aldehydes, and three volatile fatty acids, it has been shown that it may be possible to explain the attractancy attributed to the triglycerides, which are involatile, in terms of the response to their volatile breakdown products.

Key Words—*Oryzaephilus surinamensis*, saw-toothed grain beelte Coleoptera, Silvanidae, attractant, carobs, *Ceratonia siliqua*, triglycerides, breakdown, volatiles.

INTRODUCTION

Beroza (1972) noted that insect attractants could be extremely useful in monitoring agricultural field pests, and subsequently Lewis (1978) remarked that such monitoring had become routine practice in North America and Europe. Whereas sex pheromones have been used to monitor populations of

moths and some beetles infesting products stored in warehouses, grain stores, and ships' holds, little practical use has been made of attractant chemicals present in, or derived from, the food of storage species (Levinson, 1974).

A bait bag comprising equal parts by volume of wheat, broken groundnuts (peanuts), and kibbled (crushed) carobs in a plastic mesh envelope, has been developed at this laboratory (Pinniger, 1975). This mixture was chosen because of its superiority over others in trials in storage premises. After being left for periods of two to seven days, bags have been found to contain both adults and larvae of many species of storage beetle, the larvae of four species of storage moth, and also two species of storage mite. The bait bags proved to be especially useful in detecting infestations which would otherwise have remained hidden because of the cryptic behavior typical of these pests. The relationship between the numbers of insects found in the bags and the numbers visible elsewhere suggested that they might contain insect attractants.

Our aims were to identify any attractant chemical in the bait bags. This paper reports preliminary studies which were confined to the rapid laboratory screening of materials derived from a single bait component, using only one insect species.

Kibbled carobs (locust beans), obtained from the tree *Ceratonia siliqua* (L.) (Leguminosae), were selected because there had been indications that their addition to the bait bags greatly increased the number of insects found (D.B. Pinniger, personal communication). Despite an earlier report of the use of ground carob pods as an insect bait (Gomez Clemente and Del Rivero, 1955) there has been no systematic study of carob constituents which might act as insect attractants.

The saw-toothed grain beelte *Oryzaephilus surinamensis* (L.) was chosen because it is the major insect pest of grain stored in the U.K. (Freeman, 1976) and because large numbers had been found in the bait bags (Pinniger, 1975). Although substances extracted from food materials have elicited attraction, aggregation, and feeding behavior in *Tribolium confusum* (Duv.) (Tamaki et al., 1971a), *Trogoderma* spp. (Spangler, 1965), and *Sitophilus zeamais* (Mots.) (Yamamoto et al., 1976), no similar study had been attempted on *O. surinamensis* until recently. While the current paper was being prepared, Pierce et al. (1981) reported that volatiles isolated from a culture medium of rolled oat flakes and brewer's yeast were attractive to *O. surinamensis* and *Oryzaephilus mercator* (Fauvel), although no details of the chemicals involved were given.

The apparatus used in the current work was similar to that described by Pinniger and Collins (1976). Since there is no contact between the insects and the test material, only vapor-phase effects are assessed. This apparatus does not require an imposed airstream, so it avoids the disadvantages inherent in

the use of most olfactometers (Kennedy, 1977). Moreover Pierce et al. (1981) stated that an airflow olfactometer was unsuitable for assessing the olfactory responses of *Oryzaephilus* spp., and they used a pitfall olfactometer. Although our apparatus has been used to assay putative components of the trail pheromone of *Monomorium pharaonis* (L.) (Edwards and Pinniger, 1978) it has not previously been used in the study of food attractants.

METHODS AND MATERIALS

Carob Extracts. Kibbled pods from Cypriot carob trees were heated to 70° C for 8 hr. After cooling, a portion (100 g) was ground to a fine power which was then successively and exhaustively extracted with hexane, diethyl ether, ethanol, and water (3 × 250 ml each) (Figure 1). Removal of the solvents gave a hexane extract, H (124 mg), an ether extract, E (924 mg), an alcohol extract, A (11.81 g), and a water extract, W (15.29 g). These and all subsequent extracts were stored at 0° C in the dark sealed under nitrogen. In a repeat extraction with hexane, powdered kibbled carobs (100 g) gave an extract, H′ (137 mg), which was identical by NMR spectroscopy with the previous hexane extract, H. A portion of hexane extract H (50 mg) was heated in refluxing hexane (100 ml) for 20 hr. Removal of solvent gave an oil, HR (50 mg), which was identical by NMR spectroscopy with extract H. Kibbled pods (150 g) were heated and ground to a fine powder as before, then extracted with hexane (250 ml) in a Soxhlet apparatus for 20 hr. Removal of solvent gave an oil, X (336 mg), which was identical by NMR spectroscopy to extracts H and H′.

Refined Carob Extracts. Carob extract X (200 mg) was chromatographed on silica gel with mixtures of hexane, diethyl ether, and methanol (Figure 2). Fractions of similar chromatographic mobility were combined to give, in order of elution and increasing polarity, five refined carob extracts, X1 (18 mg), X2 (52 mg), X3 (47 mg), X4 (20 mg), and X5 (54 mg). A repeat separation of X (100 mg), isolating only the fraction equivalent to X2, gave an oil X2′ (24.2 mg), which was identical by IR and NMR spectroscopy with refined carob extract X2. After storage for several months, X2′ (20 mg) was separated by elution with chloroform on a chromatography plate into three components: X2′1 (8 mg) R_f 0.9, X2′2 (5 mg) R_f 0.7, and X2′3 (6 mg) R_f 0.35. TLC comparison with 12 triglycerides in eight solvent systems on silica plates, some impregnated with silver nitrate, showed that X2′2 contained components which cochromatographed with OOO, LLL, PLL, OLL, PγLnP, and PLnP but did not contain POO, PSS, PLP, POP, PLeP, or PLO. (The structure code is as shown in the next section.) TLC comparison of X2′3 with four 1,3-diglycerides (Sigma London Chemical Co. Ltd.) in acetone–hexane (1:3) gave the following R_f values: dipalmitin 0.60, diolein 0.60, dilinolein 0.58, X2′3 0.54, and di-9,12,15-linolenin 0.52.

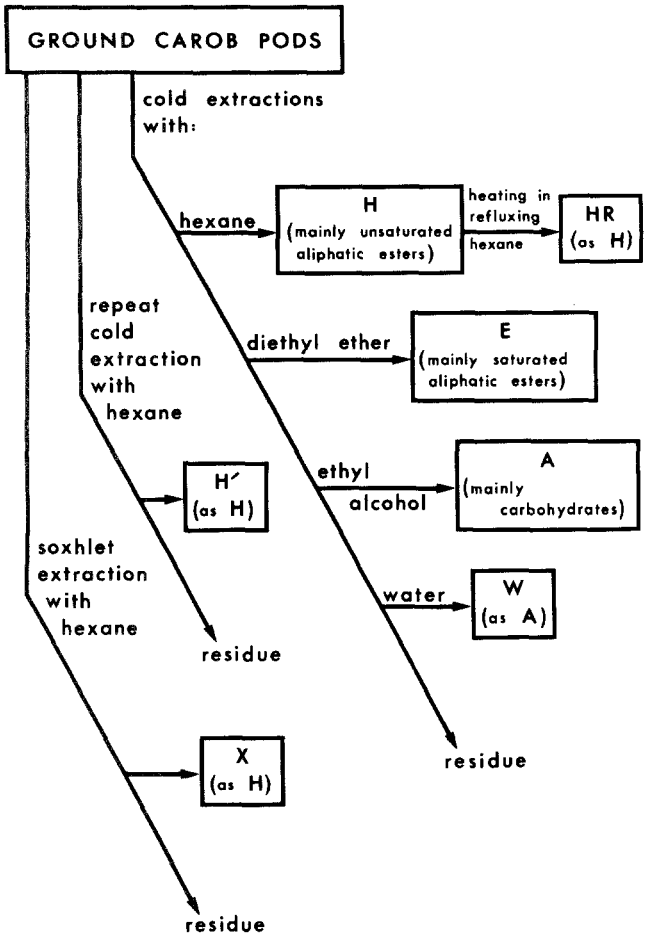

FIG. 1. Carob extracts (with spectroscopic assignments). Key: H = extracted with hexane, E = extracted with diethyl ether, A = extracted with ethyl alcohol, W = extracted with water, R = heated in refluxing solvent, X = hexane soxhlet extract, ' = repeat.

Triglycerides, Glyceride Acids, Aldehydes, and Volatile Fatty Acids. Where possible, commercially available materials were used (Aldrich Chemical Co. Ltd., B.D.H. Chemicals Ltd., or Sigma London Chemical Co. Ltd.). Those triglycerides which were not readily obtainable were synthesized by standard methods similar to those outlined by Mattson and Volpenhein (1962). The triglyceride structure code is: p = palmitoyl, C 16:0; S = stearoyl, C 18:0; O = oleoyl, C 18:1 *cis* Δ^9; L = linoleoyl, C 18:2 *cis* $\Delta^{9,12}$; Le = linoelaidoyl, C 18:2 *trans* $\Delta^{9,12}$; Ln = linolenoyl, C 18:3 *cis* $\Delta^{9,12,15}$; γ-Ln = γ-linolenoyl, C 18:3 *cis* $\Delta^{6,9,12}$.

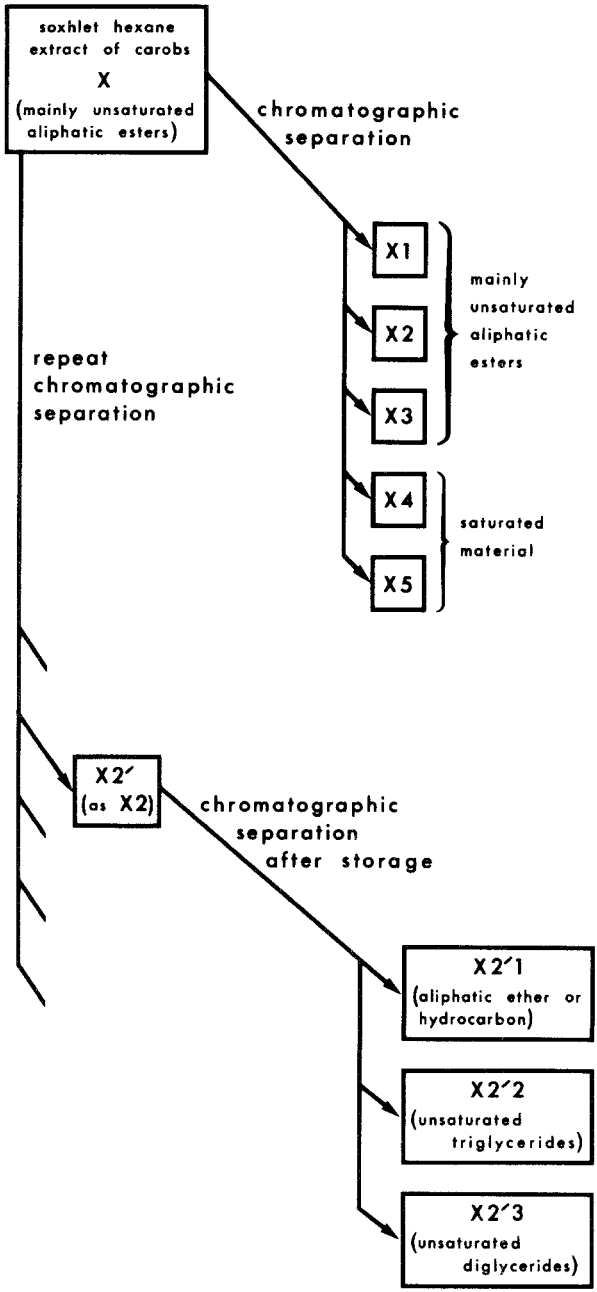

FIG. 2. Refined carob extracts (with spectroscopic assignments). Key: X = hexane soxhlet extract, ′ = repeat; numerals are chromatographic fraction numbers.

Full details of the extractions and syntheses together with chromatographic and spectroscopic data for all the materials tested are contained in a separate report available from one of the authors (J.C.).

General Bioassay Method. The chemicals were assayed against unsexed adult *O. surinamensis* of an insecticide-susceptible strain. The insects were bred at 25° C, 70% relative humidity in constant darkness on a food mixture of rolled oats, whole wheat flour, and powdered yeast in the ratio 5:5:1 by weight. All the bioassays were carried out using apparatus based on the VLDIC insect activity detector described by Pinniger and Collins (1976) and by Edwards and Pinniger (1978). This detector makes use of an overhead light source, giving a light intensity of 350 lux at test arena level, and light-dependent resistors (LDRs), which are triggered when an insect crosses a target area above an LDR in a test arena. The apparatus counts the number of times the insects cross such a target area.

Carob Extracts Bioassay. Insects of adult age between 0 and 3 weeks were removed from an 8-week-old culture, put in a subculture jar containing the food mixture, and kept at 20° C, 70% relative humidity for at least 24 hr prior to each test. One hour before each test began, 10 insects were placed in the test arena.

A section of a test arena is shown in Figure 3. To achieve the necessary sensitivity, the LDRs were masked to leave exposed a central circular area 1 mm in diameter. The aluminium ring, 95 mm inner diameter and 12 mm high, was coated on its inner surface with fluon (an aqueous suspension of polytetrafluoroethylene) which confined the insects to the filter paper. The glass plate, filter paper, and aluminum ring were positioned so that the centers of the LDRs, which were 40 mm apart, lay on a diameter of the aluminium ring. The arena lid comprised an inverted Petri dish base, 120 mm × 14 mm,

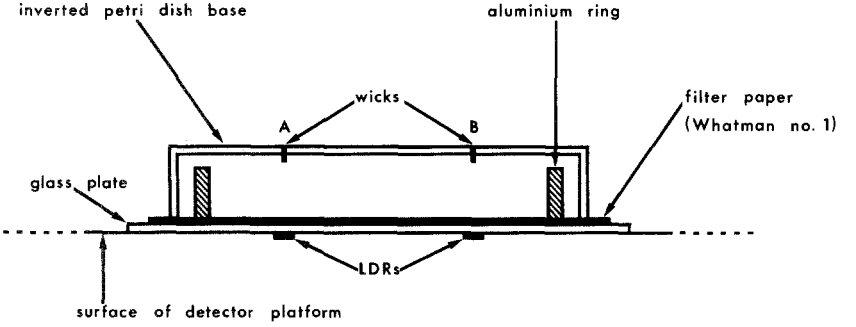

FIG. 3. Section through assay arena for the carob extracts, the refined carob extracts, and the triglycerides. For the glyceride acids, the aldehydes, and the volatile fatty acids, the two LDRs and the two wicks were replaced with a single central LDR and a single central quarter wick as described in the text.

which had two 1.5-mm-diam holes drilled through it, 40 mm apart on a diameter of the dish. A 5-mm length of wick, 2 mm in diameter, was fitted into each hole in the lid. The porous polyethylene wicks used were obtained from Schleicher and Schuell (Keene, New Hampshire), and were of two porosities, one suitable for aqueous and the other for organic solvents.

After the insects had been placed within the aluminium ring, the lid was positioned so that the two wicks were immediately above the target areas. The light was turned on and the insects were allowed to settle for 1 hr. The sensitivity was then adjusted so that an insect crossing a target area registered as one crossing on the digital display. The test was then started, and the numbers of crossings over each target area were noted during two successive 10-min intervals. Immediately after the last of these readings, 500 μg of the candidate attractant dissolved in 5 μl of the solvent used to extract the material from the carobs was introduced onto the top of wick A using a 10-μl microsyringe. Similarly, 5 μl of the solvent alone was introduced onto wick B. The sensitivity was readjusted as necessary, and then the numbers of crossings under each wick were recorded over a further two consecutive 10-min periods. The insects, filter paper, and both wicks were discarded after the test, and the other components of the arena were thoroughly washed before reuse. Each material was tested in this way four times.

Refined Carob Extract Bioassay. The solvent used in these tests was hexane, except when testing X5, which was insufficiently soluble and for which tetrahydrofuran was used.

Triglyceride Bioassay. The wicks used in these tests were 5-mm lengths of polyporus strip, and the solvent used in all tests was diethyl ether. In addition to testing each chemical 4 times, eight blank tests were carried out when 5 μl of diethyl ether alone was applied to both wicks A and B, four at the beginning of the series of tests on the triglycerides (B) and the other four at the end (B′). Also readings of the numbers of crossings were taken every 10 min for 40 min before and 40 min after the application of material to the wicks.

Glyceride Acid Bioassay. The 10 insects to be used in a test were removed from an 8-week-old culture 24 hr before the test was due to begin, and kept at 25° C, 100% relative humidity for 23 hr. Conditioning at the maximum possible humidity was intended to minimize any stress due to lack of water and thus improve uniformity between tests. The insects were then transferred to the test arena (25° C, 50% relative humidity).

The arena had only one central LDR, and the arena lid had a single, central hole which held the wick. Polyethylene wicks of porosity suitable for organic solvents were used for all the tests. Whole wicks were trimmed to a length of 10 mm, including the knob at one end, and were cut longitudinally into quarters.

After the insects had been placed in the arena, one (quarter) wick was dropped into the hole in the arena lid. The quarter of the knob held the wick in

place. The lid was then positioned over the target area. The insects were allowed to settle for 1 hr, as in previous tests. Thirty minutes before the insects were due to be exposed to the candidate attractant, another (quarter) wick, suspended on a wire support, was treated with the chemical as follows. Diethyl ether (5 μl) containing the test chemical (500 μg) was applied to the lower end of the wick. At the end of the hour allowed for the insects to settle in the arena, the numbers of crossings under the wick were recorded for two periods of 5 min each. Then the wick was replaced with the treated wick. The numbers of crossings under the wick were recorded after a further 50 min. Each chemical was tested in this way four times. In addition, four blank tests were carried out using the same method except that diethyl ether alone was applied to the wick.

Aldehyde and Volatile Fatty Acid Bioassay. Since all these chemicals are liquids at 25° C, the use of a solvent was unnecessary and thus the test chemical (1 μl) was applied to the wick immediately prior to the wick transfer, since it was not necessary to allow time for the solvent to evaporate. In addition, nonanal and propionic acid were tested at 0.1 and 0.02 μl, applied using a 1-μl microsyringe and a microcapillary, respectively. Each chemical was tested four times, and in these series the blank tests used untreated wicks.

RESULTS AND DISCUSSION

Improvements were made to the bioassay technique during these studies but the technique was held constant throughout the tests for any one class of materials so that the results within classes could be compared. Although it might be considered that the amounts of materials applied to the wicks are very high, it must be emphasized that the doses received by the insects are considerably lower because of the low release rate and presumed radial distribution of the vapors. In those tests where the numbers of crossings were high, there was no sign of the response decreasing markedly with time. Where the numbers of crossings were low, all the materials except two (see later) showed no sign of eliciting any visible behavioral response even immediately after introduction. Thus there was no evidence for sensory adaptation or habituation. Since this work was intended as a preliminary screen for a large number of candidate attractants, each material was tested at just one dose (500 μg or its approximate equivalent for organic liquids, 1 μl). The results show that this dose was sufficiently large to elicit responses to some materials, but the points mentioned above confirm that it was only rarely too high. To provide the fullest possible assessment of the results, and to help suggest where changes in the bioassay technique would be advantageous, the results were subjected to detailed scrutiny using analysis of variance methods.

Carob Extracts. The kibbled carob pods used in bait bags are heated before use to kill any insects already present. Thus in the current study the

TABLE 1. MEAN NUMBERS OF CROSSINGS WITH 95% CONFIDENCE LIMITS FOR CAROB EXTRACTS

	Wick A (test material)		Wick B (control)	
Material	Preapplication	Postapplication[a]	Preapplication	Postapplication
X	0.63 ± 0.89	17.88 ± 7.81 a	0.63 ± 0.63	1.75 ± 1.47
HR	4.38 ± 1.68	15.00 ± 4.40 ab	2.13 ± 1.45	5.00 ± 2.06
H	2.63 ± 1.79	10.63 ± 4.86 bc	1.50 ± 0.78	3.63 ± 2.65
H′	3.00 ± 1.74	7.75 ± 3.34 c	3.13 ± 1.52	3.13 ± 1.23
A	2.50 ± 0.90	6.75 ± 6.83 c	3.38 ± 1.55	3.38 ± 2.50
W	3.00 ± 2.80	5.88 ± 2.75 c	3.50 ± 1.49	2.00 ± 1.49
E	2.25 ± 0.75	4.38 ± 2.09 c	1.38 ± 1.09	3.25 ± 2.09

[a]Values followed by the same letter are not significantly different ($P \leq 0.05$).

carob pods were similarly heated. After grinding, the exhaustive sequential solvent extraction gave extracts H (0.1% by weight), E (0.9%), A (12%), and W (15%) as shown in Figure 1, and the bioassay results are presented in Table 1. Neither here, nor in any of the other results obtained in the current work, was there any obvious relationship between the pre- and postapplication scores for each test. The largest mean number of postapplication crossings on wick A was obtained with the least polar extract (H) and thus this fraction was studied further. The repeat hexane extract (H′) gave reasonably comparable results in the bioassay while the high score for HR showed that heating H in refluxing hexane did not destroy its biological activity. Hence it was possible to extract the carobs in a Soxhlet apparatus and thereby improve the yield. X (0.2% by weight) did not differ from H spectroscopically, but it did give a larger number of crossings.

The data recorded during these tests were subjected to a nested two-factor analysis of variance (ANOVAR) which had been suggested by Dr. R.W. Howe (personal communication). The use of a hierarchal method permitted partitioning of the variance between the insect groups into that between the materials and that within the groups subjected to each material. This was necessary because each group was used only once. The pre-application data were recorded to provide a test of uniformity. This was largely satisfactory despite some indication that there could have been some long-term variation in the behavior of the insects.

After application there is a very highly significant difference ($P \leq 0.001$) between the seven materials using the data from wick A (test material and solvent), while there is no significant difference in the data obtained from wick B (solvent) ($P > 0.05$). Thus there is no advantage to be gained by analyzing anything other than the data for wick A when assessing the differences

between the materials tested. Although the apparatus with two wicks (Figure 3) had to be used for some of the following work, it was possible to obtain all the results needed by considering only the data from wick A. This difference between the wicks confirms that the response of the insects is of a directional nature and that the increase in the number of crossings under wick A is not due to an increase in undirected movement. This was also the case for the other series of tests using arenas with two wicks (refined carob extracts and triglycerides).

The results of a Student–Newman–Keuls test (Sokal and Rohlf, 1969) for ranking the seven materials according to the data obtained from wick A alone are included in Table 1. Although the two hexane extracts H and H′ did give larger mean numbers of crossings than A, E, and W, Table 1 shows that they did not differ from them significantly. The improvement gained by heating a portion of H in refluxing hexane was sufficient to make HR significantly better than H′, A, W, and E, while the Soxhlet hexane extract X was significantly better than all of the other extracts except HR.

Refined Carob Extracts. When a portion of X was refined into five fractions (Figure 2) it was found that the second fraction, X2, gave the largest mean number of crossings (Table 2), closely followed by X1 and X3, both of which had marginally higher means than the two fracitons which contained only saturated material, X4 and X5. None, however, gave such large numbers of crossings as X itself.

X2 and the repeat equivalent extract, X2′, both constituted about 0.05% of the original carob weight. Their spectroscopic and chromatographic properties strongly suggested that they might be derived from glycerides. After storage for several months at 0° C sealed under nitrogen, while reference glycerides were obtained, X2′ was tested against the insects and found to give a far higher mean number of crossings than any other extract.

Statistical analysis of the data for all six extracts listed in Table 2 gave satisfactory results for the preapplication uniformity trials. The postapplica-

TABLE 2. MEAN NUMBERS OF CROSSINGS WITH 95% CONFIDENCE LIMITS FOR REFINED CAROB EXTRACTS

Material	Preapplication	Postapplication
X1	4.13 ± 2.99	6.00 ± 3.36
X2	2.50 ± 2.06	7.63 ± 5.42
X3	4.50 ± 3.99	5.00 ± 3.81
X4	1.63 ± 1.42	4.00 ± 2.20
X5	2.63 ± 2.24	2.50 ± 2.25
X2′	0.50 ± 0.64	27.50 ± 14.35

tion data gave variance ratios which were not significant, apart from that for the difference between the materials. This was significant at only the $P \leqslant 0.05$ level, and thus no attempt was made to rank the different refined extracts.

Subsequent chromatographic analysis showed that despite the careful storage, X2′ had degraded and contained three components X2′1, X2′2, and X2′3. Regrettably the small amounts of material available did not permit a full bioassay, but it seemed that some activity resided in X2′2, with less in X2′3 and none in X2′1. Confirmation that X2′2 contained glyceride material was obtained from the NMR spectrum. Mass spectral analysis showed fragments which confirmed the presence of palmitoyl, stearoyl, oleoyl, linoleoyl, and linolenoyl and/or γ-linolenoyl residues. Comparison by TLC with the 12 authentic triglycerides showed that X2′2 consisted of at least four components which were not separated in eight solvent systems from triglycerides with three or more double bonds per molecule. Similarly X2′3 seemed to consist of one or more diglycerides. X2′1 appeared to be an aliphatic ether or hydrocarbon.

Although these results show that carob pods contain glyceride-based materials which are attractive to *O. surinamensis*, there is no published information on the identity of the glycerides present in carobs. In view of the chemical complexity of the refined carob extracts and the limited amounts available, it was not felt worthwhile to pursue their chemical analysis. As a resonable alternative attention was turned to the behavioral effects of the 12 authentic triglycerides. Although relatively involatile, triglycerides were selected for further study because of the firm link which had been established between them and a behavioral response in the bioassay apparatus.

Triglycerides. Certain unsaturated triglycerides can elicit aggregation behavior from adult *T. confusum* (Starratt and Loschiavo, 1971, 1972; Tamaki et al., 1971a,b), attract adult *Sitophilus granarius* (L.) (Nawrot and Czaplicki, 1978), and exert an arrestant effect on larvae of *Sitotroga cerealella* (Oliver) (Chippendale and Mann, 1972). There are, however, no reports on the effect of triglycerides on the behavior of *O. surinamensis*.

The 12 authentic triglycerides used were examples from a wide range of patterns and degrees of unsaturation. The mean numbers of crossings are presented in Table 3. As was the case for the carob extracts, in the preapplication data there was some suggestion of a long-term variation in the behavior of the different insect groups. Nevertheless, again there was no apparent relationship between the pre- and postapplication scores, suggesting that the postapplication difference between the scores from the insect groups, which was very highly significant ($P \leqslant 0.001$) was indeed due to the materials tested rather than the insects themselves.

The results of the ranking test are included in Table 3. There was no significant difference between the ether tests carried out before (B) and after

TABLE 3. MEAN NUMBERS OF CROSSINGS WITH 95% CONFIDENCE LIMITS FOR TRIGLYCERIDES

Material[a]	Preapplication	Postapplication[b]
PγLnP	2.88 ± 1.18	16.31 ± 3.13 a
LLL	3.69 ± 1.12	10.56 ± 4.31 b
PLP	2.63 ± 0.84	10.19 ± 2.85 b
PLL	2.44 ± 0.75	8.44 ± 2.88 bc
POP	3.50 ± 1.08	6.81 ± 2.18 cd
OLL	2.63 ± 0.97	6.19 ± 2.49 cd
POO	3.44 ± 0.89	5.06 ± 1.39 cde
PLnP	1.81 ± 0.70	4.38 ± 1.30 de
PLO	2.25 ± 0.76	4.06 ± 1.22 de
B′	2.94 ± 0.81	3.81 ± 1.26 de
PLeP	2.13 ± 0.75	3.50 ± 1.56 de
OOO	2.31 ± 1.00	3.38 ± 1.11 de
B	1.88 ± 1.25	1.88 ± 0.98 e
PSS	1.63 ± 0.86	1.56 ± 0.70 e

[a] B and B′ refer to the diethyl ether blank tests, the remaining abbreviations refer to triglycerides by the standard code (see Methods and Materials).
[b] Values followed by the same letter are not significantly different ($P \leq 0.05$).

(B′) the triglyceride tests. It would seem then that no unexpected changes in response occurred over the relatively long period required to test so many materials, confirming that the triglyceride results can be compared. Several triglycerides did not differ significantly from B and B′; those which did differ significantly (PγLnP, LLL, PLP, and PLL) contained either γ-linolenoyl or linoleoyl residues, although the converse was not always true. Geometrical isomerism seemed to be important since PLeP, the isomer of PLP in which the two double bonds are *trans* rather than *cis*, did not differ significantly from B and B′. Moreover the location of the double bonds was also relevant: there was a very large difference between PLnP ($\Delta^{9,12,15}$), which did not differ significantly from B and B′, and PγLnP ($\Delta^{6,9,12}$) which was significantly better than every other triglyceride tested. The only totally saturated triglyceride tested (PSS) gave the lowest scores of all.

Implications of the Results. In considering the results from all the materials presented so far, there are two recurring themes. First, certain unsaturated triglycerides, whether derived from carob pods, synthesized, or purchased, are capable of attracting *O. surinamensis*. Second, the attractive effect appears to be improved by either heat (compare H with HR and X) or storage (compare X2 with X2′). Triglycerides are essentially involatile, yet the effect is observed in an apparatus where the insects cannot touch the wicks. These points suggest that the attractive effect is caused not by the triglycerides

themselves, but by volatile degradation products derived from them. A similar conclusion has been reached recently by Nara et al. (1981), who believe that the aggregation effect of wheatgerm oil on *Trogoderma glabrum* (Herbst) larvae is elicited by a volatile fraction of (presumably) lipid degradation products. Similarly, Levinson and Kanaujia (1981) hypothesize that the ability of wheat to exert an arrestant effect on *S. granarius* may be related to the formation during storage of long-chain (C_{16}–C_{18}) fatty acids. There is, however, no literature on either lipid breakdown in or formation of volatiles by carobs.

Lipid degradation is often complex, usually involving breakdown of either the glyceride ester itself, or of the glyceride acid formed by prior hydrolysis, by oxidation at the unsaturated bonds to form hydroperoxides (Keeney, 1962). These cleave to volatile pungent carbonyl compounds. For example, the major most volatile autoxidation products of oleic, linoleic, and linolenic acids are nonanal, hexanal, and propanal, respectively (Hoffmann, 1962). These aldehydes can further autoxidize giving the corresponding volatile fatty acids.

Levinson and Levinson (1978) reviewed the effects of some individual lipid degradation products on the behavior of certain stored-product insects. In general the glyceride acids (C_{16}–C_{18}) seem to be aggregants for *S. granarius* or phagostimulants for *Dermestes maculatus* (Deg.) while the volatile fatty acids (C_5–C_{11}) are repellents for *Tribolium castaneum* (Herbst.), *D. maculatus*, and *Trogoderma granarium* (Everts). In contrast, *S. zeamais* shows little response to glyceride acids but is attracted by some of the volatile fatty acids, especially hexanoic acid (Yamamoto et al. 1977). There is, however, no information on the effect of any of these possible lipid degradation products on *O. surinamensis*.

As a preliminary investigation in this complex area, it was decided to test a few compounds which are likely to be formed in the degradation of carobs. It was hoped that this would elucidate the effects of the various carob extracts and triglycerides on *O. surinamensis*. The compounds chosen were five glyceride acids, the three aldehydes likely to be their most important oxidation products, and the three corresponding volatile fatty acids (Figure 4).

Glyceride Acids, Aldehydes, and Volatile Fatty Acids. Since having two wicks brought no advantage in the interpretation of the results, the bioassay tests for all these materials were carried out in the single wick arena. In the hope of reducing the slight variation in behavior observed earlier between the groups of insects prior to the application of the carob extracts or the triglycerides, a conditioning period of 23 hr was introduced into the bioassay technique. In none of the current bioassay tests was there any apparent difference between the insect groups before application, although the

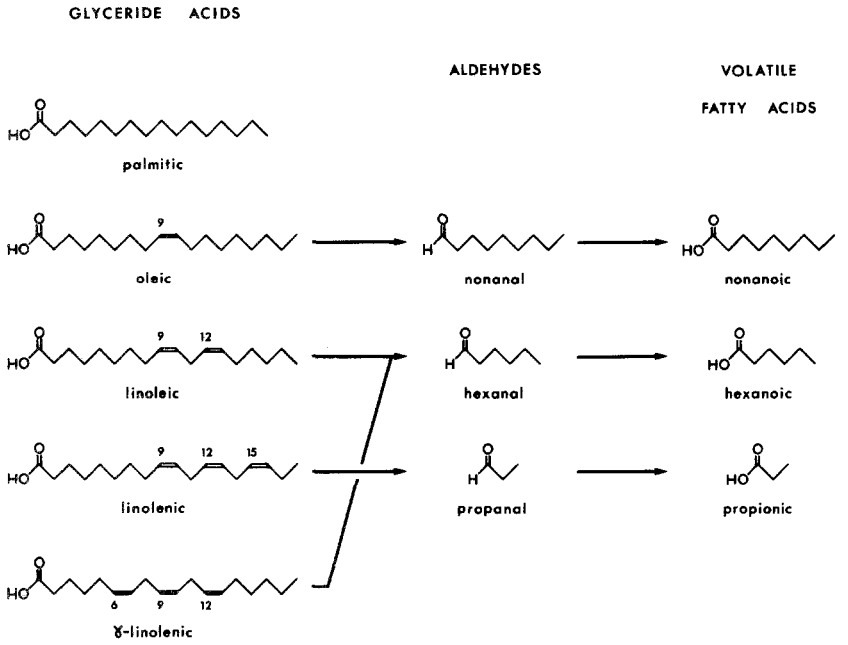

FIG. 4. The five glyceride acids tested and a partial scheme for their oxidative degradation.

numbers of crossings were very low because of the short preapplication recording period and were not subjected to statistical analysis. The effects of the glyceride acids seemed to take time to be observable, so in all the current tests the post application test period was extended to 50 min. The mean numbers of crossings are shown in Table 4. Recording of the results over a single period permitted simplification of the statistical analysis to a one-factor ANOVAR (Sokal and Rohlf, 1969). The differences between the materials were very highly significant ($P \leqslant 0.001$) in all three series.

The results of a Student–Newman–Keuls ranking test (Sokal and Rohlf, 1969) for the glyceride acids are shown in Table 4. Linoleic and γ-linolenic acids were significantly ($P \leqslant 0.05$) more attractive than oleic and linolenic acids which, although moderately attractive subjectively, were not significantly different from either palmitic acid or the diethyl ether blanks, which were virtually without effect. It is interesting that this order is similar to that observed earlier where the same acid residues are esterified in the form of triglycerides (Table 3). This lends more support to the theory that the attractancy of the triglycerides is due to the glyceride acids from which they are composed or to products of further breakdown.

Propanal was found to be highly attractive to *O. surinamensis*, signifi-

TABLE 4. MEAN NUMBERS OF CROSSINGS WITH 95% CONFIDENCE LIMITS FOR GLYCERIDE ACIDS, ALDEHYDES, AND VOLATILE FATTY ACIDS

Series	Material	Postapplication[a]
Glyceride acids	Linoleic	66.75 ± 62.90 a
	γ-Linolenic	62.25 ± 23.38 a
	Oleic	30.50 ± 22.51 b
	Linolenic	22.75 ± 8.94 b
	Palmitic	4.00 ± 3.18 b
	Blank	0.75 ± 1.52 b
Aldehydes	Propanal	82.25 ± 48.15 a′
	Hexanal	52.25 ± 16.39 b′
	Nonanal	5.25 ± 4.92 c′
	Blank	5.00 ± 4.68 c′
Volatile fatty acids	Nonanoic	84.00 ± 16.06 a″
	Hexanoic	25.50 ± 18.01 b″
	Blank	11.00 ± 6.87 c″
	Propionic	5.00 ± 5.03 c″

[a] Values followed by the same letter are not significantly different (for significance levels see text).

cantly more so ($P \leqslant 0.05$) than hexanal, which was moderately attractive. Hexanal was significantly better ($P \leqslant 0.01$) than nonanal which did not differ significantly from the blanks (Table 4). The three corresponding volatile fatty acids showed the opposite order of attractancy. Nonanoic acid was highly attractive and significantly different ($P \leqslant 0.01$) from hexanoic acid, which was moderately attractive and significantly different ($P \leqslant 0.05$) from propionic acid, which did not differ significantly from the blanks.

As mentioned earlier, all the materials studied in this work have been tested at just one dose. It would be dangerous to generalize from these limited results. For example, propionic acid and nonanal caused few crossings and so might be considered to be inactive. However, when either material was tested, the insects raised their antennae and started moving around. If they approached within about 1 cm of the wick, they would turn sharply and run away. This may have been because the dose was too high. At lower doses (0.1 and 0.02 μl) propionic acid had little observable effect (6.00 ± 3.89 and 1.00 ± 1.83 crossings respectively) whereas nonanal was attractive (39.75 ± 22.16 and 59.75 ± 8.55). These two were the only compounds which gave low numbers of crossings at 1 μl yet appeared to elicit some behavioral response. Therefore there is no need to test at lower doses the other compounds which gave low numbers of crossings at 1 μl.

These points show that it would be unwise to conclude anything about the relative attractancy of the different breakdown products tested without a full study of each over a range of doses. However, this work does show that

some triglyceride breakdown products can attract *O. surinamensis* and may be responsible for the effects which triglycerides appear to have on the insects. It follows that it would be very difficult to rationalize the behavioral effects on *O. surinamensis* which result from application of the different triglycerides without knowing the composition of the associated vapor and the action of each constituent odor. Moreover, although the triglyceride PSS and palmitic acid were not attractive when tested individually, it has been pointed out that saturated materials such as these can autoxidize in the presence of unsaturated compounds giving many chemicals which may be important contributors to aroma (Brodnitz, 1968). Thus in a study of this type, the refining of mixtures and the testing of pure materials could give rise to misleading results.

Although the apparent attractancy of triglycerides and glyceride acids containing linoleoyl or γ-linolenoyl residues may be because these seem to be essential acid residues in the diet for insects (Downer, 1978), it should not be overlooked that the suitability of foodstuffs for *O. surinamensis* is largely determined by the presence of carbohydrates (Levinson and Levinson, 1978), that carobs are rich in sugars, and that warming sugars can generate many important aroma compounds (Oldfield et al., 1980). For these reasons we are now analyzing the headspace vapor above carobs. Early results indicate the presence of a large amount of hexanoic acid (an ultimate oxidation product of linoleoyl and γ-linolenoyl acid residues) and lesser amounts of furfuraldehyde (a degradation product of some sugars) (J.P.G. Wilkins, personal communication).

CONCLUSIONS

The bioassay test method proved to be easy to use even with insects as small as adult *O. surinamensis* (about 3 × 1 mm). Since there was no contact between the insects and the wicks, the influence of the test material was confined to vapor-phase effects. Kennedy (1978) distinguishes between vapor-phase effects which result in undirected movement (a special case of which is termed arrestancy) and directed movement (attractancy). In those tests which were conducted using arenas with two target areas, the results demonstrate conclusively that the response of *O. surinamensis* to our test material was of a directed nature. Our subjective observations led us to believe that this was also the case during later tests with a single target area. After application, the differences between the insect groups treated with the various materials were generally very highly significant. The behavioral response appeared to persist throughout the duration of each test suggesting that, despite its small size, the air-space above the arena was not saturated. We feel that this laboratory method provides a useful preliminary screen for attractant chemicals which may be responsible for the effectiveness of the bait bags in storage premises. The present work has shown that glyceride-based material found in the bait

bags can elicit attractancy. It may be possible to explain this response in terms of the effects of the glyceride breakdown products.

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DETERMINATION OF THE VAPOR PRESSURES OF MOTH SEX PHEROMONE COMPONENTS BY A GAS CHROMATOGRAPHIC METHOD

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Abstract—The vapor pressures of decyl acetate, five decenyl acetate isomers, (*Z*)-7-dodecenyl acetate, and (*Z*)-9-tetradecenyl acetate have been determined at three to six temperatures in the interval 25–45°C by a gas chromatographic method suitable for accurate measurements of the low vapor pressures of moth sex pheromone components at biologically relevant temperatures. The vapor pressure values at 30.5° C are 3.80 Pa for decyl acetate, 4.08–5.40 Pa for the decenyl acetate isomers, 0.562 Pa for (*Z*)-7-dodecenyl acetate, and 0.094 Pa for (*Z*)-9-tetradecenyl acetate. The vapor pressures of the decenyl acetates show a small but significant dependence on the double bond position. Four of the compounds in this study, 10:Ac, *Z*5-10:Ac, *Z*7-12:Ac, and *Z*9-14:Ac have recently been identified as sex pheromone components of the turnip moth, *Agrotis segetum*. Large differences between the mole percentages of the component as found in liquid extracts of female abdominal tips and the corresponding mole percentages in the vapor phase are predicted.

Key Words—Vapor pressure, gas chromatography, sex pheromone, olefinic acetates, turnip moth, *Agrotis segetum*, liquid–vapor equilibrium.

INTRODUCTION

The volatility, measured as the saturated vapor pressure, of pheromone components is an important factor in studies on the physicochemical aspects of insect pheromones. Hirooka and Suwanai (1976) have, for instance, derived an equation which relates the rate of pheromone release by a female moth to the vapor pressure, the diffusion coefficient, and the size and form of

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the pheromone gland. For moth species the pheromone generally is a mixture of several compounds, mainly olefinic acetates with varying chain lengths. In many cases geometrical and/or positional isomers are also present in the pheromone blend (Ritter, 1979). The female moths produce a well-defined ratio of the different compounds and the component ratio has been found to play an important role for optimal attraction of males in the field (Roelofs, 1978). However, when the vapor pressures of the pheromone components differ significantly, the ratio of the components in the vapor phase is different from that on the female gland. The former ratio depends on the relative saturated vapor pressures of the components and their relative mole fractions in the liquid phase (Hirooka and Suwanai, 1978).

In connection with field trapping studies more basic work concerning the release rates of pheromones from controlled release systems are needed (Weatherston, 1981). In such investigations physical data on the volatility relationships should be of great value.

In electrophysiological studies on dose–response relationships, the volatilities of the test compound should be taken into account to make it possible to determine the amount of substance to be used in the experiment.

Although the vapor pressures of the compounds used by insects as sex pheromone components thus enter into many different types of pheromone studies, very few experimental values are available. Hirooka and Suwanai (1978) used a gas saturation method to determine the vapor pressure of a few compounds used as pheromone components by moth species.

In this paper we report on vapor pressures for some pheromone components and related compounds determined by a gas chromatographic method, developed by two of us (Olsson et al., 1976). It has previously been used for several applications (Jönsson et al., 1980; Jönsson and Pscheidl, 1981).

Four of the compounds chosen for this work, decyl, (*Z*)-5-decenyl, (*Z*)-7-dodecenyl and (*Z*)-9-tetradecenyl acetate, have been identified as sex pheromone components of the turnip moth, *Agrotis segetum*, (Tóth et al., 1980; Arn et al., 1980; Löfstedt et al., 1981). To investigate the influence of the position of the double bond on the vapor pressure, (*Z*)-3-, (*Z*)-4-, and (*Z*)-6-decenyl acetate were included in the study. Finally, the vapor pressure of a geometrical isomer, (*E*)-5-decenyl acetate, was determined.

METHODS AND MATERIALS

Principles. The substance under study is used as the stationary liquid phase (SLP) in a gas–liquid chromatographic column. A suitable sample compound, the "probe," is repeatedly injected into the gas chromatograph and the net retention volume V_N is carefully measured. According to basic gas chromatographic theory, the following equation applies:

$$V_N = V_R - V_M = K \cdot V_L \quad (1)$$

Here V_R is the total retention volume, usually calculated as the product of the retention time and the carrier gas flow rate. V_M is the volume of the empty space in the column, V_L is the volume of the SLP, and K is a constant, the partition coefficient. V_M can be measured as the retention volume of methane as K for methane can be neglected under normal conditions.

Due to the evaporation of the SLP, V_L decreases linearly with the total amount of gas, V_{acc}, which has passed the column since the start of the experiment. As is seen from equation (1), this means that V_N also decreases in the same way. An experimental example of such a line is shown in Figure 1. The slope, $-dV_N/dV_{acc}$ is proportional to the evaporation rate and thus to the vapor pressure of the SLP if the carrier gas is saturated with the evaporated

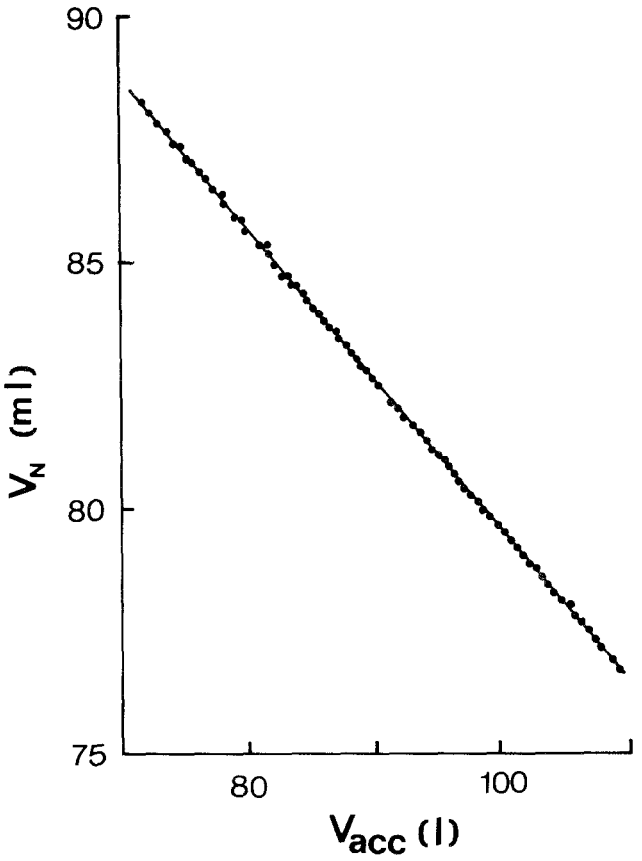

FIG. 1. The retention volume, V_N, of isobutyl acetate on a (Z)-7-dodecenyl acetate column vs. the accumulated carrier gas volume, V_{acc}, at 35.1°C.

substance. This is normally the case, as was shown by Olsson and coworkers (1976) and Olsson and Jönsson (1981).

From a knowledge of the initial weight of the SLP W_L^B and the initial retention volume V_N^B, the vapor pressure p of the SLP can be calculated from the equation (Olsson et al., 1976):

$$p = \frac{dV_N}{dV_{acc}} \cdot \frac{W_L^B}{V_N^B} \cdot \frac{RT}{M} \tag{2}$$

Here, T is the absolute temperature, M is the molecular weight of the SLP, and R is the gas constant. Equations (1) and (2) are valid if contributions from adsorption effects to the retention volume of the probe can be neglected. This can be achieved by selection of a probe which is chemically similar to the SLP.

Chemicals. The following acetates were used in the study: decyl (10:Ac), (*Z*)-3-decenyl (*Z*3–10:Ac), (*Z*)-4-decenyl (*Z*4–10:Ac), (*Z*)-5-decenyl (*Z*5–10:Ac), (*E*)-5-decenyl (*E*5–10:Ac), (*Z*)-6-decenyl (*Z*6–10:Ac), (*Z*)-7-dodecenyl (*Z*7–12:Ac), and (*Z*)-9-tetradecenyl (*Z*9–14:Ac).

The monoolefinic compounds were all synthesized from alkynes and ω-bromoalkyl tetrahydropyranyl ethers. The resulting alkynyl tetrahydropyranyl ethers were hydrogenated over Lindlar catalyst to give the corresponding (*Z*)-alkenyl compounds or were treated with sodium in liquid ammonia to give the (*E*)-alkenyl compound. Finally they were converted to acetates by acetyl chloride in acetic acid.

After purification by argentation chromatography using a cation-exchange resin impregnated with silver nitrate (Houx et al., 1974), the compounds were >99.5% pure except for *Z*9–14:Ac which was about 97% pure.

Experimental Procedure. For each substance, one gas chromatographic column was prepared, according to the method described by Conder and Young (1979, p. 595). The solid support was Chromosorb W-AW-DMCS (Johns-Manville, Denver, Colorado). About 600 mg of packing, containing about 60 mg of the substance was filled into each column. The glass columns were V-shaped and had the dimensions 800 mm × 2 mm ID. The initial amount of liquid was determined by careful weighings during the column preparation procedure.

All retention volume measurements were performed using a high precision gas chromatograph, coupled online with a mini-computer. This system, described in detail elsewhere (Jönsson et al., 1975), permits the direct measurement of retention volumes, without involving the concept of retention time.

The carrier gas was hydrogen in order to minimize the pressure gradient in the column. The flow rate was ca. 30 ml/min. The column volume V_M, was measured by injection of methane (99.95%). As "probe" we used isobutyl

acetate (Eastman, Rochester, New York) which is chemically similar to the substances studied, thus minimizing adsorption.

Vapor samples of the probe were repeatedly injected at specified time intervals under computer control. For each injection the retention volume was automatically calculated by the computer and stored for further computer processing. The evaporation of a substance was traced at each temperature, until the 95% confidence interval for the slope dV_N/dV_{acc} was less than 1% relative. This is of the same magnitude as other sources of error and, consequently, the precision cannot be improved by further measurements. Depending on the vapor pressure, the time needed to reach enough high precision was 5–75 hr. The same column was used for several temperatures as in an earlier work (Jönsson et al., 1980) and the temperatures were run in random order.

RESULTS AND DISCUSSION

In Table 1 and Figures 2 and 3 the results of the vapor pressure measurements are summarized. In the figures, the values of $\ln p$ are plotted vs. the reciprocal temperatures. In a limited temperature interval, a straight line for each substance should be obtained according to Clausius–Claperon's equation:

$$\frac{d \ln p}{d(1/T)} = -\frac{\Delta H_{vap}}{R} \tag{3}$$

In a few cases the measurements were repeated with a new column. Such data are indicated in Table 1 and are not plotted. It can be seen that repeated measurements give values which closely agree with each other. This reflects the good reproducibility in the entire measurement procedure, including the preparation of the column.

In an earlier study (Jönsson et al., 1980) vapor pressures for 1-chloroalkanes were found to agree well with literature data, generally within 5%, and we conclude that the method gives reliable vapor pressure values. It is also a suitable method for the study of moth sex pheromone components as it is possible to accurately measure the low vapor pressures of such compounds at biologically relevant temperatures. Extrapolations of vapor pressure data over large temperature intervals may give grossly erroneous values. The method is best suited for the determination of vapor pressures in the range 0.01–100 Pa.

In Figures 2 and 3 the slopes of the lines for all decyl and decenyl acetates are approximately the same, and the slope increases with increasing chain length. From this slope, the enthalpy of vaporization, ΔH_{vap}, can be calculated according to equation (3).

TABLE 1. VAPOR PRESSURES (p) OF SUBSTANCES STUDIED[a]

Substance	Temperature (°C)	p (Pa)[b]
10:Ac	25.9	2.48 ± 0.01
	26.1	2.52 ± 0.04[c]
	30.4	3.80 ± 0.05
	34.8	5.66 ± 0.03
	39.4	8.86 ± 0.03
	39.5	8.70 ± 0.11[c]
Z3-10:Ac	26.0	3.02 ± 0.04
	30.4	4.65 ± 0.04
	34.8	6.76 ± 0.07
	39.4	10.40 ± 0.11
Z4-10:Ac	34.9	8.26 ± 0.06
	34.9	7.95 ± 0.06[c]
	39.4	12.12 ± 0.09
Z5-10:Ac	26.2	2.93 ± 0.04
	30.6	4.65 ± 0.04
	35.1	6.91 ± 0.06
	39.6	10.13 ± 0.11
E5-10:Ac	26.0	2.84 ± 0.02
	30.4	4.08 ± 0.04
	34.9	6.44 ± 0.08
	39.3	9.58 ± 0.10
Z6-10:Ac	25.9	2.91 ± 0.03
	30.4	4.38 ± 0.03
	34.8	6.83 ± 0.06
	39.4	10.08 ± 0.06
Z7-12:Ac	30.5	0.562 ± 0.005
	34.8	0.83 ± 0.01
	35.1	0.89 ± 0.01[c]
	39.2	1.33 ± 0.01
	44.0	2.01 ± 0.02
	44.1	2.07 ± 0.01[c]
Z9-14:Ac	30.5	0.094 ± 0.001
	35.0	0.163 ± 0.001
	39.5	0.267 ± 0.002
	44.0	0.428 ± 0.004

[a]The uncertainty is given as a 95% confidence interval. The abbreviations of the names of the substances are explained in the text under Chemicals.
[b]1 Pa = 0.987×10^{-5} atm = 7.50×10^{-3} mm Hg.
[c]New, independent measurement. See text.

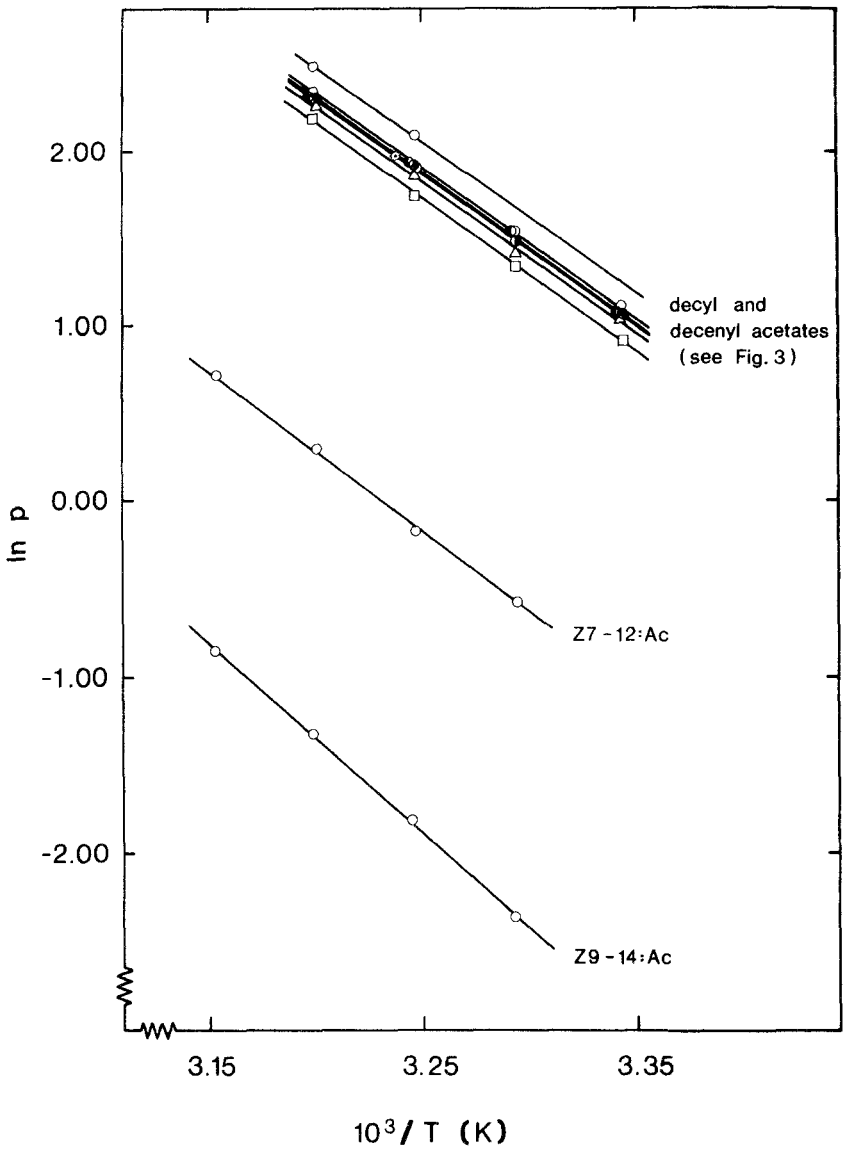

FIG. 2. The logarithm of the vapor pressure for pheromone substances vs. the reciprocal temperature. The abbreviations for the names of the substances are explained in the text under Chemicals.

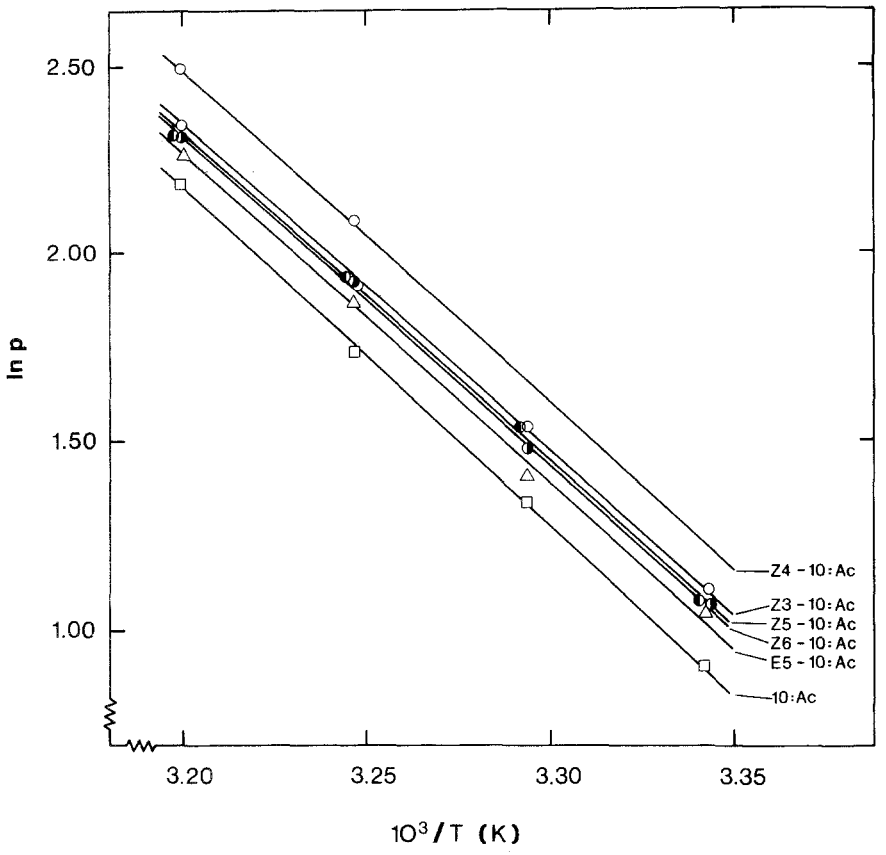

FIG. 3. Expansion of the upper part of Figure 2.

The results calculated from our data are: 72 kJ/mol for decyl and decenyl acetates, 77.5 kJ/mol for *Z*7–12:Ac, and 90 kJ/mol for *Z*9–14:Ac. These data agree well with vaporization enthalpy data obtained for 1-chloroalkanes of similar molecular sizes (Jönsson et al., 1980).

Hirooka and Suwanai (1978) measured vapor pressures of compounds similar to those in this study. Only one of them is the same (*Z*9–14:Ac). The agreement with our values for this compound is within 10% at 40°C and 50°C, but at 30°C their value is ca. 50% higher than ours. This compound is one of the least volatile in their study. The vaporization enthalpy for *Z*9–14:Ac calculated from the data of Hirooka and Suwanai is 72 kJ/mol, which is unrealistically low for a molecule of this size. Their method seems to suffer from systematic errors, especially at low vapor pressures. No other sources of literature data of vapor pressures at biologically relevant temperatures for the

substances under study have been found, not even in a very comprehensive compilation (Dykyj and Repáš, 1979).

Effects of Position of Double Bond. From measurements on computer-generated models of the different (*Z*)-decenyl acetates, the midpoint of the molecule is found to be near the 4 position. Thus *Z*4–10:Ac is the most symmetrical compound with respect to the double bond position. As (*Z*)-olefins are folded at the double bond, a more centrally located double bond gives a smaller molecular size. The vapor pressure mainly depends on intramolecular forces in the liquid phase. Therefore, a molecule with a smaller effective size should have a higher vapor pressure than one which is effectively larger. Thus *Z*4–10:Ac should have the highest vapor pressure, followed by *Z*3–10:Ac and *Z*5–10:Ac which is in agreement with the data obtained.

This effect can also be seen in vapor pressure data for small olefins as hexenes (Camin and Rossini, 1956) and heptenes (Eisen and Orav, 1970). (*Z*)-3-Hexene and (*Z*)-3-heptene both have higher vapor pressures than (*Z*)-2-hexene and (*Z*)-2-heptene, respectively. Also, Butler and McDonough (1979) found higher evaporation rates for more symmetric *Z*-olefin acetates from rubber septa. This was explained by less retardation by the rubber matrix. Our results indicate that at least part of this effect may be explained by differences in vapor pressure. The logarithms of the half-lives ($t_{\frac{1}{2}}$) as determined by Butler and McDonough and the corresponding vapor pressures reported in the present work show an excellent linear relationship.

Effects of Chain Length. The vapor pressure ratio at 35°C for the homologs studied in this work are 7.8 for *Z*5–10:Ac/*Z*7–12:Ac and 5.4 for *Z*7–12:Ac/*Z*9–14:Ac. Thus there is no strict additivity in ln p for the homologs in this series as is found for more simple compounds, e.g., normal alkanes. As was shown above, the vapor pressures of olefinic compounds depend on the position of the double bond. Strict additivity should thus only be expected for a series of homologs in which the compounds have nearly the same relative length of the two hydrocarbon chains separated by the double bond.

Effects of Double Bond Configuration. In the only pair which we have studied, *Z*5–10:Ac has a higher vapor pressure than *E*5–10:Ac. The difference is less than 10%.

Effects of Double Bond. As expected, all decenyl acetates have higher vapor pressures than the saturated decyl acetate. Discussions of the relative amounts of compounds in a pheromone blend in terms of major and minor components are generally based on the relative amounts found in extracts of female glands. When the vapor pressures of the pheromone components are significantly different, the relative amounts in the vapor phase differ from those in the liquid phase. As an example, four of the compounds in this work, 10:Ac, *Z*5–10:Ac, *Z*7–12:Ac, and *Z*9–14:Ac, identified as sex pheromone

components of *Agrotis segetum*, may be considered. The amounts of substances found in female abdominal tip extracts (Löfstedt et al., 1981) correspond to the mole percentages 5.7, 4.3, 62.7, and 27.3 for 10:Ac, Z5–10:Ac, Z7–12:Ac, and Z9–14:Ac, respectively, if only these four components are considered. Using Raoult's law and the vapor pressures of the compounds at 20°, obtained by extrapolation of the data in Table 1, the corresponding mole percentages in the vapor phase become 28.6, 25.5, 43.2, and 2.7, respectively. Thus the minor components in the extract, 10:Ac and Z5–10:Ac, drastically increase their relative amounts in the vapor phase, due to higher vapor pressures. Instead Z9–14:Ac (27.3 mole % in the liquid phase) with a lower vapor pressure becomes a minor components (2.8 mole %) in the vapor phase.

If the technical problems involved in quantifying sex pheromones volatilized from female glands can be solved, an analysis of the female effluvium directly gives the vapor phase composition of the pheromone blend. This method should give a more biologically relevant picture of pheromone compositions than the generally used analysis of female gland extracts. The vapor pressure data of pheromone components presented in this work should then be useful in connection with research on dispensing pheromone components as an optimal blend.

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CHEMOTAXIS OF LARVAL SOYBEAN CYST NEMATODE, *Heterodera glycines* RACE 3, TO ROOT LEACHATES AND IONS

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Abstract—Second-stage larvae of the soybean cyst nematode *Heterodera glycines* Race 3 were attracted in an in vitro bioassay to pooled leachates of soybean roots that were expressed as root gram-hours (1 g of root/hr/vol). Their responses were dosage dependent with maximal attraction to a 5 root g-hr/ml source. Optimal bioassay conditions used 24 hr for gradient formation and 1.5 hr for larval movement. Individual plants produced leachate with little variation in biological activity. Production was constant for five days of preparation. The attractive activity of root leachate declined with storage at 4° C and heating over 30° C, but loss of activity was reduced by freezing. Extraction and Sep-Pak fractionation indicated that the attractions(s) was water-soluble. Larvae were attracted also to several ionic solutions.

Key Words—Soybean cyst nematode, nematode chemotaxis, larval attraction, Nematoda, host location.

INTRODUCTION

A number of plant stimuli are proposed to mediate the larval behavior of nematodes for host location. Croll (1970) and Green (1971) have reviewed the role of various physical and chemical factors. Several species of *Heterodera* larvae have been studied to determine the chemical basis of their orientation to host plants.

H. schachtii are attracted to carbon dioxide as a gas or carboxyl ion, oxygen, potassium permangante, and reducing agents. Host plants and

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microorganisms were attractive while nonhost plants elicited only weak responses by larvae. *H. avenae* and *H. rostochiensis* respond also to exudates of host plants. The plant compounds are largely unknown, but aqueous components are implicated. Additionally, host attraction may occur over a 40-cm distance (Luc, 1961).

Orientation to ionic gradients has been little studied. *H. rostochiensis* larvae were attracted to ammonium nitrate (Rode, 1965); however, electrical potentials are implicated for several other species of nematode larvae (Croll, 1970). Additionally many ions stimulate hatching of *Heterodera* eggs (Clarke and Shepherd, 1966).

Chemotaxis of larvae of the soybean cyst nematode, *Heterodera glycines*, has not been examined despite its economic importance. Thus, preliminary investigations of the larval response to exudates of host plants and ions were conducted to further our understanding of chemically mediated events in the host-parasite association.

METHODS AND MATERIALS

Heterodera glycines Race 3 were maintained on soybean plants as previously reported (Rende et al., 1982). Cysts were opened manually and second-stage larvae were collected for bioassay within 48 hr after hatching from eggs.

Bioassay was performed in 10 × 1.5-cm plastic Petri dishes which were coated with a thin file of 1.5% agar in reagent-grade (18 mΩ) water from a Milli-Q purification system. Filter paper circles (Whatman No. 1) with a 7-mm diameter were placed in the center of the dish. Two circles were stacked in each dish and 1.5 μl of the test solution was placed on the paper. The bioassay was modified from Schmidt and All (1978).

After a 24-hr diffusion period, larvae were positioned 8 mm from the paper disk. Three larvae, placed equidistant from one another, were used on each plate. Attraction was determined as the percentage of larvae that reached the filter paper in the response period, according to analysis of their tracks.

Initially, larval responses to root leachate were examined. Concentrations were prepared according to Tefft et al. (1982) and expressed as root gram-hours/unit volume. Six to eight plants were used for preparation of a standard solution in distilled water, based on estimated root mass. Various dosages from 0.5 to 15 root g-hr were tested with at least 30 replicates per dose. Preliminary trials examined larval responses at 10-min intervals from 10 to 120 min after 24 hr of diffusion to determine the time of maximal responsiveness. Subsequent experiments employed 1.5 hr for response. Distilled water was tested as a control.

The influence of diffusion period on the larval responses to roots was

investigated. Leachate was prepared at 3 root g-hr for bioassay after 2, 3, 6, 8, 24, and 48 hr of chemical diffusion. Thirty replicates were done for each period of diffusion.

Other experiments studied various factors that potentially influenced the production, stability, and characterization of the chemoattractant from soybean roots. Stability of the chemotactic activity of the leachate was examined by storage of solutions at 4° C for 18 days. Bioassay was done at a dosage of 7.3 root g-hr to evaluate remaining attractancy for larvae after 1, 4, 6, 11, and 18 days. Leachate was held also under vacuum for 15 min to assay any volatility of the attractants by similar bioassay procedures at a dosage of 15 root g-hr. Thermal lability of the attractant from leachate was determined by heating at 30, 35, 40, 50, and 60° C for 15 min. Bioassay at a dosage of 6 root g-hr was done after cooling for comparison to the standard dose–response line at ambient (20–22° C) temperature. Frozen leachate was tested also by bioassay at dosages of 2 and 5.6 root g-hr after 14 days of storage to further assess stability.

Preliminary fractionation of root leachate was initiated by extraction with a threefold volume of chloroform–methanol (2:1). After nitrogen evaporation of the organic layer, the residue was dissolved in distilled water. This reconstituted material and the aqueous layer were tested for biological activity by dosage assay.

Reverse-phase Sep-Paks (Waters Associates) were also employed for separation of leachate components. Five milliliters of solution of 3.8 root g-hr/ml were eluted through the C-18 cartridge. Retained compounds were then eluted with distilled water or 25, 50, 75, and 100% methanol. After solvent evaporation and reconstitution in distilled water, these fractions were assayed at 3.8 root g-hr to determine larval responses with 30 replicates per fraction.

The attractancy of individual plants was determined also. After preparation of leachate, the resultant solutions were adjusted to 2.25 or 5.6 root g-hr concentrations with distilled water. Larval responses were examined by bioassay of 30 replicates per plant. Six plants were tested at each of the above doses for comparison to the standard dose–response line from pooled leachate.

The release or production of the attractant in root leachate was studied by incubation of root mass for 1, 2, 3, 4, or 5 days. Each leachate was then adjusted to a 3.9 root g-hr dosage for bioassay of 30 replicates of the larval response.

Additionally, the orientation of larval *H. glycines* to ionic solutions was studied. Tested solutes included sodium sulfate, sodium chloride, magnesium sulfate, magnesium chloride, zinc sulfate, zinc chloride, ferric sulfate, ferric chloride, calcium sulfate, and calcium chloride. A range of at least five dosages from approximately 2 to 250 mM were done for each solution with 30

replicates per dose. Bioassay procedures were similar to those for root leachate. Distilled, reagent-grade (18 mΩ) water was used as a control.

Data were analyzed by linear regression and analysis of variance. The 0.05 probability was considered significant.

RESULTS

Responses of larval *H. glycines* to dosages of soybean roots are shown in Figure 1 for a 24-hr diffusion period and a 1.5-hr period for larval orientation. Larvae were significantly attracted to the root solution, based on dosage ($r = 0.80$). Maximal response (63%) was caused by a 3 root g-hr/ml dosage in comparison to the 24% responses to the distilled water or filter paper controls. Dosages over 0.5 g-hr were significantly different from zero (SEM = 5.57) Assay of dosages to 15 root g-hr elicited responses similar to 3–5 g-hr concentrations.

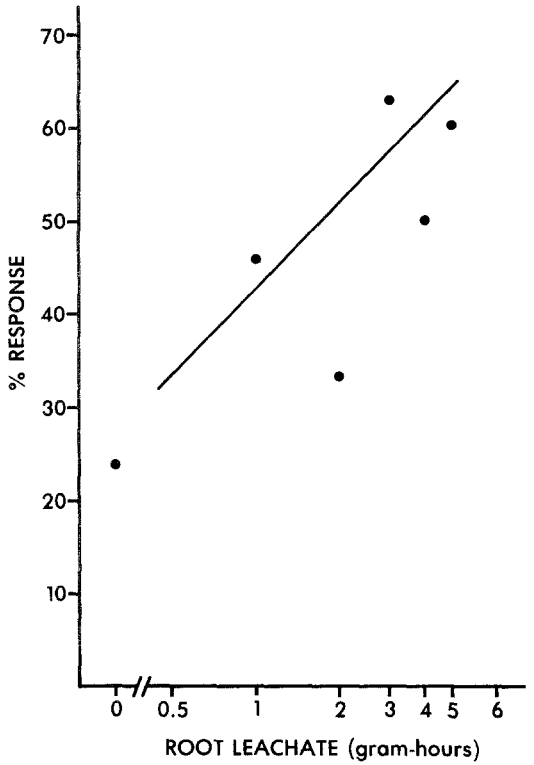

FIG. 1. Responses of *H. glycines* larvae to dosages of soybean root leachate in gram-hours after 24-hr diffusion and 1.5-hr response periods (SEM = 5.57).

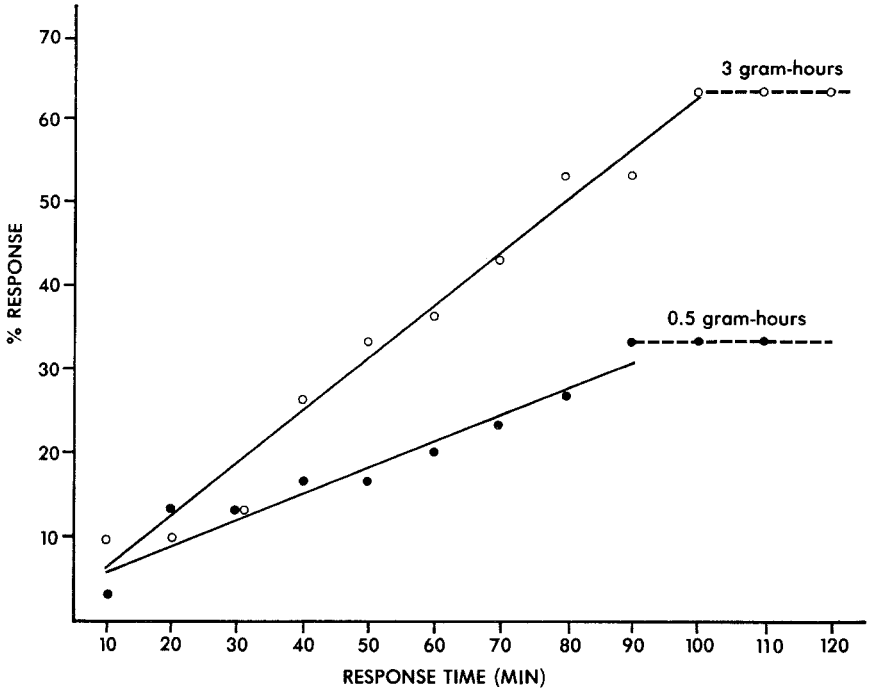

FIG. 2. Timed responses of *H. glycines* larvae to selected dosages of soybean root leachate at 0.5 (•) g-hr (SEM = 2.72, r = 0.96) and 3 (0) g-hr (SEM = 5.77, r = 0.98).

Figure 2 gives the effect of root dosage on the larval response time for selected dosages. Generally, maximal responses were obtained after a 90- or 100-min period. The percent of larval attraction increased linearly from 10 to 90 min, but showed little change with additional time. Lower dosages were intermediate to those in Figure 2.

Diffusion time for the root solution influenced larval responsiveness (Figure 3). Insignificant responses were found with 8 hr or less for chemical diffusion when compared to controls (SEM = 3.7). Maximal responsiveness by larvae occurred in a 24-hr gradient, while a 48-hr period reduced larval attraction slightly presumably due to gradient equilibrium.

The attractiveness of the root leachate for larvae declined as storage time increased (Figure 4). Insignificant responses were obtained with 10-day or older leachate (SEM = 6.2) when compared to the controls.

Larval responses to the original and evacuated leachates were similar at the examined dose. Thus volatility of the root attractant(s) appears negligible.

Increased temperature reduced linearly (r = 0.98) the attractiveness of the root leachate according to bioassay (Figure 5). No remaining activity was found after heating at 60° C for 15 min. Moderate temperature (30° C),

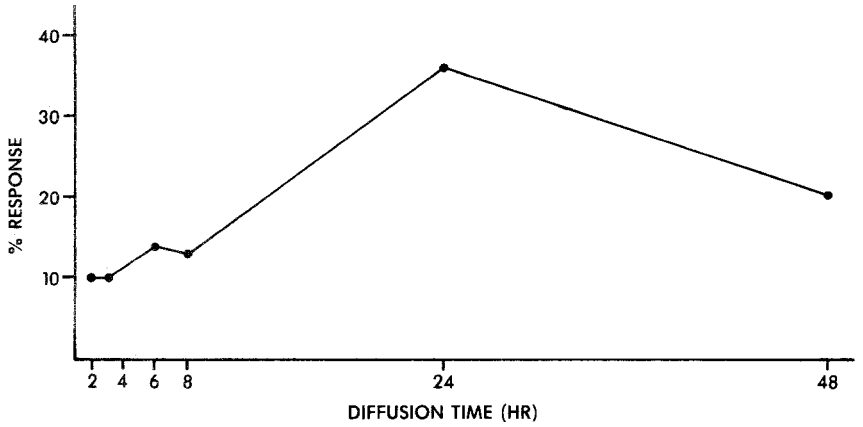

FIG. 3. Larval responses of *H. glycines* to a 3 root g-hr dosage after the indicated diffusion periods and a 1.5-hr response period (SEM = 3.7).

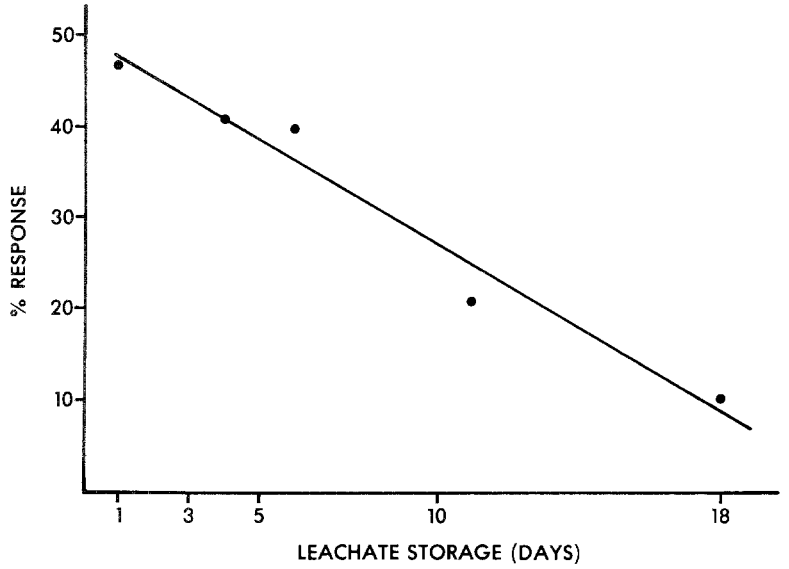

FIG. 4. Larval responses of *H. glycines* to soybean root leachate after storage at 4°C for the indicated periods (SEM = 6.2).

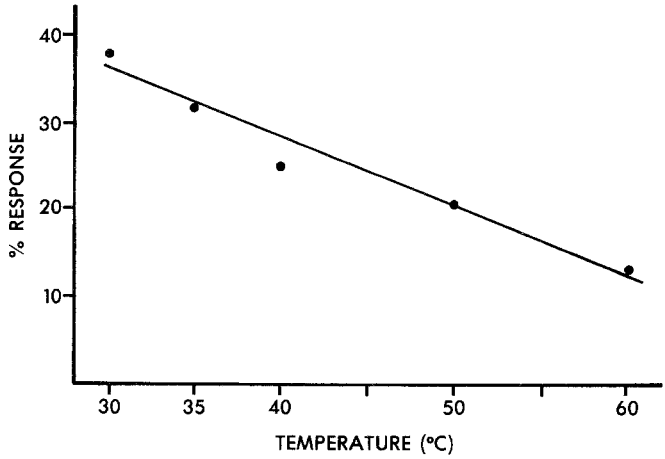

FIG. 5. Larval responses of *H. glycines* to soybean root leachate after heating for 15 min at the indicated temperatures (SEM = 3.86).

however, caused no appreciable change when compared to previous results from bioassay at ambient temperatures.

Bioassay of frozen leachate after 14 days of storage revealed no appreciable change in attractiveness according to the larval responses. Thus, a 5.6 root g-hr dose elicited an initial response of 38% and an elevated 52% response after freezing. Therefore, freezing may partially reduce the loss of leachate attractancy that occurs at 4° C or elevated temperatures.

Extraction of the root leachate with organic solvents revealed that the biological activity for larval attraction remained in the aqueous fraction. This fraction caused a dosage-dependent attraction ($r = 0.81$) of the larvae with a maximal 39% response to a 10 root g-hr dosage. This decreased response, however, suggests some activity was lost. In contrast, the reconstituted organic fraction did not differ from the control responses (24%) until a 36 root g-hr dose was exceeded. A 47% response occurred at 45 root g-hr of the organic fraction. This represents only 4% of the expected activity and therefore may have resulted from inadequate extraction or manipulative error.

Similar results were obtained by Sep-Pak fraction. Elutions showed that the aqueous fraction contained most of the biological activity of the original leachate and caused a 67% response by larvae. The methanol eluants (mean = 25.5%) did not differ significantly from the control responses to distilled water. Thus, the attractant is probably water-soluble, based on extraction and separation data.

Little variation was found in the larvae's response to leachate that was prepared from individual plants. The percent response to a 2.25 g-hr dose

ranged from 29 to 34% for five tested plants while the sixth plant caused only a 17% attraction. The mean response (29.3%, SEM = 2.32) agreed with that from pooled samples. At 5.6 g-hr the larval responses to five individual hosts were 36–43% except for a single plant that elicited a 17% response. The mean (34.5%, SEM = 3.35) was similar to that from pooled leachate (Figure 1).

No significant decrease in the production of attractant in leachate solution was observed during 1–5 days of preparation ($r = 0.59$) (Figure 6). A tendency to decreased activity, however, was suggested at longer periods. Other studies of this investigation employed a 1- or 2-day preparation of leachate. Little difference occurred at these times.

Based on the responses to distilled water versus reagent-grade water as controls (24 and 13%, respectively), larval orientaiton to ions was examined also. Larvae showed no significant response to the sulfates or chlorides of sodium and iron. Dosage-dependent responses by larvae, however, were found for zinc sulfate, zinc chloride, calcium sulfate and magnesium chloride ($r = 0.96, 0.79, 0.79$, and 0.83, respectively). The threshold for responsiveness was lowest for calcium sulfate (1.5 mM) and highest for zinc chloride (36 mM). Increased responsiveness did not occur beyond a 60- to 70-mM concentration of the ionic solution. Thus, the larvae of *H. glycines* are

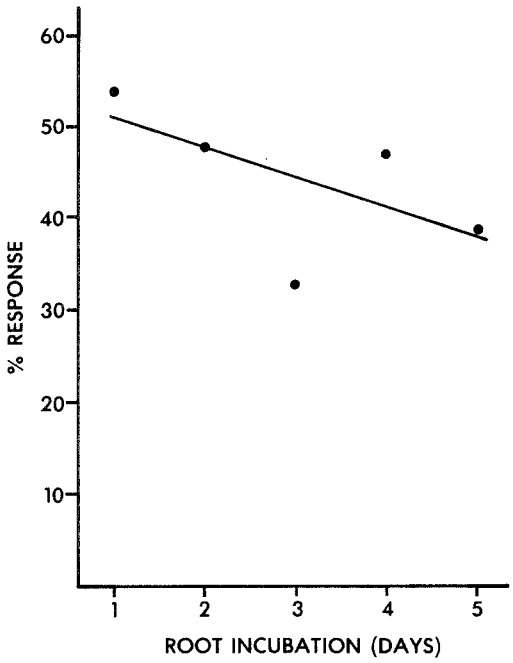

FIG. 6. Larval responses of *H. glycines* to soybean root leachate that was prepared by plant incubation for the indicated periods (SEM = 3.29).

attracted to various ions which may account for the twofold difference in the control responses of this study.

DISCUSSION

Location of a host plant by larval *Heterodera glycines* is partially mediated by chemicals from roots. The compound is apparently water-soluble, nonvolatile, and heat-labile. These features are probably consistent with its chemical function in a subterranean environment. The loss of activity with increased temperature may be minor in reduced soil temperatures. Also, we suspect that the loss of activity with storage and incubation time of the plant pose no problem in the natural ecology of parasitism; these features are probably more important during experimental treatments. Ionic gradients may be somewhat involved, but the lability of root leachates reinforces the presence of additional biochemical components.

The larvae's attraction to certain ions is particularly interesting since many of these substances serve as hatching stimuli in various species of cyst nematodes. The response of the soybean cyst nematode to zinc is intriguing due to the role of this ion as one of the few known stimuli that induce hatching. If zinc is involved to some degree in both egg hatching and host location, its role as a biological messenger becomes quite plausible. However, further studies that are directed toward isolation of other active material are needed for more comprehensive knowledge.

The consistent production of the compound(s) by individual plants for a period of time may contribute to plant susceptibility or levels of nematode infection. Examination of the orientation of larvae to root leachates of nonhost plants or resistant cultivars of soybeans may offer additional insight into host–parasite interaction with a resultant agricultural impact.

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ECOLOGICAL PATTERNS IN THE GLUCOSINOLATE CONTENT OF A NATIVE MUSTARD, *Cardamine cordifolia*, IN THE ROCKY MOUNTAINS

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Abstract—Insect use of native crucifers may be related to patterns in mustard oil content. Consequently, in 1979 we measured glucosinolate content of Rocky Mountain bittercress, *Cardamine cordifolia* (Cruciferae), using paper and gas chromatography, in relation to: plant organ, phenology, elevation, habitat, leaf position and weight, and plant consumption by two adapted insect herbivores. Results for each are as follows. (1) The predominant constituent in all vegetative organs was 2-butylglucosinolate; concentration of isothiocyanate-yielding glucosinolates (IYG) was highest in roots (1.11 mg/g fr. wt) and lowest in stems (0.07 mg/g). (2) Concentration of IYG appeared to be higher in plants lacking oxazolidinethione-yielding glucosinolates (OYG) than in those with OYG. (3) Terminal cauline leaves had a higher content of IYG than leaves in other positions on a plant. (4) Heavy leaves had significantly higher concentrations of IYG than did lighter leaves. (5) IYG concentrations were not directly related to elevation. (6) Leaves of plants occurring naturally in the sun had concentrations of IYG similar to those of plants in the usual shaded habitat. However, experimental removal of overhanging willows caused a significant, stress-induced increase in IYG concentrations. Finally, (7) feeding by two adapted herbivores, chrysomelids and psyllids, was associated with lower, rather than higher, IYG concentrations. The results demonstrate significant variation in glucosinolate content in a native crucifer and suggest that some of this variation can be partitioned in relation to the ecological and environmental axes examined.

Key Words—Cruciferae, chemical defense, gradient, glucosinolate, herbivory, insect–plant interactions, *Cardamine cordifolia*, Coleoptera, Chrysomelidae, Homoptera, Psyllidae.

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INTRODUCTION

Many secondary plant metabolites function as antiherbivore defenses (Fraenkel, 1959; Ehrlich and Raven, 1965; Whittaker and Feeny, 1971; Feeny, 1976; Levin, 1976; Rhoades and Cates, 1976; Seigler, 1977; Swain, 1977; McKey, 1979). Consequently, individual variation in the composition, concentration, distribution, and phenology of these compounds should have significant implications for plant–insect interaction. The evidence available on the role of variation in secondary compounds in these interactions in the field, however, is scarce (Janzen, 1979) and conflicting. For example, Moore (1978a,b) found that variation in seed loss to insect predators in the African legume *Crotalaria pallida* was high, consistent among individual plants between seasons, and, unexpectedly, positively correlated with pyrrolizidine alkaloid concentration. In a study of two insect predators on cocklebur, *Xanthium strumarium*, Hare and Futuyma (1978; Hare, 1980) found that variation in one (unidentified) compound correlated positively with one insect but negatively with the other. For naturally occurring lupines, Dolinger et al. (1973) detected significant intra- and interspecific variation in alkaloid composition of three cooccurring species in the Rocky Mountains, with a complex pattern of correlations with seed-eating lycaenid larvae. These researchers proposed that variation in secondary compounds within plant populations contributes to persistence over time despite significant insect herbivore pressure. More studies of intraspecific variation in allelochemics of native plants and its relation to insect herbivory are necessary to determine the frequency and generality of these opposing outcomes (cf. Janzen, 1968; 1979; Rodman and Chew, 1980; Woodhead, 1981).

Plants of the mustard family (Cruciferae) provide an exceptional opportunity to examine the details and dynamics of a coevolving insect-plant–secondary compound system. Crucifers are characterized by distinctive compounds, the glucosinolates or mustard oil glucosides (Ettlinger and Kjaer, 1968; Kjaer, 1960, 1976; Feeny, 1977). Whereas considerable information exists on glucosinolate composition of cultivated and introduced mustard species (Slansky and Feeny, 1977; Feeny, 1977; VanEtten and Tookey, 1978, 1979), as well as native species (Al-Shehbaz, 1973; Rodman, 1974, 1976, 1980, 1981; Rodman and Chew, 1980; Rodman et al., 1981; Feeny and Rosenberry, 1982), most information is focused on agronomic and taxonomic, rather than ecological, patterns. Glucosinolates are used as a classic example of toxic, low-dosage compounds that function as chemical defenses for ephemeral plants and tissues (Whittaker and Feeny, 1971; Feeny, 1975, 1976, 1977; Rhoades and Cates, 1976; Blau et al., 1978; Lubchenco and Gaines, 1981).

Furthermore, crucifers maintain an adapted, or relatively specialized,

insect fauna (e.g., Bonnemaison, 1965; Root, 1973) that utilize them despite the potentially toxic glucosinolates (Blau et al., 1978) and sometimes even because of them (David and Gardiner, 1966; Schoonhoven, 1967, 1969, 1972; Bogawat and Srivastava, 1968; Nielsen, 1977, 1978; Dethier, 1980; Matsumoto, 1980). These adapted insects show differential attraction to glucosinolates (see Schoonhoven, 1967, 1969; Wieczorek, 1976) and to native crucifers (Chew, 1975, 1977; Courtney, 1981) and high but differential tolerance to glucosinolates (e.g., Nayar and Thorsteinson, 1963; Hicks, 1974). Since cooccurring crucifers differ in their glucosinolate profiles, Rodman and Chew (1980) have extended Janzen's (1968) general hypothesis and suggested that interspecific variation may mediate crucifer–herbivore dynamics within a community. Further understanding of these interactions requires quantitative data on glucosinolate content in relation both to plant development, phenology and habitat and to insect herbivore use.

We report here on paper- and gas-chromatographic analyses of glucosinolate content in the native mustard, *Cardamine cordifolia* (bittercress), in relation to abiotic and biotic environmental factors that have been predicted to be important for insect use of plants. We address two main questions: (1) What is the extent of quantitative variation in glucosinolate content among *C. cordifolia* individuals in the field? (2) Is the variation associated with parameters of plant position or phenology, i.e., plant organ, leaf position, leaf weight, elevation, sun versus shade habitat, or plant use by two insect herbivores?

We focus on *C. cordifolia* for several reasons. First, observations suggest that plants in contrasting sun and shade habitats suffer differential insect herbivore damage (Chew, 1977). Second, leaf glucosinolate profiles differ qualitatively among individuals (Rodman and Chew, 1980). Third, over 30 species of insects commonly feed on *C. cordifolia* in the southern Rocky Mountains, and individual plants vary extensively in the amount of insect damage experienced (Louda, 1983b). Finally, the plants are amenable to experimental manipulation.

METHODS AND MATERIALS

Cardamine cordifolia A. Gray (bittercress) occurs in the Rocky Mountains from Idaho and Wyoming south to Arizona and New Mexico (Harrington, 1954). It grows between 2150 and 3550 m elevation, typically in moist ground along streams or in meadows, usually in the shade of spruce or willow stands (Barrell, 1969; personal observations). Our research was conducted in the southern Rockies in the Cooper Creek drainage (2990–3550 m) above the Rocky Mountain Biological Laboratory (RMBL), Gothic,

Gunnison County, Colorado. Plants were locally abundant in the area, growing as herbaceous perennials from short, slender rhizomes, sending up shoots 30–75 cm high. Flowering occurs from late June through July, and fruit set and maturation occur in August and early September in the Gothic area. The frost-free growing season varies at Gothic, but usually it starts in late May and ends in late August (Chew, 1975; Louda, personal observation).

Field Collection of Plant Material. Two collections of plants were made in 1979 ($N = 94$): a preliminary, first sample June 28–July 13 ($N = 35$ plants) and a second sample August 18–25 ($N = 59$ plants). Collections were made primarily along the 7-km Copper Creek drainage above RMBL; however, the second collection included six plants sampled from one site in Virginia Basin, which runs adjacent and parallel to Copper Creek, and six plants sampled from one site on the nearby Upper Slate River. The first set of samples, in which leaf and stem material for an individual plant were combined, was collected in lower Copper Creek; samples were stratified in relation to three conditions of plant growth: (1) shaded versus unshaded habitat, (2) initiation of a shade removal experiment, and (3) presence or absence of chrysomelid beetle adults. The second set of plant samples was collected along the whole Copper Creek drainage and stratified in relation to three environmental conditions: (1) elevation, (2) habitat, and (3) termination of the shade removal experiment for 1979; in addition, the six samples from Virginia Basin were collected in relation to the presence or absence of psyllids.

A plant sample involved clipping one or more stems from individual, randomly selected rootstocks, placing them into a sealable Ziploc® plastic bag, and returning them to the laboratory. Samples were then processed within 4 hr of collection. First, stems were measured and leaves were counted. Second, sample material was weighed. The first collection samples were small and were not partitioned; the second collection samples were larger and were separated into leaf and stem subsamples. In three cases root subsamples were also taken (root = rhizome plus associated roots). Third, samples were preserved for glucosinolate analysis by boiling them in 70% methanol for 10–15 min, denaturing the endogenous myrosinases. Plant material was stored in 70% methanol until glucosinolate analysis was carried out in the fall.

Field Collection of Insects. During collection of the first set of plant samples, adult beetles (Coleoptera: Chrysomelidae) were the most conspicuous insect herbivores on *Cardamine cordifolia.* Consequently, ten plants at one site along Copper Creek (= First Streamlet site) were selected for presence (1–10 beetles) or absence (0) of leaf beetles on July 5, 1979, and then collected for analysis. The most abundant chrysomelid beetle was identified as *Phaedon* sp. nr. *oviformis* (Lec.); others present were *Phyllotreta ramosa*, and *Phyllotreta* sp. nr. *polita* (R. White, personal communication). Psyllids were not common at this time at any sites. During collection of the second set of

plant samples in late August, adult chrysomelid beetles were not observed. However, psyllids (*Aphalara* sp. nov., Homoptera: Psyllidae; D.R. Miller, personal communication) were exceptionally abundant on some plants. Consequently, at one site in Virginia Basin random samples of plants were collected from among those where psyllids were present (>100/plant stem) or absent (1–10/plant stem). Voucher specimens of insects have been deposited in the collections of the United States National Museum/U.S.D.A. Insect Identification Bureau, Beltsville, Maryland.

Glucosinolate Analysis. Glucosinolate analyses were performed on the samples in 70% methanol after two to five months of storage. Storage under these conditions does not affect analytical results (Rodman and Chew, 1980). Glucosinolates were identified from the paper- and gas-chromatographic behavior of their hydrolysis products, isothiocyanates (mustard oils) and oxazolidinethiones (Figure 1), following treatment with exogenous (*Sinapis alba*) myrosinase (Ettlinger and Thompson, 1962; Rodman, 1978). Previous analyses of *Cardamine cordifolia* from Colorado and New Mexico revealed the presence of up to nine different glucosinolates (Rodman and Chew, 1980), and we detected the same compounds in this study (Table 1). They are separated into two operationally defined groups (cf. Josefsson, 1967a, 1970): 1) glucosinolates that yield volatile isothiocyanates (IYG) upon myrosinase hydrolysis, which were analyzed and quantified by gas chromatography, and 2) glucosinolates that yield nonvolatile, cyclic oxazolidinethiones (OYG), which were separated by paper chromatography and visualized with Grote's

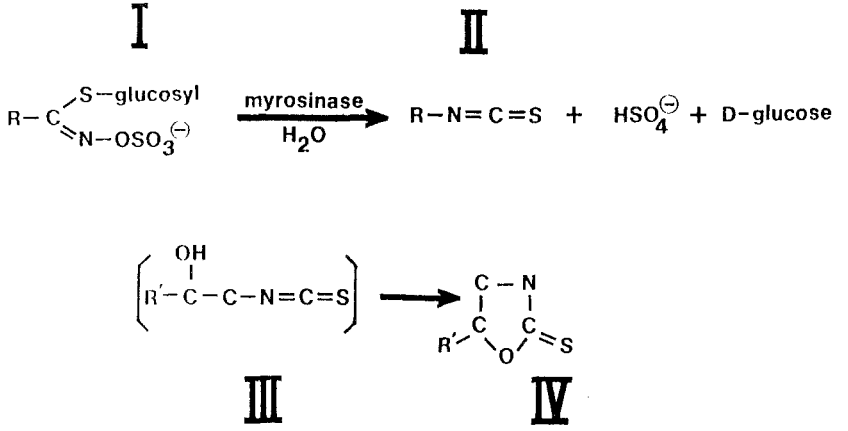

FIG. 1. General reaction for the enzymatic hydrolysis of glucosinolates (I) to their corresponding isothiocyanates (II); 2-hydroxyglucosinolates give rise to 2-hydroxy-isothiocyanates (III) that are unstable and cyclize spontaneously to oxazolidinethiones (IV).

TABLE 1. GLUCOSINOLATE HYDROLYSIS PRODUCTS DETECTED BY PAPER AND GAS CHROMATOGRAPHY IN *Cardamine cordifolia* FROM STUDY SITES AROUND GOTHIC, COLORADO

Product of myrosinase hydrolysis	Gas chromatographic retention time (min)	Paper chromatographic mobility (R_{Ph})[b]	
		I[c]	II[d]
	At 80° C		
1. Isopropyl[a]	4.1–4.4	0.32	0.43
2. 2-Butyl[a]	7.9–8.3	0.65	0.73
3. Isobutyl[a]	9.0–9.6	0.65	0.73
	At 160° C		
4. Benzyl[a]	6.6–7.3	0.82	0.92
5. 2-Phenylethyl[a]	10.4–11.3	1.25	1.08
6. 4-Methyloxazolidine-2-thione[e]	n.a.	0.73	0.90
7. 4-Ethyloxazolidine-2-thione[e]	n.a.	1.33	1.20
8. 5,5-Dimethyloxazolidine-2-thione[e]	n.a.	1.15	1.15
9. 5-Phenyloxazolidine-2-thione[e]	n.a.	2.15	1.35

[a] Isothiocyanate; converted to corresponding thiourea by ethanolic–ammonia treatment for paper chromatography.
[b] In chromatographic solvent system I or II with mobility relative to phenylthiourea standard.
[c] I = "toluene–acetic acid" system (Rodman, 1978).
[d] II = "chloroform" system (Rodman, 1978).
[e] Generated from corresponding β-hydroxyglucosinolate.

spray reagent (Ettlinger and Thompson, 1962; Ettlinger et al., 1966; Rodman, 1978).

Each weighed plant sample in 70% methanol was blended in a Waring blender, filtered through cheesecloth, and boiled down to an aqueous slurry for 15–20 min. Cooled extracts in 50-ml Erlenmeyer flasks were brought to pH 7 by the addition of sodium bicarbonate and overlaid with 9 ml of ethyl ether; myrosinase and ascorbate solution were then added for hydrolysis (Ettlinger and Thompson, 1962; Rodman, 1978). Flasks were left overnight in the dark at room temperature, with occasional manual shaking. The following morning, the ether layer was pipetted into a 10-ml volumetric flask. Aliquots of 1–3 μl were injected for gas chromatography. Subsequently, the ether solution was evaporated to dryness under nitrogen over a steam bath, and the residue was redissolved in 10 drops of absolute ethanol. The nonvolatile, cyclic oxazolidinethiones released by myrosinase hydrolysis were detected by paper chromatography of these extracts. Samples were run by ascending paper chromatography in "chloroform" and "toluene–acetate acid" systems (described by Rodman, 1978).

For gas chromatography, a Perkin-Elmer model 3920 instrument was used, with hydrogen flame ionization detectors and a Perkin-Elmer model 56 strip chart recorder. Retenton times and peak areas were computed by an interfaced Perkin-Elmer model 1 integrator. A 1.8m × 3-mm ID stainless-steel column packed with 6% DC-560 plus 2% EGSP-Z on 100/120 GasChrom Q was used, with a carrier gas (helium) flow rate of 50 ml/min. Samples were run isothermally at 80° and 160°C, one subsample at each temperature, through glass injector and detector ports set at 225°. Identification of peaks was based on retention times compared with those of commercially obtained standards or to extracts from other crucifers known to produce particular glucosinolates (Kjaer, 1960). Identifications were corroborated by paper chromatography of extracts (Rodman and Chew, 1980).

Quantity of each isothiocyanate-yielding glucosinolate (IYG) was determined by gas chromatography from the empirical relationship of peak area to known concentration of standards. Our standards were 0.1 mM ether solutions of three commercially obtained mustard oils (K & K Laboratories, Plainview, New York) that were structurally similar to the compounds in *Cardamine cordifolia*. A least-squares regression line forced through the origin was computed for each. Quantity of isopropyl isothiocyanate was estimated from the equation for an allyl standard (both possess a 3-carbon side chain), whereas quantities of 2-butyl and isobutyl isothiocyanates were estimated using an *n*-butyl standard curve and those of benzyl and 2-phenylethyl isothiocyanates using a benzyl standard curve. For each compound, the "isothiocyanate equivalent" was converted to the corresponding "glucosinolate anion equivalent" by multiplying by the ratio of the molecular weights of the two forms. The sum of the glucosinolate anion equivalents for each sample thus provides an estimate of total concentration of isothiocyanate-yielding glucosinolates (IYG) per gram fresh weight of plant material.

RESULTS

Variation Associated with Plant Organ. As shown in Table 2, roots had the highest concentrations of isothiocyanate-yielding glucosinolates (IYG) (1.11 mg/g, SE = 0.34, fresh wt), stems had the lowest (0.07, SE = 0.02), and leaves were intermediate (0.32, SE = 0.04). The same relative pattern was found in a subset of our data, the 10 plants sampled on one day (August 18) from a single site (First Ford Meadow) in the Copper Creek drainage: roots = 1.11 mg/g (SE = 0.34, $N = 3$), stems = 0.06 (SE = 0.01, $N = 10$), and leaves = 0.16 (SE = 0.05, $N = 10$). Comparable results have been obtained for the distribution of glucosinolates within cultivated *Brassica* plants (Josefsson, 1967a). In addition to possessing the greatest amounts of

TABLE 2. TOTAL AND CONSTITUENT CONCENTRATIONS (mg/g) OF ISOTHIOCYANATE-YIELDING GLUCOSINOLATES (IYG) IN RELATION TO PLANT ORGAN AND TIME OF COLLECTION FOR *Cardamine cordifolia*, GOTHIC, COLORADO, 1979[a]

Collection		Total IYG	Constituent IYG[b]					% With OYG[c]
			a	b	c	d	e	
First[d]								
Leaf and stem	$\bar{X}$	0.28	0.02	0.15	0.08	0.01	0.01	29
($N = 35$)	SE	0.04	0.01	0.02	0.01	0.002	0.003	
	%		9.0	55.0	30.2	2.5	3.6	
Second[e]								
Leaf	$\bar{X}$	0.32	0.05	0.20	0.05	0.01	0.01	54
($N = 59$)	SE	0.04	0.01	0.04	0.01	0.001	0.002	
	%		15.0	63.2	16.2	2.5	3.1	
Stem	$\bar{X}$	0.07	0.01	0.03	0.02	0.01	0.004	75
($N = 12$)	SE	0.02	0.002	0.01	0.004	0.002	0.001	
	%		11.1	45.8	26.4	11.1	5.6	
Root	$\bar{X}$	1.11	0.30	0.64	0.10	0.01	0.06	100
($N = 3$)	SE	0.34	0.18	0.21	0.08	0.01	0.02	
	%		27.0	57.7	9.5	0.7	5.0	

[a] Mann-Whitney U tests of selected comparison are discussed in text.
[b] Presented as glucosinolate anion equivalents in fresh material (mg/g), with: a = isopropylglucosinolate; b = 2-butylglucosinolate; c = isobutylglucosinolate; d = benzylglucosinolate; and e = 2-phenylethylglucosinolate.
[c] Proportion of samples containing oxazolidinethione-yielding glucosinolates.
[d] June 28–July 13, 1979.
[e] August 18–25, 1979.

IYG, roots also consistently possessed oxazolidinethione-yielding glucosinolates (OYG), whereas stems and leaves were variable for the presence of these compounds (Table 2).

Variation Associated with Time of Collection. The difference in concentration of IYG between the first collection samples (June 28–July 13) of leaf + stem material (0.28 mg/g, SE = 0.04), and the second collection samples (August 18–25) of leaf material (0.32 mg/g, SE = 0.04) was not significant (Mann-Whitney U test, $P > 0.10$). Because stem material had only one third the IYG concentration of leaf material, at least in late August (Table 2), this result initially suggested that there was no major change in glucosinolate concentration in leaves between early July and late August. However, the proportion of samples containing OYG approximately doubled between the first and second collections: 0.29 to 0.54 (Table 2). OYGs were not quantifiable with our gas chromatographic column, so we have no data on the relative contribution of OYG to total concentration of glucosinolates in plant tissues. The data demonstrate, however, that average chemical profile

changed with respect to the frequency of OYG between the two sampling dates. This increase in the presence of OYG occurred without an obvious decrease in IYG concentrations; hence, an increase in total glucosinolate content may have occurred.

In both collections and for all tissues, 2-butylglucosinolate was the dominant constituent, comprising 46–63% of total IYG (Table 2). In the first collection, isobutylglucosinolate was codominant (30%). In the second, later collection, isobutylglucosinolate remained the second highest IYG in leaf (16%) and stem (26%) material, but in root material isopropylglucosinolate was the second highest constituent (27%).

Variation Associated with Presence of OYG. The OYGs were found to be present in a higher proportion of samples from the second collection (Table 3). This raised the question of whether presence of OYG affected the concentrations of IYG. For the first collection, significantly higher concentrations of IYG occurred in samples without OYG than in samples with them (Table 3). The quantity of three of the four constituent IYGs with hydroxylated analogs was significantly lower when its OYG form was present (Table 4). The pattern for the fourth constituent, isobutyl, was similar but not quite significant (Mann-Whitney U test, $P = 0.08$, Table 4).

For the second collection, the pattern of IYG concentration in relation to presence or absence of OYG was ambiguous. Concentration of IYG in leaf

TABLE 3. TOTAL CONCENTRATION OF ISOTHIOCYANATE-YIELDING GLUCOSINOLATES (IYG) IN PLANT MATERIAL (mg/g) IN RELATION TO PRESENCE OR ABSENCE OF OXAZOLIDINETHIONE-YIELDING GLUCOSINOLATES (OYG) BY PLANT ORGAN AND TIME OF COLLECTION FOR *Cardamine cordifolia*, GOTHIC, COLORADO, 1979

			Total IYG in Samples (mg/g):						
			With OYG			Without OYG			
Collection	Total *N*	% With OYG	*N*	$\bar{X}$	SE	*N*	$\bar{X}$	SE	*P*
First[a]									
Leaf + stem	35	28.6	10	0.10	0.02	25	0.35	0.04	0.002[c]
Second[b]									
Leaf	59	54.2	32	0.38	0.07	27	0.25	0.04	0.095[c]
Stem	12	75.0	9	0.05	0.01	3	0.14	0.01	0.050[d]
Root	3	100.0	3	1.11	0.34	0			0.125[e]

[a] June 28–July 13, 1979.
[b] August 18–25, 1979.
[c] Mann-Whitney U test.
[d] Mann-Whitney U test (1-tailed).
[e] Conditional probability of occurrence.

TABLE 4. COMPARISON OF CONCENTRATION OF SPECIFIC ISOTHIOCYANATE-YIELDING GLUCOSINOLATES (IYG) IN PLANT SAMPLES (mg/g) WITH COMPOUND PRESENT AS HYDROXYLATED, OXAZOLIDINETHIONE-YIELDING (OYG) OR NONHYDROXYLATED, ISOTHIOCYANATE-YIELDING (IYG) FORM[a], GOTHIC, COLORADO

	IYG Only			OYG Present Also			
Collection	N	$\bar{X}$	SE	N	$\bar{X}$	SE	P^b
First[c]							
Leaf + Stem							
Isopropyl-	24	0.03	0.01	5	0.01	0.002	0.01
2-Butyl-	25	0.20	0.02	10	0.03	0.01	0.001
Isobutyl-	27	0.10	0.01	5	0.05	0.02	0.08
2-Phenylethyl-	20	0.02	0.004	2	0.003	0.003	0.04
Second[d]							
Leaf							
Isopropyl-	37	0.06	0.01	13	0.02	0.004	0.01
2-Butyl-	36	0.25	0.05	17	0.14	0.03	n.s.
Isobutyl-	29	0.05	0.01	23	0.06	0.01	n.s.
2-Phenylethyl-	31	0.01	0.003	10	0.02	0.01	0.05
Stem							
Isopropyl-	9	0.01	0.003	2	0.002	0.000	0.01
2-Butyl-	6	0.06	0.02	6	0.01	0.004	0.01
Isobutyl-	8	0.02	0.005	4	0.01	0.002	n.s.
2-Phenylethyl-	9	0.005	0.001	0			0.10

[a] Benzylglucosinolate does not have a hydroxylated analog.
[b] Mann-Whitney U test, $P \leq 0.10$ values shown.
[c] June 28–July 13, 1979.
[d] August 18–25, 1979.

material appears higher in samples *with* OYG than in those without them ($P = 0.10$, Table 3). This relationship, of higher IYG in leaves in the presence of OYG late in the season, was significant for a homogeneous subset of plant samples ($N = 28$ samples collected on August 18, 1979, along Copper Creek, Figure 2). These data show that by late summer concentrations of IYG are higher in leaves of plants with OYG than in those without OYG (Mann-Whitney U test, $P < 0.05$) and suggest that the pattern is accentuated as leaf weight increases (Figure 2). In contrast, there were higher concentrations of IYG in samples of stem material without OYG than in those with OYG ($P = 0.05$, Table 3).

The data thus imply a tradeoff between accumulation of IYG and biosynthesis of their hydroxylated analogs, at least among the early samples. Biosynthesis of 2-hydroxy glucosinolates is known to require the enzymatic conversion, possibly in a single step, of a precursor glucosinolate (Josefsson, 1971; Underhill and Kirkland, 1972; Underhill et al., 1973). The tradeoff was

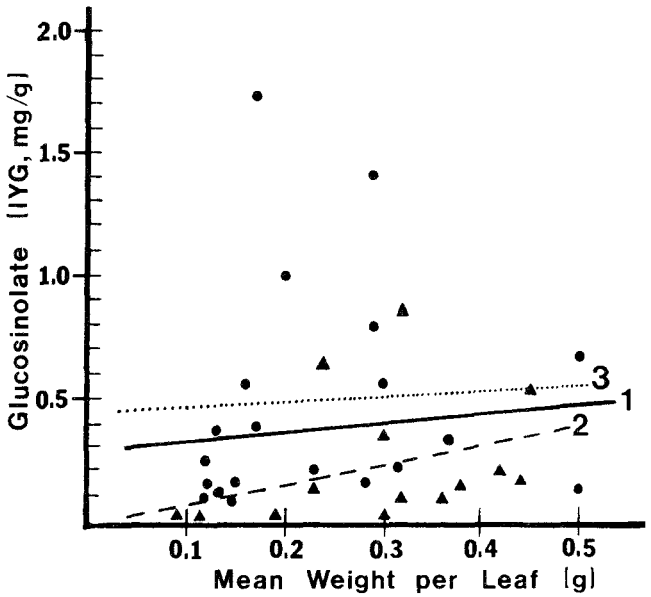

FIG. 2. Relationship of total isothiocyanate-yielding glucosinolate concentration to the average weight per leaf for samples of *Cardamine cordifolia* collected in Copper Creek drainage, Gothic, Colorado, August 1979, for (1) all samples ($Y = 0.289 + 0.345\ X$, $N = 34$, $r = 0.10$); (2) only samples without oxazolidinethione-yielding glucosinolates (▲ ; $Y = -0.001 + 0.838\ X$, $N = 14$, $r = 0.38$); and (3) only samples with oxazolidinethione-yielding glucosinolates (●; $Y = 0.417 + 0.267X$, $N = 20$, $r = 0.07$).

most evident for isopropylglucosinolate and its analog, 2-hydroxy-1-methylethylglucosinolate, that yields the cyclic 4-methyloxazolidine-2-thione upon hydrolysis with myrosinase. Concentration of isopropylglucosinolate was significantly higher when the OYG form was absent, in both collections and for all tissues examined (Table 4). Similar trends appear for: 2-butyl and 2-phenylethyl glucosinolates and their OYG analogs in the first collection and for 2-butylglucosinolate in stem material in the second collection (Table 4). However, the pattern for 2-phenylethylglucosinolate in leaves reversed in the second collection, with higher IYG concentration in samples with the OYG form than in those without it (Table 4). Finally, concentration of isobutylglucosinolate appeared to be independent of presence or absence of its hydroxy analog (Table 4).

Variation Associated with Leaf Position. There was a definite gradient in concentration of IYG in leaves by position along a stem and, consequently, by developmental age. Analyses of glucosinolates in leaves by position along the stem, or basal to the stem, are shown in Table 5 for 10 plants sampled at the First Ford Meadow site on Copper Creek on August 18, 1979. The leaves for

TABLE 5. CONCENTRATION OF ISOTHIOCYANATE-YIELDING GLUCOSINOLATES (IYG) FROM LEAVES OF *Cardamine cordifolia* (mg/g) IN RELATION TO POSITION ON PLANT AND DEVELOPMENTAL AGE, AUGUST 18, 1979, AT FIRST FORD MEADOW SITE, COPPER CREEK, GOTHIC, COLORADO

Attachment		Total	Constituent IYG[a]					% With OYG
			a	b	c	d	e	
Top 3 cm	$\bar{X}$	0.35	0.03	0.19	0.12	0.01	0.001	50
of stem	SE	0.21	0.03	0.11	0.07	0.005	0.001	
($N = 2$)	%		9.3	53.4	34.2	2.8	0.3	
Midstem	$\bar{X}$	0.19	0.02	0.08	0.04	0.01	0.04	50
($N = 2$)	SE	0.05	0.01	0.03	0.04	0.01	0.04	
	%		10.7	40.4	22.3	6.9	19.7	
Bottom 3 cm	$\bar{X}$	0.12	0.02	0.05	0.04	0.01	0.0004	50
of stem	SE	0.03	0.01	0.01	0.01	0.001	0.0004	
($N = 2$)	%		15.1	42.3	35.1	7.2	0.3	
From rhizome[b]	$\bar{X}$	0.06	0.004	0.03	0.02	0.01	0.000	50
($N = 4$)	SE	0.02	0.002	0.01	0.01	0.003	0.000	
	%		7.2	46.4	35.7	10.7	0.0	

[a] Constituent IYG labeled as in Table 2.
[b] Small basal leaves emerge from rhizomes or rootcrown at ground level throughout the season; those sampled were generally comparable in age to young, terminal cauline leaves.

each stem position were bulked and then divided into two samples for each stem position and into four samples for the basal position. Cauline leaves from the top 3 cm of upright stem had twice the concentration of IYG as those from the middle 3 cm of stem, three times the concentration in leaves from the bottom 3 cm of stem, and six times the concentration in basal leaves, recently emerged from rhizome or rootcrown. Young cauline leaves in the top 3 cm of stem had the highest concentrations (0.35 mg/g, SE = 0.21), but basal leaves from the rhizome, also generally young leaves, had the lowest concentrations (0.06 mg/g, SE = 0.02). Presence or absence of OYG did not influence the contrast with respect to leaf position since half the samples in all classes had OYG.

The distribution of constituent IYG in the profile showed three trends with leaf position. First, there was a linear gradient along the stem in the contribution of benzylglucosinolate; the relative amount of benzyl to total IYG was greatest in basal leaves (10.7%) and least (2.8%) in the terminal cauline leaves (Table 5). Second, isopropylglucosinolate was greatest in the lowest (oldest) cauline leaves whereas 2-phenylethylglucosinolate was greatest in intermediate leaves and 2-butylglucosinolate was greatest in terminal leaves. Third, the relative distribution varied in some cases from the general pattern presented in Table 2. The dominant compound was always 2-

butylglucosinolate, varying from 40 to 53%; however, the relative contribution of isobutylglucosinolate was twice as great in plants at this site as it was in overall late-season leaf samples (31% compared to 16%). Furthermore, the relative contributions of benzyl and 2-phenylethylglucosinolate (Table 5) were approximately double in these plants compared to overall late-season samples (2.5–5.8% for benzyl; 3.1–5.1% for 2-phenylethyl).

Variation Associated with Leaf Weight. Average leaf weight and associated insect damage vary among individual plants in different habitats (Louda, 1983c). Consequently, we examined the hypothesis that concentraionof IYG was correlated with leaf weight per plant, using the subset of data for which number and weight of leaves were recorded ($N = 34$ samples, August 18–25, 1979, Copper Creek). Plant samples were grouped into three significantly different leaf-weight classes: heavy = greater than 0.35 g/leaf, light = less than 0.20 g/leaf, and intermediate = 0.20–0.35 g/leaf (Table 6).

Two main results were evident. First, plant samples with OYG tended to have a higher concentration of IYG than did those without OYG, independent of leaf weight class; this difference was highly significant for samples in the lightest weight class (Table 6) and consistent with the late-season pattern for leaves (Table 3). Second, as leaf weight increased, concentration of IYG increased significantly in samples without OYG (Figure 2, Kruskal-Wallis test, $P > 0.05$); light leaves had significantly lower IYG than did heavy leaves (Table 6, Mann-Whitney U test, $P = 0.01$). For samples with OYG, concentrations of IYG were so variable within leaf weight classes that the differences in concentration of total IYG among weight classes were not significant (Kruskal-Wallis test, $P > 0.10$).

Variation Associated with Habitat Exposure. Observational and exper-

TABLE 6. TOTAL CONCENTRATION OF ISOTHIOCYANATE-YIELDING GLUCOSINOLATES (IYG) FOR *Cardamine cordifolia* PLANTS WITH LEAVES IN DIFFERENT AVERAGE WEIGHT CLASSES, COPPER CREEK, GOTHIC, COLORADO,[a] 1979

	Weight/leaf (g)		Total IYG (mg/g) in samples with						
			IYG form only[b]			OYG form also[c]			
Leaf weight class	$\bar{X}$	SE	N	$\bar{X}$	SE	N	$\bar{X}$	SE	P^d
Heavy (>0.35 g)	0.43	0.02	4	0.24	0.10	3	0.38	0.15	0.20
Intermediate	0.28	0.01	6	0.34	0.13	8	0.60	0.15	0.11
Light (<0.20 g)	0.14	0.01	4	0.05	0.03	9	0.41	0.17	0.03

[a] August 18–25, 1979.
[b] Kruskal-Wallis analysis of variance by ranks, $P < 0.05$.
[c] Kruskal-Waurs Analysis of variance by ranks, $P > 0.10$.
[d] Mann-Whitney U test for pairwise comparisons, one-tailed.

TABLE 7. PATTERNS OF OCCURRENCE IN TOTAL ISOTHIOCYANATE-YIELDING GLUCOSINOLATES (IYG) IN *Cardamine cordifolia* IN RELATION TO SPECIFIC ENVIRONMENTAL PARAMETERS

Parameter	Percent with OYG	Total IYG (mg/g) N	$\bar{X}$	SE	P
Natural exposure[a]					
Shade	33.3	16	0.37	0.07	n.s.[b]
Sun	18.2	9	0.32	0.04	
Elevation (m)[c]					
3070	40.0	5	0.19	0.10	
3200	42.9	7	0.47	0.14	0.05[d]
3325	66.7	6	0.77	0.26	
3450	80.0	5	0.17	0.06	
Herbivore Presence					
Chrysomelidae[e]					
Present	0.0	5	0.38	0.11	n.s.[b]
Absent	0.0	5	0.48	0.11	
Psyllidae[f]					
Many	0.0	2	0.45	0.38	0.06[g]
Few	100.0	2	0.52	0.05	

[a] Leaf + stem material: Gothic site, Copper Creek drainage; June 28–July 13, 1979. In samples without OYG forms.
[b] Mann-Whitney U test, $P > 0.10$ = n.s.
[c] Leaf material; along Copper Creek; August 18–20, 1979.
[d] Kruskal-Wallis analysis of variance by ranks test.
[e] Leaf + stem material; First Streamlet site, Copper Creek drainage; July 5, 1979; present = 1–10 beetles; absent = 0 beetles per plant.
[f] Leaf material; one site in Virginia Basin; August 23, 1979; few = 0–10 psyllids; many = >100 psyllids per plant. No difference between the two samples of stems (0.16, 0.15).
[g] Binomial probability of ranked order among the two sets of samples.

imental data were collected to test the hypothesis that habitat exposure, such as sun versus shade, influences glucosinolate content in *Cardamine cordifolia*. The observational data consisted of comparisons of leaf-plus-stem material from plants sampled in relation to exposure at the time of collection at the Gothic site, Copper Creek drainage, on June 28, 1979. As shown in Table 7, plants occurring naturally in the sun had IYG concentrations which were similar to those of plants occurring in the more typical shade habitat.

An experiment was performed to test plant performance and insect herbivory in relation to exposure (Louda, 1983c). The data include glucosinolate profiles from plants in naturally shaded control plots and from plants in treatment plots that had been exposed to direct sun by severe pruning of overhanging willows. The experiment ran for six weeks in 1979: July 11 to August 25. Plant samples at the beginning of the experiment were collected

adjacent to treatment plots; six weeks later random plant samples were collected from within the treatment plots.

Three main points emerged from the glucosinolate data associated with this experimental modification of exposure for in situ plants (Table 8). First, plants in shaded plots and in newly exposed plots at the start of the manipulation did not differ significantly in concentration of total IYG or, in August, in the frequency of OYG occurrence (Table 8). The only significant difference found initially was a higher concentration of 2-phenylethylglu-

TABLE 8. CONCENTRATION (mg/g) OF ISOTHIOCYANATE-YIELDING GLUCOSINOLATES (IYG) IN LEAVES OF *Cardamine cordifolia* IN SHADE-REMOVAL EXPERIMENT, FIRST FORD MEADOW, COPPER CREEK DRAINAGE, GOTHIC, COLORADO 1979, $\bar{X}$ (SE)

			Constituent IYG[a]				
	N	Total IYG	a	b	c	d	e
July 11, 1979							
Leaf & Stem							
Shade control[b,c]	6	0.12	0.02	0.04	0.04	0.015	0.005
		(0.03)	(0.00)	(0.01)	(0.02)	(0.008)	(0.001)
Sun treatment[d,e]	6	0.17	0.03	0.07	0.06	0.008	0.002
		(0.08)	(0.02)	(0.04)	(0.03)	(0.005)	(0.001)
P[f]		0.47	0.53	0.22	0.38	0.29	0.05
August 25, 1979							
Leaf							
Shade control[b,c]	6	0.17	0.02	0.08	0.05	0.010	0.000
		(0.04)	(0.01)	(0.02)	(0.01)	(0.001)	(0.001)
Sun treatment[c,d]	6	0.35	0.10	0.16	0.08	0.009	0.008
		(0.09)	(0.05)	(0.03)	(0.02)	(0.004)	(0.005)
P[f]		0.03	0.24	0.01	0.12	0.35	0.20
Stem							
Shade control[b,g]	4	0.07	0.01	0.02	0.02	0.013	0.006
		(0.02)	(0.00)	(0.01)	(0.01)	(0.004)	(0.002)
Sun treatment[c,g]	4	0.04	0.01	0.02	0.01	0.003	0.001
		(0.02)	(0.01)	(0.01)	(0.01)	(0.002)	(0.001)
P[f]		0.17	0.17	0.17	0.24	0.02	0.14

[a] Constituent compounds labeled as in Table 2.
[b] Shaded control plots = in situ plants in willow shade (i.e., in usual habitat).
[c] 83% of samples with OYG.
[d] Sun treatment plots = in situ plants above which willows were pruned July 11, 1979.
[e] 50% with OYG.
[f] Mann-Whitney U test; significant differences ($P \leq 0.05$) between treatments are underlined.
[g] 100% with OYG.

cosinolate in plants in shade control plots compared to those in sun treatment plots. This difference disappeared by August (Table 8). Second, after six weeks the leaves in the sun had significantly higher levels of total IYG than did those in the shade (Mann-Whitney U test, $P = 0.03$), but equal frequency of OYG (83%). However, total concentration of IYG and frequency of OYG in stems were similar between treatments in August (Table 8). Third, the relative proportion of component glucosinolates varied between the two treatments. Exposed leaves had significantly greater amounts of 2-butylglucosinolate (Mann-Whitney U test, $P = 0.01$) and perhaps more isobutylglucosinolate than did shaded leaves but similar amounts of the other constituents by the end of the season (Table 8). Stems in the sun had significantly lower concentrations of benzylglucosinolate than did those in the shade (Mann-Whitney U test, $P = 0.05$), but concentrations of other constituents were similar.

Variation Associated with Elevation. We collected samples to test the hypothesis that a cline in glucosinolate concentration occurred along an elevational gradient. Plants were sampled from 3070 m to 3450 m in the Copper Creek drainage on August 18–20, 1979. Two significant results are of interest (Table 7). First, the proportion of plants producing OYG increased with elevation, doubling in the 360-m gain over a 6-km distance. Second, despite considerable variation within elevations, total concentration of IYG differed significantly among elevations (Kruskal-Wallis test, $P < 0.05$). The pattern, however, was not linear along the gradient. Leaves from the highest and lowest elevation samples (3070 and 3450 m) had significantly lower concentrations of total IYG than did samples from the two intermediate elevations (Mann-Whitney U test: $P = 0.02$ for 3200 m and $P = 0.04$ for 3325 m). The low levels of IYG plus high frequency of OYG in leaves of high elevation plants at the end of the season were in contrast to the trend toward higher levels of IYG in the presence of OYG in August leaf samples overall (Table 3).

Variation Associated with Insect Herbivores. The two types of insects which were most abundant at our sites were leaf-feeding chrysomelid beetles early in the season and phloem-feeding psyllids late in the season. For both, feeding was heaviest upon plants containing lower levels of IYG and lacking OYG (Table 7).

Plants without chrysomelid beetles may have had higher concentrations of IYG (0.48 vs. 0.38 mg/g), but the difference was not significant (Mann-Whitney U test, $p = 0.34$) given our sample sizes (Table 7). Of the ten plants sampled in relation to chrysomelid feeding at the First Streamlet Site, Copper Creek, on July 5, 1979, none had OYG. Additionally, there was only one significant difference in the distribution of constituent IYG between plants with and without beetles (Table 9). Plants with adult beetles had significantly

TABLE 9. CONSTITUENT ISOTHIOCYANATE-YIELDING GLUCOSINOLATE (IYG) CONCENTRATIONS (mg/g) FOR *Cardamine cordifolia* INDIVIDUALS WITH AND WITHOUT CHRYSOMELID LEAF-FEEDING BEETLES ON JULY 5, 1979, FIRST STREAMLET SITE, COPPER CREEK DRAINAGE, GOTHIC, COLORADO,[a] $\overline{X}$ (SE).

	N (plants)	Total IYG (mg/g)	Constituent IYG[b]				
			a	b	c	d	e
No beetles (0)	5	0.48 (0.11)	0.06 (0.02)	0.26 (0.06)	0.15 (0.023)	0.00 (0.00)	0.02 (0.01)
Beetles (1–10)	5	0.38 (0.11)	0.05 (0.01)	0.18 (0.06)	0.14 (0.04)	0.00 (0.00)	0.005 (0.004)
P[c]			0.50	0.27	0.50	—	0.01

[a] Leaf + stem material.
[b] Labeled as in Table 2.
[c] Mann-Whitney U test, significant differences ($P < 0.05$) underlined.

lower concentrations of 2-phenylethylglucosinolate (0.004 mg/g, SE = 0.004; Mann-Whitney U test, $P = 0.01$) than did plants without (0.03 mg/g, SE = 0.009). Neither set of plants, with or without beetles, had any benzylglucosinolate recorded.

Psyllid feeding showed a similar pattern to chrysomelid feeding. Leaves of plants with large psyllid colonies lacked OYG and had a lower total IYG concentration (Mann-Whitney U test, $P = 0.06$) than did leaves of plants with few psyllids (Table 7). Only two stem samples were collected, one for each condition, and these did not differ in total concentration of IYG (0.16 mg/g vs. 0.15 mg/g), although they did differ with respect to the presence of OYG. Additional information on stem glucosinolate constituents would be of interest because psyllid densities were highest on stems and leaf petioles (Louda, personal observation).

DISCUSSION

This study examined the glucosinolate profile of the native mustard, *Cardamine cordifolia*, in order to address two main questions. The first was: does significant quantitative variation in glucosinolate composition occur among individual plants in the field? Our results show that quantitative variation was even more extensive than expected based on previous work (Rodman and Chew, 1980). This finding made our second question even more relevant: could the variation be partitioned so that implications of the variability for insect–plant interactions and for plant chemical defense theory

could be deduced? We focused on parameters hypothesized to influence plant performance and insect herbivore utilization of host plants: developmental and structural distribution of secondary compounds as well as habitat and elevational patterns of plant occurrence (e.g., Feeny, 1970, 1975, 1976, 1977; Rhoades and Cates, 1976; Levin, 1976; Rosenthal and Janzen, 1979).

The five IYGs in *Cardamine cordifolia* provide it with a unique secondary compound profile among crucifers in its plant community (Rodman and Chew, 1980). The predominant compound in all vegetative organs of *C. cordifolia* was 2-butylglucosinolate. The next most common was isobutylglucosinolate in leaves and stems and isopropylglucosinolate in roots. These results are consistent with those of Rodman and Chew (1980), who collected their material in mid-July 1978 in the same study region. Although we converted peak areas from gas chromatograms to the glucosinolate anion equivalents whereas they did not, the results on relative contribution of constituent IYGs were similar. In 1978, the relative contribution of 2-butylglucosinolate in leaf material was 62.0% (SE = 4.97, $N = 13$), compared to 58.3–63.2% in our 1979 leaf samples.

Variation in Relation to Developmental Age and Leaf Position. Our results for this native plant are consistent with published data on the distribution of glucosinolates in root and green parts of commercial mustards (Josefsson, 1967a; VanEtten and Tookey, 1978, 1979). As was the case in our data, root concentrations were 2–3 times higher than leaf concentrations in several cultivars of *Brassica oleracea* (Josefsson, 1967a; 0.27 mg/g vs. 0.07 mg/g, determined as "isothiocyanate equivalents"). Josefsson (1967a) found nearly equal concentrations of isothiocyanates in stems compared to leaves, whereas our data showed much higher concentrations in leaves, a trend consistent with results summarized by VanEtten and Tookey (1979) for other cultivars of *B. oleracea*.

Glucosinolate content is known to vary between older and younger parts of a plant (Josefsson, 1967b; Cole, 1980). One general pattern is low concentrations in very young leaves, increasing concentrations with development, leading to highest concentrations in newly matured leaves, and decreased concentration in old senescing leaves (Cole, 1980). Two sets of factors have been proposed as driving variables in the patterns of occurrence and change in potentially toxic secondary compounds between leaves and tissues of different ages: (1) change in internal physiological demands related to carbon fixation (e.g., Mooney and Chu, 1974) with constraints on storage of allelochemics in young, rapidly growing tissues, and (2) variation in external herbivore pressure related to probability of discovery and relative cost of loss among leaves of different ages (Feeny, 1970; Cates, 1975; Rhoades and Cates, 1976). McKey (1979) points out that the observed distribution of compounds must accommodate both of these pressures.

The glucosinolate distribution within *C. cordifolia* plants is consistent with McKey's predictions. For *C. cordifolia* we examined two sets of young leaves with different estimated value to the plant. One set was the smaller, terminal leaves on the vertical stems. These represent rapidly growing tissues of premium value to the plant because *C. cordifolia* produces terminal flowering racemes. Loss of these leaves and of terminal meristematic tissue eliminates reproduction by that stem (Louda, unpublished data). These leaves had the highest concentrations of glucosinolates (0.35 mg/g), providing high levels of potential defenses against unadapted herbivores. The second set of young leaves which we examined were those newly emerged from the root crown and rhizomes. These leaves, which are produced throughout the season (Louda, personal observation) and which could be considered less costly to lose, had the lowest concentrations of glucosinolates observed (0.06 mg/g). There may be a developmental component to this latter result. Cotyledon leaves and young primary leaves of cultivated mustard seedlings also have low concentrations of glucosinolates which later increase (Cole, 1980). In our case, mature, actively photosynthesizing leaves near the middle of the stem had higher concentrations of IYG (0.19 mg/g) than did the oldest leaves on the lowest 3 cm of stem (0.12 mg/g) (Table 5).

IYG Concentrations in Relation to Elevation and Habitat. Our results suggest that some of the variation in glucosinolate content in *C. cordifolia* occurs in relation to environmental variation. First, as mentioned, the frequency of OYG production increased between the early July and the late August sets of samples, i.e., as the season progressed. Second, we found a linear increase in the frequency of OYG in leaf material and a nonlinear relationship in total IYG concentration with increase in elevation from 3070 to 3450 m. The elevational gradient in the Rocky Mountains integrates variation in temperature and radiation. Both of these have been shown important in the physiology of another alpine mustard, *Thlaspi* (*alpestre*) *montanum* (Rochow, 1970). Furthermore, a plant's herbivore load can change over a gradient and influence plant performance (Handel, 1976; Louda, 1978, 1982, 1983a). Rochow (1970), in fact, found that insects and pathogens contributed to decreased performance of *Th. montanum* at the lower portions of the elevational gradient on which he worked in the Rocky Mountains of Wyoming. However, alternately, the nonlinear pattern in IYG over the gradient may represent spatial patchiness in glucosinolate phenotypes of *C. cordifolia.*

Third, we found no difference in total IYG concentration between plants occurring naturally in a sun exposure and those occurring under the more usual, shaded conditions. However, with experimental reduction of naturally occurring shade over plants in situ, we recorded a significant increase in total IYG and component 2-butylglucosinolate in leaves and a significant decrease

in benzylglucosinolate in stems of exposed plants compared to shaded ones after six weeks. These data lead us to hypothesize that the physiological stress accompanying sudden change in environmental conditions caused increased accumulation of IYG by *Cardamine cordifolia*. The frequency, consistency, and magnitude of this response to stress in *C. cordifolia* and in mustards in general are presently unknown. Given the correlation between plant stress and insect-caused damage (White, 1969, 1974, 1976; Lewis, 1979), this result is significant and merits further investigation.

Tradeoff of IYG and OYG Analogs. Our data on occurrence of OYG and concentrations of IYG for samples with and without OYG (Tables 3 and 4) suggest that there is either a temporal sequence of glucosinolate production by some plants or a tradeoff in the accumulation of IYG and biosynthesis of related OYG within individuals. Additionally, the hydroxylated (OYG) analogs of the principal nonhydroxylated (IYG) glucosinolates occur in some individuals but not in others, so this variation could represent a genetic polymorphism (Rodman and Chew, 1980). First, IYG concentration and OYG occurrence tended to be inversely related; total IYG concentration was higher in the absence of OYG for both leaf + stem material of the first collection and stem material of the second collection. These results are consistent with published information for cultivated mustard species. Namai and Hosoda (1975) reported a negative correlation between goitrin (5-vinyloxazolidine-2-thione) content and volatile isothiocyanates in seeds of *Brassica* species, except *B. campestris*. Ju et al. (1980) substantiated this pattern for *B. napobrassica* (rutabaga) and *B. rapa* (turnip). They also demonstrated a delayed accumulation of progoitrin, 2-hydroxy-3-butenyl-glucosinolate, compared to the production of indolyl and volatile (IYG) glucosinolates in growth of these two species from seed.

Second, there was a possible reversal in this tradeoff in leaf material in late August. This finding lends support to the hypotheses that there are two groups of plants with respect to temporal sequence of or tradeoff between accumulation of IYG and biosynthesis of OYG and that a genetic polymorphism in this capacity may exist. Average total IYG in this case was higher, not lower, for samples with OYG, and this difference was at least potentially significant ($P = 0.10$). In addition, average concentration of 2-phenylethylglucosinolate was significantly higher when its OYG form was present than when the form was absent. However, the opposite was true for isopropylglucosinolate. The concentration of isobutylglucosinolate was independent of OYG occurrence. Furthermore, total IYG increased significantly more rapidly in samples with OYG than in samples without them as sample leaf weight increased (Figure 2), as might have been expected under the tradeoff hypothesis. Genetic polymorphism in the capacity to produce and accumulate hydroxylated analogs (OYG) of IYG over the season is a viable hypothesis for this variation in content (Rodman and Chew, 1980).

Glucosinolates and Herbivore Utilization. Feeny (1977) summarized arguments for the hypothesis that glucosinolates represent qualitative defensive compounds in the Cruciferae. Glucosinolates or their breakdown products are toxic and deterrent for unadapted insects (Lichtenstein et al., 1962; Erickson and Feeny, 1974; Blau et al., 1978) but may be attractants or stimulants for species which have detoxification or avoidance mechanisms (Thorsteinson, 1953; Gupta and Thorsteinson, 1960; Schoonhoven, 1967, 1969; Nair and McEwen, 1976; Nair et al., 1976; Hawkes and Coaker, 1979). The growth of crucifer-feeding insects, such as *Pieris rapae* (Slansky, 1974; Slansky and Feeny, 1977; Blau et al., 1978) or *Brevicoryne brassicae* (van Emden, 1972), has been demonstrated to be independent of glucosinolate concentrations except at artificially boosted levels (Hicks, 1974; Marsh and Rothschild, 1974). Yet differential feeding responses occur among crucifer-feeding insects, and these may be mediated by glucosinolate composition and concentration (Thorsteinson, 1953; David and Gardiner, 1966; Nayar and Thorsteinson, 1963; Hicks, 1974). Rhoades and Cates (1976), in fact, have argued that glucosinolates operate as both qualitative defenses against unadapted herbivores and quantitative defenses against partially adapted insect herbivores. Perhaps adaptation is seldom "complete" and responses to qualitative defensive compounds by most insect species can be placed along a quantitative continuum.

Our results are consistent with the hypothesis that glucosinolates function as quantitative defenses against partially adapted, crucifer-feeding insect herbivores. Both the chrysomelids and the psyllids are characteristic crucifer feeders (Bonnemaison, 1965; Root, 1973). Hicks (1974) found that 0.15 mg/g allylglucosinolate concentration was required to stimulate feeding of the related chrysomelid, *Phyllotreta cruciferae*; the effect increased up to 46 mg/g and dropped at 61 mg/g. Consequently, we expected a positive relationship between glucosinolate concentration and overall insect, and especially chrysomelid, occurrence. Our data, however, showed the opposite; insect occurrence was associated with lower total IYG concentrations and with the absence of OYG. These results support the hypothesis that higher levels of IYG, or of particular glucosinolates such as oxazolidinethione-yielding ones, may reduce feeding by otherwise adapted crucivores (Hicks, 1974; Rodman and Chew, 1980).

An alternative hypothesis, based on the observations of crucifer-feeding insect response to glucosinolates, was that the relative distribution of constituent IYG would correlate with pattern of use. In our case, the hydroxylated compounds (OYG) would be implicated because they were absent in plants utilized by chrysomelids in early July; they also were absent in plants utilized at high levels by psyllids in late August. Additionally, 2-phenylethylglucosinolate was significantly lower in plants with beetles than in those without beetles. There may be an inverse relationship, repellency

perhaps, between presence and concentration of particular glucosinolates in *C. cordifolia* and crucivore feeding. While seemingly anomalous, these results are consistent with some previously published work (e.g., Nayar and Thorsteinson, 1963) and with the Rhoades and Cates (1976) hypothesis of dual function. The extensive quantitative variation in the glucosinolate profile of *C. cordifolia* may contribute to persistence of the crucifer–beetle interaction in a manner similar to that proposed by Dolinger et al. (1973) for the lupine–lycaenid system. Variation in glucosinolate concentrations should spread chrysomelid utilization differentially among *C. cordifolia* plants at different times, making tracking by beetles difficult and limiting the tightness of potential coevolution.

Further data are warranted for the relationship between psyllid feeding and glucosinolate levels. Our sample sizes were small, and an alternative hypothesis to account for psyllid feeding and numbers exists. White (1969, 1974, 1976, 1978) suggested that psyllid populations specifically, and insect herbivores generally, are limited by the availability of soluble nitrogen, particularly for the young stages. The review and work of McNeill and Southwood (1978) provide support for the nitrogen hypothesis. This hypothesis would be consistent with our results if psyllid occurrence were related to nitrogen availability as well as glucosinolate profile. Work is in progress to test the alternative hypothesis in relation to glucosinolate patterns over time and to evaluate the generality of these results for the insect–glucosinolate interaction. The patterns found so far, however, provide substantial support for the hypothesis that glucosinolate variation plays a role in the dynamics of the insect–plant interaction for this native crucifer species.

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ANALYSES OF AN EGG RECOGNITION KAIROMONE OF *Telenomus heliothidis* (HYMENOPTERA: SCELIONIDAE)
Isolation and Host Function

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Abstract—Two large proteins (>330 K) present in the accessory gland of *Heliothis virescens* F. (Lepidoptera: Noctuidae) serve as an egg recognition kairomone for the parasitoid *Telenomus heliothidis* Ashmead (Hymenoptera: Scelionidae). Glass beads coated with the isolated proteins were examined and probed by the parasitoid while uncoated glass beads were not. Electrophoretic analysis of the contents of the *H. virescens* accessory gland suggests that the gland's contents serve as an egg adhesive. Comparison of the gland proteins revealed that the *T. heliothidis* host *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) accessory gland contained the kairomonally active proteins while the nonhost *Spodoptera frugiperda* (Lepidoptera: Noctuidae) did not.

Key Words—*Telenomus heliothidis*, *Heliothis*, Hymenoptera, Scelionidae, accessory gland, colleterial gland, host recognition, kairomone, parasitoid.

INTRODUCTION

The importance of kairomones in host selection is well documented for larval parasitoids (Vinson, 1976, 1981; Weseloh, 1976; Arthur, 1981). With the exception of the research performed on *Trichogramma* spp. (Salt, 1935; Jones et al., 1971; Lewis et al., 1975), very little is known of the involvement of similar chemical stimuli in host selection by egg parasitoids.

Strand and Vinson (1982a) showed that the parasitoid *Telenomus heliothidis* Ashmead recognized its host, the eggs of *Heliothis virescens* F., by the presence of a secretion found on deposited eggs. This secretion stimulated examination of eggs as well as initiation of ovipositor drilling. The source of

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this secretion was the accessory (colleterial) gland of adult female *H. virescens*. Glass beads approximately equal in size to *H. virescens* eggs and coated with the accessory gland material were readily attacked by *T. heliothidis* while uncoated glass beads were not. Based upon enzymatic studies and the presence of a large amount of protein stored in the accessory gland, it was suggested that the kairomone is proteinaceous. Preliminary work further indicated that the accessory gland serves as the source of an adhesive for the attachment of eggs to a given substrate. *T. heliothidis* also attacks the eggs of *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae), but does not attack the eggs of *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae).

We report here the isolation of the *H. virescens* recognition kairomone, its presence in *H. zea*, its absence in *S. frugiperda*, and the demonstration of a host function for the accessory gland material.

METHODS AND MATERIALS

Rearing of Insects. T. heliothidis were reared by a method similar to that of Morrison (1970). Adults were maintained on honey in 36.5 × 5-cm tubes at 25° C. *H. virescens* eggs were offered daily for parasitization. Parsitized eggs were placed in clean glass tubes and incubated at 30° C under 16 hr light–8 hr dark photoperiod. Emergence occurred in ca. 13 days.

H. virescens and *H. zea* larvae were reared on the medium of Vanderzant et al. (1962). Adult moths were kept in 4-liter glass jars covered with paper towels. Moths were fed a 5% aqueous sucrose solution. *S. frugiperda* larvae were reared on bean diet, with the adults maintained as previously described for *H. virescens* and *H. zea*.

Preparation of Samples for Gel Electrophoresis. Prior to electrophoretic analysis of accessory gland proteins, experiments were designed to determine whether the biological activity of accessory gland material could be recovered after exposure to disruption buffer solution. The contents of two *H. virescens* accessory gland reservoirs were placed in buffer [0.5 M Tris aminomethane (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10 M urea. 1% mercaptoethanol]. The solution was then dialyzed in 10 K dialysis tubing. The retenulate was lyophilized to dryness, reconstituted with distilled water to 30 μl, and applied to 0.56-mm glass beads.

For gel samples, accessory glands were dissected under saline from *H. virescens*, *H. zea*, and *S. frugiperda* females, 48–60 hr hold. The accessory gland was cut, separating the actual gland, or filament, from the gland reservoir. The gland reservoir was placed on a broken fragment of glass coverslip and pierced with forceps, releasing the reservoir's contents (ca. 1–1.5 μl) onto the coverslip fragment. The reservoir was then discarded. This

procedure was necessary because the great viscosity of the gland material precluded easy separation of the reservoir from its contents. The glass fragment and the associated gland material were placed in electrophoresis disruption buffer. The gland filaments of each species were placed directly into disruption buffer.

Based upon the previous determination of 200 mg/ml protein present in the gland reservoir of *H. virescens* (Strand and Vinson, 1982a), and the approximately equal size of the accessory glands of *H. zea* and *S. frugiperda*, the contents of two gland reservoirs for each species were placed in 100 μl of disruption buffer. This adjusted the protein content of each analytical sample to ca. 4 μg/μl, and each preparative sample to 24 μg/μl. The egg adhesive of *H. virescens* was collected by wetting (with distilled water) pieces of paper towel onto which eggs had been deposited. The water rehydrated the adhesive, allowing removal of the eggs with the aid of forceps. The sticky, adhesive substance remaining on the paper was collected by scraping with forceps and was placed in disruption buffer. Enough material was collected in this manner to obtain a concentration of 2–4 μg/μl.

High-molecular-weight standards (Pharmacia Fine Chemicals, Uppsala, Sweden) consisting of 50 μg each of thryoglobulin (330 K), ferritin (220 K), albumin (67 K), catalase (60 K), and lactate dehydrogenase (36 K) were placed into 100 μl of aqueous 1% mercaptoethanol, 1% SDS disruption buffer. Standards were heated to 60° C for 15 min prior to gel application. All samples of gland reservoir, gland filament, and egg adhesive were treated similarly prior to gel application.

Polyacrylamide Gel Electrophoresis and Sample Elution. The polyacrylamide gel system of Laemmli (1970) was used for protein separations. Ten to 15 μl of sample protein was applied per well on analytical gels. On preparative gels, the entire 100-μl sample was applied across the top of the gel. Electrophoresis was performed at a constant current of 7.5 V on 6–7% polyacrylamide slab gels 1.0 mm thick. After electrophoresis, the analytical gels were removed, prestained in 25% isopropyl alcohol and 10% acetic acid, stained with Coomassie blue, and destained in 7.5% acetic acid.

After electrophoresis of preparative gels, a 4-mm vertical strip was cut, notched for reference from the slab, and stained as previously described. After inspection of the stained strip, the unstained slab was cut into appropriate sections. The cut sections were placed in collecting cups (Lazarides and Weber, 1974) and the proteins were electroeluted from the gel at 9 V. Collected protein solutions were dialyzed for ca. 6 hr using 10 K dialysis tubing, and the retenulate was lyophilized to dryness. The proteins were then resuspended in 30 μl of distilled water. This procedure of protein separation, collection, dialysis, and reconstitution was repeated several times until bioassays revealed the active protein.

Bioassay Procedure. Lyophilized protein fractions were resuspended in 30 μl distilled water, and placed in 2-cm-diam watchglasses. Glass beads, 0.56 mm diameter, were coated separately with the protein solutions by gently rotating them with forceps. After coating, the beads were removed from the watchglass and allowed to dry. An estimated 10–20 μg of protein was applied to each bead.

All bioassays were conducted in the round depression of a flat-bottomed (17 mm diam × 3 mm deep) depression slide covered with a glass cover slip. Observations were made with the aid of a stereoscopic microscope and timed with a stopwatch. Groups of five beads, coated with the same protein solution, arranged in a circle on the bottom of the observation chamber were used for each trial of a given bioassay. The presence of more than one bead per trial served only to increase the likelihood of host encounter by *T. heliothidis*.

Only female parasitoids 72–96 hr old which had previously oviposited into *H. virescens* eggs were used in bioassays, since previous ovipositional experience may affect host selection (van Lenteren 1976; Klomp et al., 1980; Strand and Vinson, 1982b). Scoring of bioassays was performed as follows: one female parasitoid was released into the observation chamber and only her first contact was scored. A "rejection" was scored if she touched a bead with her antennae but exhibited no examination behavior (Winburn and Painter, 1932; Strand and Vinson, 1982a). An "examination" was scored if a female mounted a bead and initiated examination but failed to attempt to drill. Examination time was recorded for 0–5 sec, 5–10 sec, and greater than 10 sec. An "acceptance" was scored if the female mounted a bead, examined it, and attempted to drill the bead with the ovipositor. Uncoated beads and beads coated with unelectrophoresed accessory gland reservoir material were used as controls.

RESULTS

Beads coated with accessory gland material which had been placed in disruption buffer, dialyzed, and lyophilized were accepted as hosts by *T. heliothidis* while uncoated beads were not (coated beads: accepted = 15; rejected = 5; uncoated beads: accepted = 0; rejected = 20; $\chi^2 = 40.3$, $df = 1$, $P < 0.001$). This indicated that the kairomonal activity could be recovered after placement in disruption buffer. In addition, the active material appeared to be greater than 10 K since smaller molecules were able to pass through the dialysis tubing. All coated beads that were not accepted were examined ($x = 13.8$ sec, SD = 9.6, $N = 7$); however, none of the uncoated beads were examined. Beads coated with only disruption buffer were also ignored by *T. heliothidis*.

Electrophoresis of the *H. virescens* accessory gland contents revealed

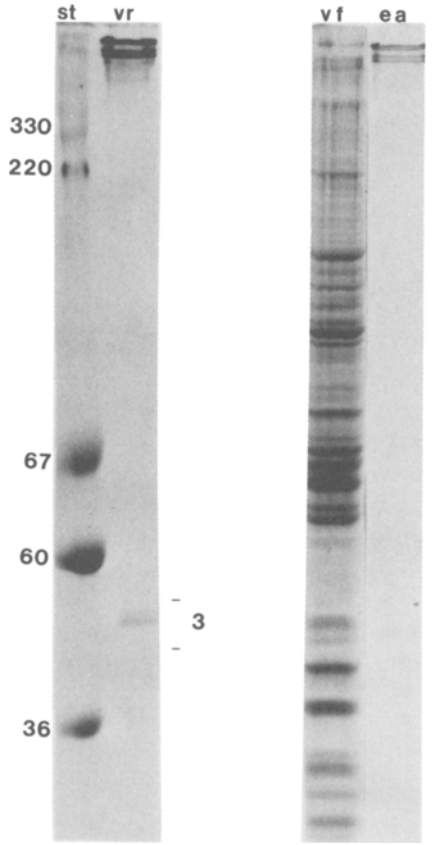

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of accessory gland proteins and egg adhesive of *H. virescens*. Abbreviations are as follows: st, standards; vr, *H. virescens* accessory gland reservoir contents; vf, *H. virescens* accessory gland filaments; ea, *H. virescens* egg adhesive. Regions of the preparative SDS–polyacrylamide gels of the *H. virescens* accessory gland reservoir were cut as indicated by the demarcated regions of the vr lane.

that while numerous proteins were present in the filaments of the gland, the material stored in the reservoir consisted of only a few proteins (Figure 1). Each of the two most prominent bands of the accessory gland reservoir actually consists of a poorly resolved doublet. The electrophoretic mobilities of the major proteins of the adhesive collected from *H. virescens* eggs were the same as those of the *H. virescens* accessory gland reservoir (Figure 1).

Preparative 6% polyacrylamide gels of the accessory gland reservoir proteins were cut into the regions indicated (Figure 1) for collection and reconcentration. Testing of the 30-μl protein solutions immediately after

lyophilization produced no positive results. All tested fractions elicited no examination or drilling response from *T. heliothidis* females. However, fractions which were stored at 20° C in a 12 hr light–12 hr dark photoperiod for 5 days elicited a behavioral response from the parasitoid, as indicated in Table 1. The first protein region was the source of activity with 41% of the parasitoids tested accepting these beads as hosts, and a total of 69% of the parasitoids at least extensively examining the beads. Only a very small percentage of beads coated with the other two fractions were accepted by *T. heliothidis*. Combinations of fractions did not enhance the overall rate of bead acceptance. Storing the fractions for a longer period (up to three weeks) before bioassaying did not elevate the rate of acceptance.

For statistical analysis, the columns "examined and accepted egg" and "examined >5 sec; rejected egg" were combined into one group, and the columns "examined <5 sec; rejected egg" and "rejected egg" were combined into one group. Such an arrangement was used because the first group indicated a strong response to the treatment while the second group indicated little or no response. A chi-square test of homogeneity of proportions (Marascuilo and McSweeney, 1977) revealed that the proteins of regions 1 were significantly more effective than regions 2 and 3 ($-0.645 < \psi_1 < -0.405$, $df = 2, \alpha = 0.05$), but were no less effective than combined equal amounts of regions 1 and 2 or regions 1, 2, and 3 ($-0.123 < \psi_2 < 0.263, df = 2, \alpha = 0.05$).

TABLE 1. ACCEPTABILITY TO *T. heliothidis* OF MODEL (0.45 TO 0.55-mm) HOST EGGS TREATED WITH SEPARATED PROTEINS FROM HOST ACCESSORY GLAND CONTENTS

		Response to target "eggs" (%)			
Gel region	N^a	Examined and accepted egg	Examined >5 sec; rejected egg	Examined <5 sec; rejected egg	Rejected egg
1	111	41	28	14	17
2	135	5	16	35	44
3	139	2	10	21	67
1 + 2	102	35	35	20	10
1 + 2 + 3	43	42	40	14	4
Control, coated[b]	50	74	14	4	8
Control, uncoated	50	0	4	12	84

[a] Number of females tested for each condition.
[b] Glass beads coated with nonelectrophoresed gland reservoir proteins.

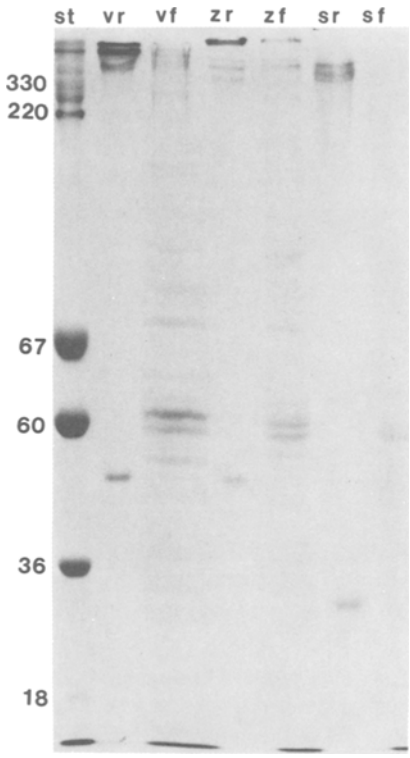

FIG. 2. Comparative SDS–polyacrylamide gel electrophoresis of the accessory gland proteins of *H. virescens*, *H. zea*, and *S. frugiperda*. Abbreviations are as follows: st, standards; vr, *H. virescens* accessory gland reservoir contents; vf, *H. virescens* accessory gland filaments; zr, *H. zea* accessory gland reservoir contents; zf, *H. zea* accessory gland filaments; sr, *S. frugiperda* accessory gland reservoir contents; sf, *S. frugiperda* accessory gland filaments.

The kairomone's molecular weight was greater than 330 K, and by extrapolation was estimated to be approximately 1100 K.

The accessory gland filament proteins of *H. virescens* and *H. zea* were very similar (see Figure 2), with major proteins at about 250, 80, 70, and 60 K present in both species. The accessory gland reservoir protein of about 50 K was also present in both *H. virescens* and *H. zea*. Based on R_f values, kairomonally active proteins of *H. virescens* were also apparently present in *H. zea*. The smaller pair of proteins of slightly smaller molecular weight than those of the active proteins of *H. virescens* were absent from *H. zea*. The accessory gland filament proteins of *S. frugiperda* were only slightly similar to

those of *H. virescens* and *H. zea*. Major proteins at 60 K were present in all three species, but proteins at 250, 80, and 70 K were different in *S. frugiperda* as compared to *H. virescens* and *H. zea*. The accessory gland reservoir proteins were also quite different. *S. frugiperda* had a protein present at about 32 K which was absent from *H. virescens* and *H. zea*, and lacked a protein at 50 K found in the other two species. The kairomonally active proteins of *H. virescens* were absent from *S. frugiperda*, even though large proteins (330–500 K) were present.

DISCUSSION

This study indicates that *T. heliothidis* recognizes its host, the eggs of *H. virescens*, through two very large proteins (>300 K) which are the primary components of the female accessory gland reservoir contents. These proteins elicited examination and attempted drilling when placed on glass beads equal in size to *H. virescens* eggs. However, previous work (Strand and Vinson, 1982a, in preparation) demonstrated that host size and shape are also important criteria in host acceptability by *T. heliothidis*.

The function of accessory glands in noctuids has not been demonstrated even though Callahan and Casio (1963) and Ferro and Akre (1975) suggest that they produce an adhesive for attaching eggs to the substratum. The current study demonstrates that protein in the accessory gland electrophoretically corresponds to adhesive found on deposited *H. virescens* eggs, strongly indicating that the accessory gland material is at least a component of the adhesive. While substances such as host frass (Arthur, 1981) and moth scales (Lewis et al., 1975) have been found to contain kairomonally active compounds, the precise host function of these compounds has not been demonstrated. Thus, this is the first reported case in which a kairomone has proven to be a nonpheromonal product useful to the host. The dual activity of the substance possibly suggests that the ability to attach eggs to the appropriate host plant is of greater importance to *H. virescens* than the selective pressure of parasitism by *T. heliothidis*.

While 0.56-mm glass beads coated with accessory gland material directly from the gland were approximately 75% accepted by *T. heliothidis* (Strand and Vinson, 1982a), only 41% of the beads coated with the separated, active proteins were accepted in the current study. Two possibilities could account for this difference: either an active factor was lost during the purification process, or the proteins which were found to be active were structurally changed during purification.

The possibility of having lost an active component cannot be totally discounted, but it does not seem to be the most likely reason for the decrease in the observed activity. The accessory gland reservoir proteins were tested to see

if the biological activity could be recovered prior to electrophoretic separation. The buffer was removed by dialysis along with any other molecules smaller than 10 K. The bioassay revealed a 75% acceptance of the beads coated with the retenulate, indicating that the material's activity could be fully recovered after placement in disruption buffer. If a smaller protein or nonprotein molecule is important to the accessory gland material's activity, it probably would have been lost during this preliminary experiment. In addition, since only the proteins from region 1 displayed any activity, it is unlikely that sample contamination could have caused the restoration of activity.

It is more likely that the loss of activity was caused by heat-induced denaturation of sample proteins, a necessary preliminary to SDS-polyacrylamide electrophoresis. After denaturation, stable proteins often partially renature (McGilvery, 1970). The lack of biological activity of the proteins immediately after separation, followed by fairly strong activity after storage for 5 days suggests that renaturation of the proteins occurred. The incomplete restoration of activity may be due to the failure of the proteins to completely reassume their original tertiary structure.

The electrophoresed proteins from the accessory gland reservoir of *H. virescens* and *S. frugiperda* show some similarities and differences when compared to the proteins of *H. virescens*. The electrophoretic mobilities of the active proteins of *H. virescens* very closely match the primary constituents of the *H. zea* reservoir. Since the eggs of *H. zea* are also an acceptable host of *T. heliothidis*, the proteins are most likely quite similar in structure and function to those of *H. virescens*. In contrast, the accessory gland reservoir proteins of *S. frugiperda* are distinctly different from *H. virescens* and *H. zea*. It would appear that the differences in these proteins are at least partially responsible for the inability of *T. heliothidis* to recognize *S. frugiperda* eggs as hosts, even though *S. frugiperda* often occurs on the same plant species as *H. virescens* and *H. zea*.

The existence of recognition kairomones could be an important link in the development of acceptable artifical hosts for more species specific parasitoids. Such factors may aid in inducing acceptance of such artificial hosts, allowing easier mass parasitoid rearing.

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SEX PHEROMONAL ACTIVITY OF (+)-BORNYL ACETATE AND RELATED COMPOUNDS TO THE AMERICAN COCKROACH

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Abstract—Many compounds related to (+)-bornyl acetate (a sex pheromone mimic of the American cockroach) were synthesized and tested for sex pheromonal activity. All compounds except for esters of (+)- and (−)-borneol were inactive, whereas (+)-bornyl acetate (Ib) and propionate (Ic) showed the activity at 0.05 mg. Although (−)-bornyl propionate (IVc) is the enantiomer of Ic, it exhibited weak activity at 0.5 mg dose. On the basis of the behavioral assay results, important chemical factors in Ib for pheromonal activity were elucidated and are discussed in connection with another mimic, (+)-*trans*-verbenyl acetate. The M/F ratio index in EAG was evaluated for both active and inactive compounds. The index demonstrated a good correlation with the behavioral activity.

Key Words—Sex pheromone mimics, American cockroach, Orthoptera, Blattidae, *Periplaneta americana*, (+)-bornyl acetate, (+)-bornyl propionate, EAG, M/F ratio, M/F ratio index.

INTRODUCTION

The first sex pheromone mimic of the American cockroach (*Periplaneta americana* L.), (+)-bornyl acetate (Ib), was discovered by Bowers and Bodenstein (1971) together with other mimics including α- and β-santalol and an unidentified sesquiterpene hydrocarbon ($C_{15}H_{24}$).

The first mimic Ib, which is a monoterpenoid, is structurally quite different from the natural sex pheromones of the insect (Persoons et al., 1979; 1982), which are sesquiterpenoids. In order to determine the chemical functional groups in Ib important for sex pheromonal activity, we synthesized several alcohols and carboxylic acids related to (+)-borneol (Ia), prepared

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their esters, and evaluated their activity by behavioral and electroantennographic assays. In the present work, we wish to report the assay results and the important factors elucidated from the biological activity–chemical structure relationships. Since the important factors of the other monoterpenoid sex pheromone mimic, (+)-*trans*-verbenyl acetate (VA) (Nishino et al., 1977), have been proposed by us (Nishino and Takayanagi, 1981a; Takayanagi and Nishino, 1982; Nishino et al., 1982; Manabe et al., 1983), the structural relationships between Ib and VA will be discussed here.

A useful supplementary index to the conventional behavioral activity is the M/F ratio index, which was used for the synthesized compounds on the basis of the results obtained from the electroantennogram (EAG) responses from the adult male and female antennae.

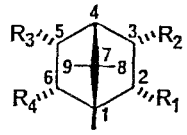
METHODS AND MATERIALS

Synthesis of Compounds. The structures of the target compounds are illustrated in Figure 1, and synthetic routes to the compounds are given in Figure 2.

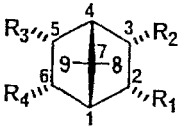
All melting points were uncorrected. Specific rotations were measured in benzene at 25° C, unless otherwise stated. IR spectra were recorded as a liquid film or KBr pellet. Samples for [^{1}H]-NMR (PMR) spectra were dissolved in carbon tetrachloride (CCl_4) or deuteriochloroform ($CDCl_3$) with tetramethylsilane (TMS, internal standard) and subjected to measurement at 60 MHz. Chemical shifts (δ ppm) were measured from the TMS signal. Mass spectra (MS) were taken with a direct inlet system or with a GC-MS system in which the column used was the same as in gas chromatographic (GC) analysis. GC analysis was performed using a 2-m × 3-mm glass column packed with 3% OV-225, and nitrogen gas (35 m./min). Retention time (t_R) was expressed in minutes. All of the compounds were estimated to be almost 100% purity by the GC analysis.

Esters of the alcohols were prepared by the usual method with acid anhydride and pyridine.

(+)-*Borneol* (*Ia*), (+)-*Bornyl Acetate* (*Ib*) *and* (+)-*Bornyl Propionate* (*Ic*). (+)-Bornyl acetate (Ib) with $[\alpha]_D$ +34.0° (c = 1.00) was kindly supplied by Dr. Bowers. Saponification of Ib afforded Ia [mp 202° C, from petroleum ether (pet. ether)]; $[\alpha]_D$ +34.0° [c = 1.00]), which was converted to Ic, $[\alpha]_D$ +32.2° (c = 1.00); IR (film): 1737, 1190 cm^{-1}; PMR ($CDCl_3$): 0.84 [3H, singlet (s)], 0.89 (3H, s), 9.02 (3H, s), 1.16 [3H, triplet (t), J = 7 Hz], 2.35 [2H, quartet (q), J = 7 Hz], 4.95 [1H, double double doublet (ddd), J = 10, 3.5, 2 Hz]; MS: m/z 210 (M^+, $C_{13}H_{22}O_2$), 154 [M^+ −56 (CH_3CH_2CO)], 136 [M^+ −74 (CH_3CH_2COOH)], 121, 95 [base peak (B^+)]; t_R: 24.7 at 100° C.



Ia:	R_1=OH, R_2=R_3=R_4=H	(+)-borneol
Ib:	R_1=OCOCH$_3$, R_2=R_3=R_4=H	(+)-bornyl acetate
Ic:	R_1=OCOCH$_2$CH$_3$, R_2=R_3=R_4=H	(+)-bornyl propionate
IIa:	R_2=OH, R_1=R_3=R_4=H	(-)-epiborneol
IIb:	R_2=OCOCH$_3$, R_1=R_3=R_4=H	(+)-epibornyl acetate
IIIa:	R_3=OH, R_1=R_2=R_4=H	(+)-epiborneol
IIIb:	R_3=OCOCH$_3$, R_1=R_2=R_4=H	(-)-epibornyl acetate
IVa:	R_4=OH, R_1=R_2=R_3=H	(-)-borneol
IVb:	R_4=OCOCH$_3$, R_1=R_2=R_3=H	(-)-bornyl acetate
IVc:	R_4=OCOCH$_2$CH$_3$, R_1=R_2=R_3=H	(-)-bornyl propionate
Va:	R_1=COOH, R_2=R_3=R_4=H	(+)-endo- and (+)-exo-
VIa:	R_1=COOH(β), R_2=R_3=R_4=H	bornylcarboxylic acid
Vb:	R_1=COOCH$_3$, R_2=R_3=R_4=H	(+)-endo- and (+)-exo-
VIb:	R_1=COOCH$_3$(β), R_2=R_3=R_4=H	bornylcarboxylic acid methyl ester
VIIa:	R_4=COOH, R_1=R_2=R_3=H	(-)-endo- and (-)-exo-
VIIIa:	R_4=COOH(β), R_1=R_2=R_3=H	bornylcarboxylic acid
VIIb:	R_4=COOCH$_3$, R_1=R_2=R_3=H	(-)-endo- and (-)-exo-
VIIIb:	R_4=COOCH$_3$(β), R_1=R_2=R_3=H	bornylcarboxylic acid methyl ester



IXa:	R_1=OH, R_2=R_3=R_4=H (R_3=OH, R_1=R_2=R_4=H)	(+)-apoborneol
IXb:	R_1=OCOCH$_3$, R_2=R_3=R_4=H (R_3=OCOCH$_3$, R_1=R_2=R_4=H)	(-)-apobornyl acetate
Xa:	R_2=OH, R_1=R_3=R_4=H (R_4=OH, R_1=R_2=R_3=H)	(-)-apoborneol
Xb:	R_2=OCOCH$_3$, R_1=R_3=R_4=H (R_4=OCOCH$_3$, R_1=R_2=R_3=H)	(+)-apobornyl acetate

FIG. 1. Structures and common names of (+)-bornyl acetate (Ib) and its related compounds evaluated for sex pheromonal activity and M/F ratio index value.

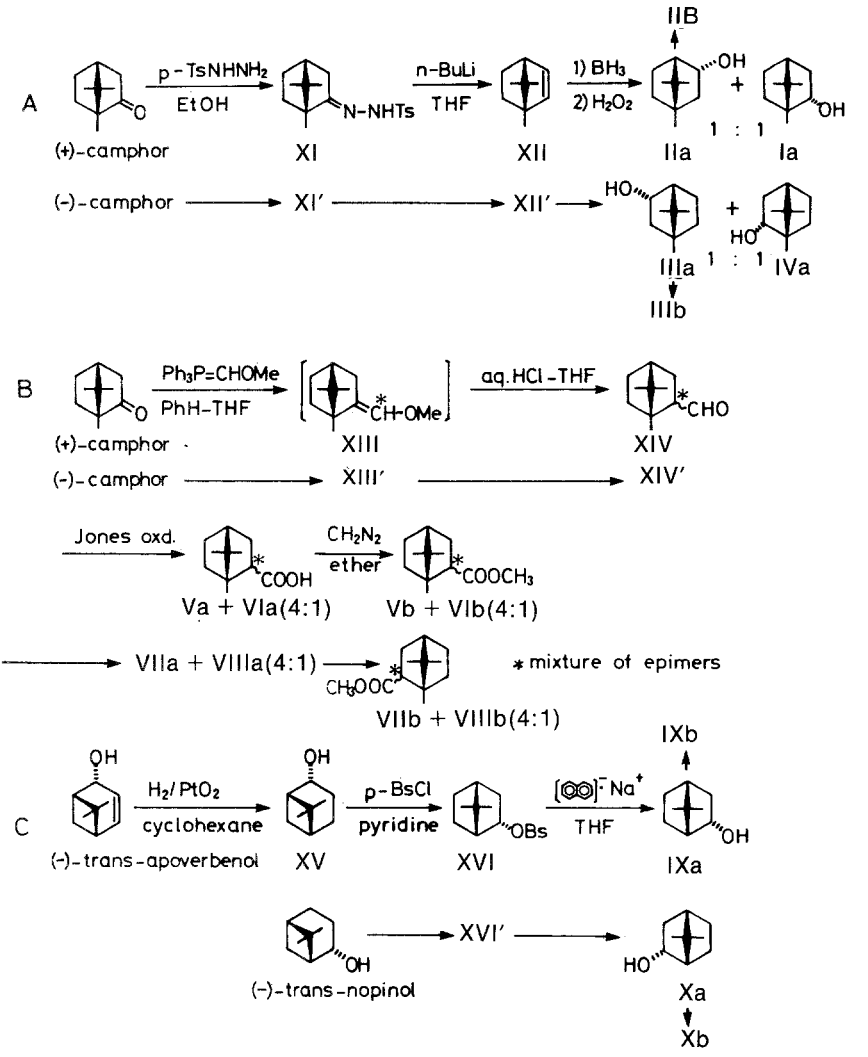


FIG. 2. Synthetic routes to testing compounds for behavioral and EAG essays. Starting materials of (+)- and (−)-bornyl analogs (I and IV series) were obtained as a gift (Ib) or commercial products (IVa and IVb).

(−)-Borneol (IVa), (−)-Bornyl Acetate (IVb) and (−)-Bornyl Propionate (IVc). (−)-Borneol (IVa) [mp 202° C (pet. ether); $[\alpha]_D$ −40.4° (c = 1.05)] was commercially obtained from Sigma Chemical Inc., while IVb ($[\alpha]_D$ −40.0° [c = 1.00]) from Aldrich Chemical Inc. IVc was prepared from IVa. IVc, $[\alpha]_D$ −40.0° (c = 1.00); the spectral data were identical with those of Ic.

Tosyl Hydrazone (XI). A mixture of (+)-camphor [10g, $[\alpha]_D$ +40.8° (c = 1.00), Tokyo Kasei Inc.] and *p*-toluenesulfonyl hydrazine (13.5 g) in absolute ethanol (15 ml) was refluxed for 30 hr. By cooling, pure XI (19g, 90% yield) was crystallized from the reaction solution. XI, mp 161–162° C (from aqueous ethanol); $[\alpha]_D$ −6.7° (c = 1.06); IR (KBr): 3190, 1660, 1600, 1335, 1165, 810 cm^{-1}; PMR ($CDCl_3$): 0.47 (3H, s), 0.82 (3H, s), 0.87 (3H, s), 2.37 (3H, s), 7.26 [2H, doublet (d), J = 8.5 Hz], 7.82 (2H, d, J = 8.5 Hz), 7.71 (1H, s, NH); MS: m/z 320 (M^+, $C_{17}H_{24}N_2O_2S$), 166 [M^+ −165 ($CH_3C_6H_4SO_2^+$), B^+], 136, 123, 121, 93, 91

(+)-2-Bornene (XII). To a solution of XI (3 g) in dry tetrahydrofuran (THF, 10 ml), *n*-butyllithium (15 ml of 1.6 N *n*-hexane solution) was added dropwise at −15° C under argon gas atmosphere. The reaction mixture was allowed to warm to room temperature, and then stirred for 2.5 hr. Subsequently, the mixture was recooled at 0° C, and quenched with water (10 ml). The mixture was poured into a mixture of water (90 ml) and *n*-pentane (150 ml). The separated aqueous layer was extracted with *n*-pentane (150 ml). The combined pentane layers were washed with water and brine, dried ($MgSO_4$), and concentrated below 0° C to 10 ml. To remove impurities, the concentrated solution was filtered with a short column packed with silica gel (solvent: *n*-pentane). The filtrate was carefully concentrated below 0° C to give crude XII which was subjected to the next step without further purification because of its extremely high volatility. XII, IR (CCl_4): 3040, 718 cm^{-1}; PMR (CCl_4): 0.76 (3H, s), 0.80 (3H, s), 1.00 (3H, s), 2.24 (1H, t, J = 3 Hz), 5.58 (1H, d, J = 5.5 Hz), 5.84 [1H, double doublet (dd), J = 5.5, 3 Hz]; MS: m/z 136 (M^+, $C_{10}H_{16}$), 121 [M^+ −15 (CH_3)], 93 (B^+), 91, 79, 77; t_R: 2.5 at 50° C (N_2 gas, 30 ml/min).

(−)-Epiborneol (IIa). To a solution of crude XII in *n*-pentane (5 ml) and THF (5 ml), 9-BBN (10 ml of 1 M THF solution) was added under argon gas atmosphere. The reaction mixture was stirred at room temperature for 24 hr, and then at 50° C for 3 hr. To the mixture, 6 M aqueous NaOH (10 ml) and 30% hydrogen peroxide (10 ml) were added successively, and heated to 60° C. After reaction at 60° C for 1.5 hr, the mixture was cooled to room temperature. Following the addition of saturated K_2CO_3 solution, the organic and aqueous layers were separated. The ether extract of the aqueous layer was combined with the original organic layer, washed with water and brine, dried, and concentrated to afford a residue which was a mixture of IIa with Ia (1 : 1 by GC) and a trace amount of other impurities. The residue was

repeatedly chromatographed over silica gel eluting with *n*-hexane–ethyl acetate (EtOAc) (10:1) to give IIa (252 mg, 17% from XI) with 99% purity. IIa was recrystallized from pet. ether. IIa, mp 180–181°C; $[\alpha]_D$ −8.2° (c = 1.00); IR (KBr): 3270 cm^{-1}; PMR (CCl_4): 0.79 (3H, s), 0.87 (6H, s), 1.92 (1H, s, OH), 4.31 [1H, double double double doublet (dddd), J = 10, 4, 3.5, ~2 Hz); MS: m/z 154 (M^+, $C_{10}H_{18}O$), 139 (M^+−15), 136 [M^+−18 (H_2O)], 121, 111, 95 (B^+), 93; t_R: 18.0 at 100°C (cf. t_R of Ia: 16.7).

(+)-*Epibornyl Acetate* (IIB), $[\alpha]_D$ +21.4°> (c = 1.07); IR (film): 1735, 1255 cm^{-1}; PMR (CCl_4): 0.83 (3H, s), 0.88 (3H, s), 0.94 (3H, s), 1.95 (3H, s), 5.08 (1H, dddd, J = 10, 4.5, 4, 2Hz); MS: m/z 196 (M^+ $C_{12}H_{20}O_2$), 126 [M^+ −60 (CH_3CO_2H)], 121 (B^+), 95, 93, (B^+); t_R:22.0 at 100°C.

(+)-*Epiborneol (IIIa) and* (−)-*Epibornyl Acetate (IIIb).* Using (−)-camphor ($[\alpha]_D$ −42.0° [c = 1.00], Tokyo Kasei Inc.) as a starting material, IIIa was synthesized through the same procedures as performed to obtain IIa. Hereafter, melting points and specific rotations and the yields of the enantiomers are expressed as follows (the other physical data were identical with those of the corresponding enantiomers): (−)-camphor → XI′ (mp 161–162°C; $[\alpha]_D$ +6.7° [c = 1.01]; 89%) → XII′ (without isolation) → IIIa (mp 180–181°C; $[\alpha]_D$ +7.9° [c = 1.00]; 19% from XI′) → IIIb($[\alpha]_D$ −21.0° [c = 1.06]; quantitative).

Aldehyde XIV (*Mixture of Epimers*). To a stirred suspension of methoxymethyl triphenylphosphonium chloride (5.4 g) in dry benzene (20 ml) and THF (20 ml), *n*-butyllithium (9.7 ml of 1.6 N *n*-hexane solution) was added dropwise at −10°C. After the reaction mixture was stirred at 0°C for 30 min, a solution of (+)-camphor (600 mg) in dry benzene (3 ml) was added at once to the resulting redish solution and further reacted at room temperature for 10 hr. The reaction mixture was poured into a mixture of ice, water, and *n*-pentane. The organic layer separated from the aqueous layer was washed with saturated $NaHCO_3$, water, and brine; dried; and concentrated at 0°C to 30 ml. This solution was passed through a short column packed with silica gel eluting with *n*-pentane to remove triphenylphosphine oxide. Enol-ether XIII was afforded by evaporating the solvent from the filtrate. XIII was treated with 10% aqueous HCl (3 ml) and THF (3 ml) at room temperature for 2.5 hr. After dilution of the reaction solution with water (10 ml), it was extracted with *n*-pentane. The combined organic layers were washed with saturated $NaHCO_3$ and brine, dried, and concentrated below 0°C. Silica gel (15 g) chromatography of the residue [eluting solvent: pet. ether–ether (19:1)] afforded an aldehyde XIV [365 mg, 56% from (+)-camphor], IR (CCl_4): 2810, 2710, 1720 cm^{-1}; PMR (CCl_4): 0.83 (3H, s), 0.86 (3H, s), 1.02 (3H, s), 2.43 (1H, broad t, J = 7.5 Hz), 9.76 (1H, d, J = 2 Hz); MS: m/z 166 (M^+, $C_{11}H_{18}O$), 151 (M^+−15), 147 [M^+−29 (CHO)], 123, 122, 109, 95 (B^+), 93; t_R: 21.5 at 100°C.

Mixture of (+)-endo- *and* (+)-exo-*Bornylcarboxylic Acid* (*Va* + *VIa*). Aldehyde XIV (250 mg) was oxidized with excess Jones reagent by the usual

manner (0° C, 2 hr) to afford a crude acid (Va + VIa). The crude acid was recrystallized from a mixture of formic acid and water. Va + VIa, mp 80–82.5° C, $[\alpha]_D^{20}$ −6.0° (c = 1.00); IR (KBr): 3200, 2600, 1690, 910 cm^{-1}; PMR (CCl_4): 0.87 (6H, s), 0.99 (3H, s), 2.63 (1H, broad t, J = 8 Hz), 12.10 (1H, broad s, COOH); MS: m/z 182 (M^+, $C_{11}H_{18}O_2$), 167 (M^+−15), 164 (M^+−18), 149, 136 [M^+−46 (HCO_2H)], 121, 110, 95 (B^+), 93. In a paper by Gream et al. (1974), the $[\alpha]_D^{20}$ value in benzene was reported to be +16.3° for Va and −96° for VIa. Based on these reported values, our $[\alpha]_D^{20}$ value was calculated to be the mixture of Va and VIa with a 4:1 mixture ratio. Therefore, the starting material for this procedure (i.e., aldehyde XIV) was expected to be a mixture of *endo* and *exo* isomers with the same ratio. All chromatographic efforts to resolve the mixture were unsuccessful for the separation of Va and VIa.

Mixture of Methyl Esters (Vb + VIb). The mixture (Va + VIa) (95 mg) was esterified with diazomethane in ether at 0° C for 20 min. Silica gel chromatography [pet. ether–ether (19:1)] of the concentrated residue yielded a mixture of pure methyl esters (Vb and VIb) (85 mg, 83%). Separation Vb from VIb was also unsuccessful. Vb + VIb (4:1), $[\alpha]_D^{20}$ −1.7° (c = 1.00); IR (film): 1730, 1170 cm^{-1}; PMR (CCl_4): 0.88 (6H, s), 0.94 (3H, s), 2.59 (1H, broad t, J = 8 Hz), 3.59 and 3.62 (singlet signal each, OCH_3); MS: m/z 196 (M^+, $C_{12}H_{20}O_2$), 181 (M^+−15), 165 [M^+−31 (OCH_3)], 164, 137, 136 (M^+−60), 121, 110, 95 (B^+), 93; t_R: 23.5 at 100° C. The ratio of the singlet signals due to OCH_3 in the PMR spectrum indicated 4:1 mixture of Vb and VIb.

Mixture of (−)-endo *and* (−)-exo-*Bornylcarboxylic Acids* (VIIa + VIIIa) *and Its Ester* (VIIb + VIIIb). (+)-Camphor → XIV′ (61%) → VIIa + VIIIa (4:1 from $[\alpha]_D$) (mp 81–83° C; $[\alpha]_D^{20}$ +5.5° [c = 1.00]; 82%) → VIIb + VIIb (4:1 from PMR) ($[\alpha]_D^{20}$ +1.1° [c = 1.00]: quantitative).

(+)-trans-*Nopinol* (XV). A mixture of (−)-apoverbenol ($[\alpha]_D$ −146.2° (c = 1.00); Takayanagi and Nishino, 1981) (600 mg) was hydrogenated with platinum oxide (80 mg) and hydrogen gas in cyclohexane (5 ml) (at room temperature for 2 hr) to afford XV, $[\alpha]_D$ +25.7° (c = 1.00); IR (film): 3320 cm^{-1}; PMR ($CDCl_3$): 0.80 (3H, s), 1.21 (3H, s), 2.36 (1H, s, OH), 4.18 (1H, broad t, J = 6 Hz); MS: m/z 140 (M^+, $C_9H_{16}O$), 125 (M^+−15), 122 (M^+−18), 107, 85 (B^+); t_R: 16.0 at 100 ° C.

Compound XVI. (+)-*trans*-Nopinol (**15**) (400 mg) was reacted with *p*-bromobenzenesulfonyl chloride (1.5 g) in pyridine (6.5 ml) at 0° C for 15 hr. The residue after workup by the usual manner was chromatographed over silica gel (40 g) eluting with *n*-hexane–EtOAc (6:1) to give brosylated compounds XVI (494 mg, 48%) which was recrystallized from *n*-hexane. XVI, mp 97.5–98° C; $[\alpha]_D$ −18.8° (c = 1.00); IR (KBr): 1580, 1370, 1190 cm^{-1}; PMR ($CDCl_3$): 0.97 (6H, s), 5.13 (1H, m), 7.76 (4H, s); MS: m/z 358 (M^+), 343 (M^+−15), 317, 219 ($BrC_6H_4SO_2^+$), 155 ($BrC_6H_5^+$). 122 [M^+−236 (BsOH), B^+]; Anal calc. for $C_{15}H_{19}O_3SBr$: C, 50.15; H, 5.33; 0, 13.36; S, 8.92; Br, 22.24. Found: C, 50.25; H. 5.34; 0, 13.55; s, 8.81; Br, 22.05.

(+)-*Apoborneol* (IXa). A mixture of naphthalene (858 mg) and pieces of sodium metal (154 mg) in dry THF (20 ml) was stirred at room temperature for 2 hr under argon gas atmosphere. The dark purple-colored reaction mixture was cooled to −78° C, a dry THF (3 ml) solution of XVI (400 mg) was added dropwise, and stirred at −78° C for 50 min under argon gas atmosphere. The reaction mixture was poured into ice-water and extracted with *n*-pentane. The organic layer was washed with water and brine, dried, and concentrated below 0° C to give a residue which was chromatographed over silica gel (50 g) with *n*-pentane–EtOAc (6:1). The obtained crude IXa (147 mg, 95%) was recrystallized from pet. ether. IXa, mp 68.5–69° C; $[\alpha]_D$ +10.7° (c = 1.00); IR (KBr): 3230 cm^{-1}; PMR (CCl_4): 1.01 (6H, s), 3.10 (1H, s, OH), 4.44 (1H, broad ddd, J = 10, 4, 3.5 ~2 Hz); MS: 140 (M^+, $C_9H_{16}O$), 125 (M^+ −15), 122 (M^+ −18), 107, 81 (B^+); t_R: 15.5 at 100° C.

(−)-*Apobornyl Acetate* (IXb). $[\alpha]_D$ −25.6° (c = 1.05); IR (film): 1738, 1250 cm^{-1}; 1.02 (3H, s), 1.10 (3H, s), 1.96 (3H, s), 5.12 (1H, broad ddd, J = 10, 4.5, ~2 Hz); MS: m/z 182 (M^+, $C_{11}H_{18}O$), 167 (M^+−15), 140 [M^+−42 (CH_3CO)], 122 (M^+ −60), 107, 81, 79, (B^+); t_R: 18.1 at 100° C.

(−)-*Apoborneol* (*Xa*) *and* (+)-*Apobornyl Acetate* (*Xb*). (−)-*trans*-Nopinol ($[\alpha]_D$ −20.0° [c = 1.00]; Nishino and Takayanagi (1980) → XVI′ (mp 97.5–98° C: $[\alpha]_D$ +18.3° [c = 1.00]; 47%) → Xa (mp 68–68.5° C; $[\alpha]_D$ −10.3° [c = 1.00]; 92%) → Xb ($[\alpha]_D$ +24.8° [c = 1.00]; quantitative).

Behavioral Assay. The assay method, including sample preparation followed Nishino et al. (1980). When a compound induced typical sexual display from the male cockroaches at 1 mg dosage, 0.5 and 0.05 mg of the compound were successively bioassayed. For the behavioral assay, a group of 25 adult males isolated from adult females for at least 1 month was used. The number of males displaying sexual behavior was counted within 3 min. Since activities of (+)- and (−)-bornyl acetates (Ib and IVb) have been evaluated in a previous work (Nishino et al., 1980), these were omitted from the present behavioral assay test data.

EAG Recording. The electroantennogram studies were carried out according to Nishino et al. (1983). The EAG response was recorded from an excised antenna using recording (glass capillary) and indifferent (stainless) electrodes. The response was run on an oscilloscope after amplification with microelectrode and biophysical amplifiers. Odor from a specified quantity of compound on a filter paper contained in a syringe was mixed with flowing air to puff onto the excised antenna.

Tests were repeated routinely at 20 to 30-sec intervals. One antenna was used for two tests of a series of the quantities (0.01, 0.02, 0.05, and 0.1 mg) of the compounds. Five antennae were used for each series. For the control, the same procedure was repeated without the compound. Camphor, which is one of the most commercially available chemicals, was employed as a reference of general odor.

Calculation of M/F Ratio and M/F Ratio Index (M/FI). The detailed method for the calculation was reported in Nishino et al. (1980) and Nishino and Takayanagi (1981b). The average EAG amplitudes from 10 repetitions for the specified quantities of a compound (the average amplitudes are in Table 2) were plotted against the logarithmic scale of the quantities. This procedure was performed for both male and female, and then induced lines were drawn for the sexes. Using the amplitudes at the 0.05-mg point on the given lines, the ratio was calculated, namely, by dividing the male amplitude by the female. The ratio index was obtained by dividing M/F ratio of a compound by the ratio obtained with camphor.

RESULTS

Behavioral Assay Results. The assay results are shown in Table 1. In the present assay, only (+)- and (−)-bornyl propionates (Ic and IVc) were active at 1 mg, and at the reduced quantities in the table. From the present and previous

TABLE 1. SEX PHEROMONAL ACTIVITY AND M/F RATIO INDEX (M/FI) OF KNOWN MIMICS (VA, Ia, AND Ib) AND PRESENT COMPOUNDS

Compound	Quantity (mg)	Number of repetitions	Activity[a]	M/FI[b]
VA	0.02	20	9 ± 3[c]	2.1[c]
Ib	0.05	15	8 ± 3[d]	1.5
Ic	0.05	20	15 ± 4	1.8
IIa	1.0	5	—[e]	0.7
IIb	1.0	5	—	1.0
IIIa	1.0	5	—	0.8
IIIb	1.0	5	—	1.0
IVb	0.1	15	4 ± 2[d]	1.2
IVc	0.1	20	2 ± 2	1.3
Va + VIa[f]	1.0	5	—	0.5
Vb + VIb[f]	1.0	5	—	1.0
VIIa + VIIIa[f]	1.0	5	—	0.6
VIIb + VIIIb[f]	1.0	5	—	0.7
IXa	1.0	5	—	0.9
IXb	1.0	5	—	1.0
Xa	1.0	5	—	0.9
Xb	1.0	5	—	1.1

[a] Average number of cockroaches showing typical sexual display within 3 min in a group containing 25 males ± SD.
[b] Calculated from the M/F ratios in Table 2.
[c] The data were from Manabe et al. (1983).
[d] The data were from Nishino et al. (1980).
[e] No activity.
[f] This expression means a mixture of two epimers.

assay data, the order of pheromonal activity among the active esters was estimated as follows: VA > Ic > Ib >> IVb ⩾ IVc. All other compounds did not cause sexual response of the males at the 1-mg dosage level.

Although the observed sexual display induced by the active esters was identical to that of the natural sex pheromones of this insect, the induction period for sexual display was much longer (1.5–2 min) compared to the immediate response to the natural pheromones. The behavior to the active esters persisted only for 30 sec after their elimination from the testing cage, which was much shorter compared with that (5–7 min) of the pheromones (Nishino et al., unpublished data; cf. Nishino et al., 1983).

EAG Results. The compiled EAG data are listed in Table 2 together with M/F ratios and M/FIs. The male antennae generally yielded larger EAG responses than the female antennae at any quantity with all compounds. In both sexes, the response increased according to increasing dosage. The mixtures (Va + IVa and VIIa + VIIIa) of carboxylic acids exhibited low M/FI values, stimulating very weakly the male antennae as well as the female antennae. This is probably due to a general odorous property of the acids, while the M/FI value (near 1.0) of the compounds other than behaviorally active esters (Ib, Ic, IVb and IVc) and the above carboxylic acids indicates that they are typical general odors.

Although the active esters showed larger M/FI values than camphor, the general odorous property implicit in the esters resembled that of the other compounds, because the variation of the female EAG amplitude in the active esters towards the increasing dose was basically similar to the variation in the other general odors.

The order of M/FI for VA and the present compounds was as follows: VA > Ic > Ib > IVc ⩾ IVb > the typical general odors including camphor > the weak general odors (acids).

DISCUSSION

It was noticed first that important chemical factors in bornyl acetate-type mimics for sex pheromonal activity were quite similar to those in VA-type mimics (Nishino and Takayanagi, 1981a; Takayanagi and Nishino, 1982; Nishino et al., 1982; Manabe et al., 1983).

In a previous study (Nishino et al., 1977), it was revealed that several alcohols with the bornane skeleton (borneols and isoborneols) were inactive, independent of configuration of the hydroxyl group. This finding was confirmed in the present work in which all of the alcohols were inactive. The acetate of *cis*-alcohol (isoborneol) had no activity (Nishino et al., 1977), whereas acetates (Ib and IVb) of *trans*-alcohols [(+)- and (−)-borneol (Ia and IVa)] were active. The parent alcohols of the active esters possess a hydroxyl

TABLE 2. EAG AMPLITUDES OF ADULT MALE AND FEMALE COCKROACHES AT VARIOUS QUANTITIES OF COMPOUNDS, M/F RATIOS, AND M/F RATIO INDICES

Compound	Quantity (mg)	EAG amplitude[a]		M/F[b]	M/FI[c]
		Male	Female		
Ib[d]	0.01	0.14 ± 0.04	0.08 ± 0.01	3.2	1.5
	0.02	0.24 ± 0.04	0.10 ± 0.04		
	0.05	0.61 ± 0.12	0.18 ± 0.05		
	0.10	0.84 ± 0.09	0.21 ± 0.07		
Ic[d]	0.01	0.09 ± 0.03	0.04 ± 0.03	3.9	1.8
	0.02	0.16 ± 0.06	0.07 ± 0.02		
	0.05	0.31 ± 0.05	0.09 ± 0.04		
	0.10	0.58 ± 0.05	0.12 ± 0.03		
IIa	0.01	0.06 ± 0.02	0.03 ± 0.03	1.5	0.7
	0.02	0.08 ± 0.03	0.05 ± 0.03		
	0.05	0.14 ± 0.03	0.11 ± 0.02		
	0.10	0.23 ± 0.04	0.15 ± 0.02		
IIb	0.01	0.09 ± 0.02	0.03 ± 0.02	2.1	1.0
	0.02	0.11 ± 0.04	0.06 ± 0.02		
	0.05	0.22 ± 0.04	0.10 ± 0.04		
	0.10	0.34 ± 0.06	0.16 ± 0.02		
IIIa	0.01	0.04 ± 0.02	0.03 ± 0.03	1.7	0.8
	0.02	0.06 ± 0.02	0.04 ± 0.03		
	0.05	0.16 ± 0.03	0.11 ± 0.04		
	0.10	0.26 ± 0.05	0.15 ± 0.03		
IIIb	0.01	0.07 ± 0.02	0.03 ± 0.02	2.2	1.0
	0.02	0.12 ± 0.05	0.07 ± 0.03		
	0.05	0.19 ± 0.03	0.11 ± 0.02		
	0.10	0.33 ± 0.07	0.12 ± 0.05		
IVb[d]	0.01	0.13 ± 0.02	0.04 ± 0.02	2.6	1.2
	0.02	0.13 ± 0.04	0.06 ± 0.03		
	0.05	0.27 ± 0.06	0.11 ± 0.02		
	0.10	0.38 ± 0.12	0.14 ± 0.04		
IVc[d]	0.01	0.10 ± 0.03	0.02 ± 0.02	2.8	1.3
	0.02	0.20 ± 0.07	0.06 ± 0.02		
	0.05	0.34 ± 0.12	0.14 ± 0.04		
	0.10	0.42 ± 0.07	0.14 ± 0.04		
Va + VIa[e]	0.01	0.01 ± 0.02	0.03 ± 0.03	1.1	0.5
	0.02	0.04 ± 0.03	0.04 ± 0.03		
	0.05	0.07 ± 0.02	0.06 ± 0.03		
	0.10	0.08 ± 0.03	0.08 ± 0.04		
Vb + VIb[e]	0.01	0.10 ± 0.03	0.04 ± 0.02	2.2	1.0
	0.02	0.13 ± 0.04	0.07 ± 0.03		
	0.05	0.24 ± 0.08	0.10 ± 0.03		
	0.10	0.39 ± 0.13	0.17 ± 0.06		
VIIa + VIIIa[e]	0.01	0.04 ± 0.03	0.04 ± 0.02	1.3	0.6
	0.02	0.04 ± 0.03	0.04 ± 0.02		
	0.05	0.08 ± 0.02	0.05 ± 0.03		
	0.10	0.08 ± 0.02	0.09 ± 0.02		

TABLE 2. *Continued*

Compound	Quantity (mg)	EAG amplitude[a] Male	EAG amplitude[a] Female	M/F[b]	M/FI[c]
VIIb + VIIIb[e]	0.01	0.06 ± 0.03	0.04 ± 0.04	1.6	0.7
	0.02	0.09 ± 0.01	0.06 ± 0.02		
	0.05	0.12 ± 0.03	0.08 ± 0.03		
	0.10	0.18 ± 0.02	0.12 ± 0.03		
IXa	0.01	0.04 ± 0.02	0.03 ± 0.03	1.9	0.9
	0.02	0.09 ± 0.02	0.06 ± 0.02		
	0.05	0.16 ± 0.03	0.09 ± 0.03		
	0.10	0.26 ± 0.05	0.11 ± 0.04		
IXb	0.01	0.21 ± 0.02	0.08 ± 0.05	2.5	1.1
	0.02	0.21 ± 0.03	0.10 ± 0.03		
	0.05	0.36 ± 0.06	0.12 ± 0.03		
	0.10	0.51 ± 0.05	0.14 ± 0.03		
(+)-Camphor[f]	0.01	0.11 ± 0.04	0.05 ± 0.02	2.2	1
	0.02	0.17 ± 0.04	0.08 ± 0.04		
	0.05	0.32 ± 0.11	0.12 ± 0.02		
	0.10	0.49 ± 0.16	0.24 ± 0.03		
Xa	0.01	0.01 ± 0.01	0.00 ± 0.00	1.7	0.9
	0.02	0.05 ± 0.03	0.01 ± 0.01		
	0.05	0.23 ± 0.14	0.14 ± 0.07		
	0.10	0.34 ± 0.11	0.16 ± 0.04		
Xb	0.01	0.10 ± 0.04	0.03 ± 0.03	1.9	1.1
	0.02	0.15 ± 0.06	0.09 ± 0.07		
	0.05	0.27 ± 0.09	0.12 ± 0.06		
	0.10	0.46 ± 0.14	0.23 ± 0.12		
(+)-Camphor	0.01	0.23 ± 0.12	0.16 ± 0.09	1.8	1
	0.02	0.33 ± 0.05	0.19 ± 0.07		
	0.05	0.53 ± 0.11	0.29 ± 0.08		
	0.10	0.84 ± 0.18	0.49 ± 0.16		

[a]Average amplitude ± SD of 10 tests (in mV).
[b]The ratio was calculated using male and female EAG amplitudes at 50 μg on the induced lines.
[c]The index was obtained by dividing M/F ratio with the ratio of camphor.
[d]Sexually active compounds (see Table 1).
[e]Mixture of two epimers.
[f]There is no difference between (+)- and (−)-camphor in EAG response.
[g]Different experiments were carried out between EAG measurements above and below the broken line.

group positioned vicinally to the bridgehead methyl group (C-1 methyl). On the other hand, when IIa and IIIa had a *trans*-oriented hydroxyl group, their acetates (IIb and IIIb) were inactive. This inactivity may result because the hydroxyl group at C-3 or C-5 is separated from the C-1 methyl group. The elimination of the C-1 methyl group (IXb and Xb) caused a complete loss of pheromonal activity.

Thus, an ester group possessing *trans* configuration has a critical effect on pheromonal activity. Furthermore, the vicinal substitution at (C-2 or C-6) of the ester group to the C-1 methyl was required for activity. The carbonyl oxygen atom in the ester group was expected to be the most reactive site to accept a proton from the corresponding active site of the receptor. Although the alcoholic oxygen atom has no role as the acceptor, the alcoholic oxygen seemed to be important in that it directly attaches to the bornane skeleton, since the methyl ester (Vb + VIb and VIIb + VIIIb) were inactive. In the methyl esters, the sequence of atoms in the acetate group was exchanged as follows, —O—CO—CH_3 (acetate) to —CO—O—CH_3 (methyl ester). This fact was evident in the VA-type analogs (Nishino and Takayanagi, 1981a).

The size of the alkyl group in the ester moiety affected the pheromonal activity. Comparison of the activity between (+)-bornyl acetate (Ib) and propionate (Ic) suggested the predominance of propionate to acetate group, as revealed in the study of VA-type mimics (Nishino et al., 1982).

The C-1 methyl group is undoubtedly a critical factor. Elimination of this methyl group (IXb and Xb) from the active acetates (Ib and IVb) caused complete loss of the activity. This methyl group may correspond to the important C-9 methyl group of VA (discussed later, Manabe et al., 1983).

In our structure–activity studies in VA- and bornyl acetate-type mimics, almost the same structural factors were elucidated. The structural correlation between Ib and VA was inspected using the Dreiding molecular model.

We attempted first to overlap the geminal dimethyl and the acetate groups between Ib and VA. When these two important groups of the mimics were overlapped, the C-1 methyl of Ib had no correlation to any important function of VA. If the C-1 methyl, C-8 methyl, and acetate groups of Ib were related to the C-9 methyl, C-4 methyl, and acetate groups of VA respectively, a good overlapping of the two molecules was observed. Figure 3 shows the overlapping from three-dimensional views. Small differences in the location of each important function from the active sites of the receptor may explain the discrepancy in activity between Ib (active at 0.05 mg) and VA (0.02 mg).

Surprisingly, weak but significant activity was discovered for the optical enantiomers (IVb and IVc) of (+)-bornyl acetate (Ib) and propionate (Ic). In our recent study (Takayanagi and Nishino, 1981), none of the optical enantiomers of the active VA analogs exhibited pheromonal activity even at high dose quantities (1–0.3 mg). It may be a resonable explanation for the lower activity of the (−)-enantiomer (IVb) than the (+)-enantiomer (Ib) that the most essential factor, carbonyl oxygen, occupies almost the same area in both Ib and IVb (Figure 4a), while the alkyl group of the acetate group locates in different area between the enantiomers (Figure 4b). The active site of the receptor may accommodate better to the alkyl group of Ib, and the group of IVb contacts partially with the site as shown in Figure 4b. Therefore, conversion of IVb to IVc did not affect the increasing pheromonal activity, which is different from the relationship in Ib and Ic.

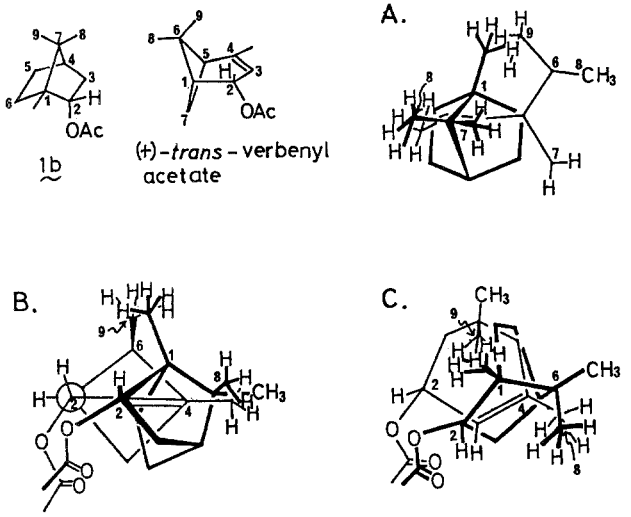


FIG. 3. Structural correlation of (+)-bornyl acetate (Ib) to (+)-*trans*-verbenyl acetate (VA). Lateral and frontal views on VA are illustrated in A and B, respectively. C shows a projection viewed from the top of VA molecule. In the illustrations, overlapping the important groups [ester groups, C-9 methyl of VA vs. methyl at C-1 of Ib, and methyl at C-4 of VA vs. C-8 methyl of Ib] is demonstrated.

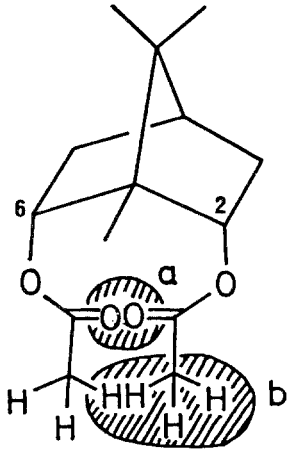


FIG. 4. Overlapped active sites between (+)- and (−)-bornyl acetates. (+)-Bornyl acetate (Ib) possesses an ester group at C-2; (−)-bornyl acetate (IVb) at C-6. The essential factor of both esters, carbonyl oxygen atom, locates almost same position (a). But the active site (b) for the methyl group of the ester group positions favorably to Ib, which may cause a stronger activity for Ib than for IVb.

In addition to sex pheromonal responses, general odorous responses are noted in the monoterpenoid sex pheromone mimics such as Ib and VA, because significant EAG responses were recorded from the male and female antennae (Nishino et al., 1980; Nishino and Takayanagi, 1981b). On the female antenna, sex pheromone receptors were expected to be lacking, while on the male antenna both sex pheromone and general odor receptors exist (Nishino et al., 1980). Accordingly, in the M/F ratio in the general odorous property of a mimic is offset. The large stimulation of the sexually active compunds to sex pheromone receptors results in great value of the M/F ratio.

The M/FI was proposed by us (Nishino and Kimura, 1981) to compensate M/F ratios in different EAG experiments. The index value in the present work has proven to be an useful indicator for the expression of sex pheromonal activity of the compounds (Table 1). In fact, in a previous work (Nishino et al., 1982), we measured stronger pheromonal activity for (+)-verbanyl propionate than for germacrene-D (both are sex pheromone mimics of the American cockroach) on the basis of comparison of the M/FI values of the two mimics.

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Erratum

STRUCTURE-ACTIVITY RELATIONSHIPS IN SEX ATTRACTANTS FOR NORTH AMERICAN NOCTUID MOTHS

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In Table 2, page 737, under *E. tristicula* (Morrison), the last component of the best known attractant should be 12:Ac, not Z7-12:Ac. (*Z*)-7-dodecenyl acetate is in fact a powerful suppressant of lures for this species.

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ISOLATION AND IDENTIFICATION OF TWO MACROLIDE PHEROMONES FROM THE FRASS OF *Cryptolestes ferrugineus* (COLEOPTERA: CUCUJIDAE)[1]

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Abstract—Two synergistic macrolide aggregation pheromones were isolated from Porapak Q-trapped volatiles obtained from the frass of *Cryptolestes ferrugineus* (Stephens). These compounds were identified as (*E,E*)-4,8-dimethyl-4,8-decadien-10-olide (I) and (3*Z*,11*S*)-3-dodecen-11-olide (II) and given the trivial names ferrulactone I and II, respectively. Analysis of captured volatiles from separated male and female adults disclosed that the pheromones are male-produced. Additional macrolides were identified in frass volatiles, but were devoid of any pheromonal activity. The structures of I and II were confirmed by comparison with synthetic materials.

Key Words—*Cryptolestes ferrugineus*, rusty grain beetle, aggregation, pheromone, macrolide, (*E,E*)-4,8-dimethyl-4,8-decadien-10-olide, (3*Z*,11*S*)-3-dodecen-11-olide, (*Z,Z*)-3,6-dodecadien-11-olide, (*Z*)-5-tetradecen-13-olide, 11-dodecanolide, 4-nonanolide, Coleoptera, Cucujidae.

INTRODUCTION

The rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), is a pest of stored food products throughout the world (Reid, 1942; Howe and Lefkovitch, 1957;

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Banks, 1979). In Canada, the beetles infest wheat during storage (Loschiavo, 1975) and transportation (Monro, 1969; Loschiavo, 1974a). Infested wheat is subjected to heating (Sinha, 1961) and suffers a loss of germinating capacity (Campbell and Sinha, 1976).

Borden et al. (1979) have shown that male beetles produce an aggregation pheromone which might have particular utility in optimizing the efficiency of survey traps such as that developed by Loschiavo (1974b). We report the isolation and identification of two synergistic aggregation pheromones I and II from *C. ferrugineus* frass volatiles. Several structurally related compounds, III–VIII, were also identified from the frass, but none had any pheromonal activity in a two-choice, pitfall bioassay.

METHODS AND MATERIALS

Insect Rearing. Initially *C. ferrugineus* cultures were maintained as described by Borden et al. (1979). The diet was later modified to contain only whole wheat (95%) and wheat germ (5%). Adjustment of the wheat moisture content to 18% followed by a 30 min autoclaving was necessary to maintain vigorous cultures.

Capture and Extraction of Beetle and Frass Volatiles. Pentane extracts of Porapak Q-captured volatiles from beetles or frass were prepared as described by Borden et al. (1979) except that activated charcoal (50/80 mesh, Fisher) was substituted for Porapak Q in the air scrubbers. For the isolation of pheromone, bioassay, and structure elucidation, beetle and frass volatiles were obtained from beetles of both sexes. Yields of 1.49×10^7 beetle hours (bh) and 4.04×10^5 gram hours (gh) of beetle and frass volatiles were obtained from typical aerations (1 bh = the volatiles from one beetle aerated for 1 hr; 1 gh = the volatiles from 1 g of frass aerated for 1 hr). Pentane extracts were concentrated to about 8 ml, transferred to a 10-ml volumetric flask, and diluted to the mark with distilled pentane. These stock solutions were stored in Teflon-lined screw-cap vials at −30° until required.

To determine the role of each sex in pheromone production, volatiles from male and female beetles were obtained by aeration of separate populations of either sex. Cold-immobilized beetles were sexed by the presence of the males' ventrolateral, mandibular tooth (Reid, 1942). Male beetles were aerated according to the following schedule: 1820 beetles (64.5 hr), 1560 beetles (71.5 hr), 1400 beetles (69.0 hr), 1220 beetles (71.5 hr), and 950 beetles (70.5 hr). Feeding periods (48 hr) were allowed between each aeration. The aeration of female beetles was carried out in a similar manner: 2580 beetles (70 hr), 2090 beetles (72 hr), 1850 beetles (68.5 hr), and 1290 beetles (88.5 hr). The aerations totalled 4.80×10^5 bh for males and 5.72×10^5 bh for females.

Bioassay Procedures. Two laboratory bioassays were used to monitor the

activity of the extracts throughout the isolation procedure. An open-arena, airflow olfactometer (Borden et al., 1979) was used in the initial phase of this study. In this bioassay, beetles responded by walking upwind toward an attractive stimulus. The isolation of pheromones was later monitored by a bioassay developed to assess olfactory response in *Oryzaephilus* spp. (Pierce et al., 1981). In this bioassay, 15 beetles per replicate were given 2 hr in the dark to respond to a stimulus in a two-choice pitfall olfactometer. Prior to each bioassay, the test beetles (mixed sex, 1–3 months of age) were held in the dark in a 25-ml glass vial at 23° C for 18 hr without food. All bioassays were performed between 0900 and 1300 hr, and all dilutions of a test solution were assayed in a single 2-hr session.

The raw data were analyzed using the t test, and the results were expressed as the mean percent response of the total number of beetles per treatment, except where otherwise indicated. Threshold is defined as the concentration of pentane extract at which significant ($P < 0.05$) positive response was first detected.

GLC Quantitation of Compounds in Beetle and Frass Volatiles. The GLC quantitation of compounds found in the pentane extracts of beetle and frass volatiles was done by the internal standard method with geranyl acetate as the internal standard. The relative weight response (RWR) of I to the internal standard was determined to be 0.86. Compounds II–VIII were assumed to have the same RWR as I.

Fractionation of Beetle and Frass Volatiles for Bioassay. The fractionation of beetle volatiles for bioassay was carried out by high-performance liquid chromatography (HPLC) on a Varian LC5000, equipped with a series 634 UV/VISIBLE spectrophotometer. A pentane extract (10 μl, 1.5×10^4 bh) of Porapak Q-captured beetle volatiles was loaded onto a MicroPak SI-10 column (30 cm × 4 mm ID) which was equilibrated with 0.1% isopropyl alcohol (IPA)/hexanes. The column was eluted with a gradient of IPA/hexanes (0.1% to 2.0% at 10 min, 10% at 30 min) at a flow rate of 2 ml/min. The effluent was monitored at 213 nm and fractions (≈2 ml) were collected to separate individual peaks.

A pentane extract (30 μl, 5.12×10^4 gh) of frass volatiles was fractionated by HPLC via the procedure described for beetle volatiles. Thirty-nine 2-ml fractions were collected.

Isolation of Pheromones for Structure Elucidation. Preparative gas chromatography was carried out on a Varian model 1200 instrument equipped with a 10:1 effluent splitter, flame-ionization detector, and a thermal gradient collector (Brownlee and Silverstein, 1968). Purification of I was achieved on a 3.0 m × 3.18 mm ID stainless-steel column of 5% Carbowax 20M on 70/80 mesh Chromosorb G (column A). The initial oven temperature of 60° C was increased at 4° C/min to a final temperature of 200° C. Under these conditions, I eluted between 72 and 76 min.

Isolation of II from the pentane extract of frass volatiles involved a combination of HPLC and preparative GLC. Initial fractionation was achieved on a Whatman Magnum 9 Partisil 10/50 column (elution gradient of IPA/hexanes: 0.2% from 0 to 8 min, 1.0% at 15 min, 2% at 25 min, 10% at 35 min; 4 ml/min; 213 nm). Material which eluted between 56 and 61 ml was collected and concentrated to 40 μl for preparative GLC. Final purification was achieved on a 3.0 m × 3.2 mm ID stainless-steel column of 10% SP-1000 on 100/120 mesh Chromosorb W (column B). The initial oven temperature of 70° C was held for 3 min and then raised to 200° C at 4° C/min. Component II was collected between 28.3 and 29.4 min.

Compounds III and IV were isolated from *C. ferrugineus* frass volatiles by the procedure used for the isolation of II. Component III was collected between 48 and 55 ml from the Magnum 9 column using the gradient elution conditions described above. Final purification of III was achieved by preparative GLC on column B (elution time 34.2–36.2 min). Component IV (100 μg) was obtained pure (>99%) after HPLC (elution volume 66.0–70.5 ml, Magnum 9) followed by preparative GLC (elution time 31.2–32.0 min, column B).

The purities of isolated compounds were determined by GLC analysis on a 33 m × 0.66 mm ID glass open-tubular column, coated with SP-1000 (column C; Hewlett-Packard Model 5830A gas chromatograph, equipped with a 18835B capillary inlet system).

Spectroscopic Methods. The IR spectra of pure isolated I and II were recorded on neat samples (≈100 μg) on AgCl plates with a Beckman IR 4230 instrument. Unit-resolution mass spectra were obtained with a Hitachi-Perkin-Elmer RMU-6 instrument coupled to a Varian model 1400 gas chromatograph or on a HP 5985B GC-MS-DS with a fused silica open-tubular column (30 m × 0.32 mm) coated with Carbowax 20M. Chemical ionization mass spectra were recorded on a MS12 instrument with NH_3 as the ionizing gas. A DS-50 mass spectrometer was used to record the high-resolution mass spectra of I, II, and III. The proton magnetic resonance (PMR) spectra in $CDCl_3$ were recorded with a Varian XL-100 instrument or a Bruker 400 MHz instrument. Chemical shifts were calculated from $CHCl_3$ [δ(TMS) = $\delta(CHCl_3)$ + 7.27 ppm]. The determination of the absolute configuration of II with $Eu(tfc)_3$ was carried out on the Bruker 400 MHz instrument. The carbon magnetic resonance (CMR) spectrum of natural I (1000 μg) was obtained on the Bruker 400 MHz instrument, while the spectrum of natural II (750 μg) was recorded on the XL-100. Chemical shifts for the CMR spectra were calculated from $CDCl_3$ [δ(TMS) = $\delta(CDCl_3)$ + 76.9 ppm]. All NMR experiments were conducted on samples sealed in 1.6 mm OD capillary tubes. For PMR the capillary tubes were supported in a 5-mm NMR tube with Teflon vortex plugs. A microprobe was available for the CMR experiments.

Microscale Hydrogenation. Microscale hydrogenations were carried out in half-dram glass vials at room temperature. Hexane solutions of the sample were stirred with a trace of 10% Pd on carbon under hydrogen for 1 hr.

RESULTS AND DISCUSSION

Biology. Volatiles captured from frass and beetles of both sexes were attractive to *C. ferrugineus* in a two-choice, pitfall olfactometer (Figure 1).

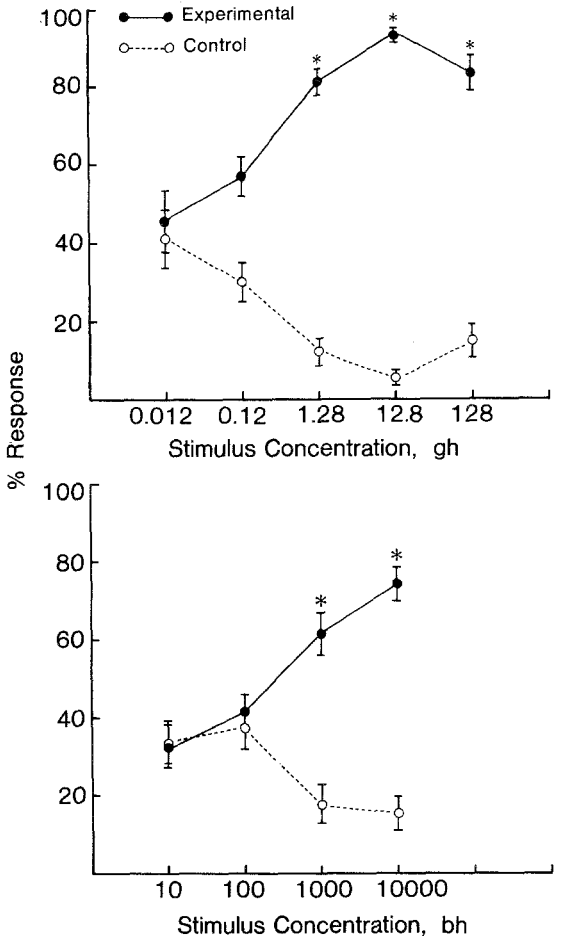


FIG. 1. Response of *C. ferrugineus* to Porapak Q-captured volatiles of frass (top) and beetles (bottom) in the two-choice, pitfall bioassay. A significant response to experimental stimulus is indicated by * $P < 0.01$. $N = 6$ replicates.

Dose-dependent responses were observed. The threshold dose (1.28 gh) for frass volatiles is similar to that determined by Borden et al. (1979) via an open-arena, airflow olfactometer (0.67 gh) and is also comparable to the threshold dose for frass volatiles of the saw-toothed grain beetle (Pierce et al., 1981). The response decrease observed at the highest dose of frass volatiles also occurs in other stored-product beetles (Pierce et al., 1981) and may indicate sensory adaptation (Seabrook, 1977) or disorientation due to saturation of the bioassay chamber by the stimulus (Nara et al., 1981). Differences between the threshold dose for beetle volatiles (1000 bh) and those reported by Borden et al. (1979) (34 bh and 2400 bh) may be due either to variations in the quantity of the pheromone captured per beetle-hour or variations in the responsiveness of the beetles. The doses of beetle volatiles tested were probably too low to cause a decrease in response, since Borden et al. (1979) reported that this occurs at 34,000 bh in the airflow olfactometer. Our results agreed with the results of Borden et al. (1979) and therfore confirm the presence of an aggregation pheromone in frass and beetle volatiles.

The GLC analysis of frass volatiles revealed the presence of many components which appeared throughout the chromatogram (Figure 2). In contrast, the chromatogram of beetle volatiles (Figure 3) was devoid of major components, except for the regions immediately following the solvent peak and between 30 and 38 min. The latter compounds, I–V, VII, and VIII, were identical to compounds found in frass volatiles on the basis of GLC retention data and mass spectral fragmentation patterns. Some of these compounds (I–IV) were also detected in male volatiles but not in female volatiles (Figure 4). Small quantities of V, VII, and VIII could also be present in male volatiles but may not have been detected by the packed-column GLC analysis. The presence of I–V, VII, and VIII in both frass and beetle volatiles suggested that some or all of these compounds could be components of the aggregation pheromone. Stronger evidence was provided by the presence of I–IV in attractive male volatiles and the absence of these compounds in unattractive female volatiles (see Borden et al., 1979, for the response of *C. ferrugineus* to separate male and female volatiles).

Fractionations of frass and beetle volatiles monitored by the pitfall olfactometer were carried out to identify the aggregation pheromone(s). Compounds I and II from beetle volatiles were completely separated into fractions 9 and 8 by HPLC. Table 1 shows the response of *C. ferrugineus* to these two fractions, the recombined mixture and unfractionated beetle volatiles. In this experiment, fraction 8 (>99% II by GLC analysis on column C) was not significantly attractive, while fraction 9 (90% I, 10% IV) was moderately attractive. However, the mixture of fractions 8 and 9 (I:II:IV, 9.5:7:1) was equal to unfractionated beetle volatiles (I:II:IV, 8.7:9.6:1) in activity. None of the other HPLC fractions were either attractive or increased

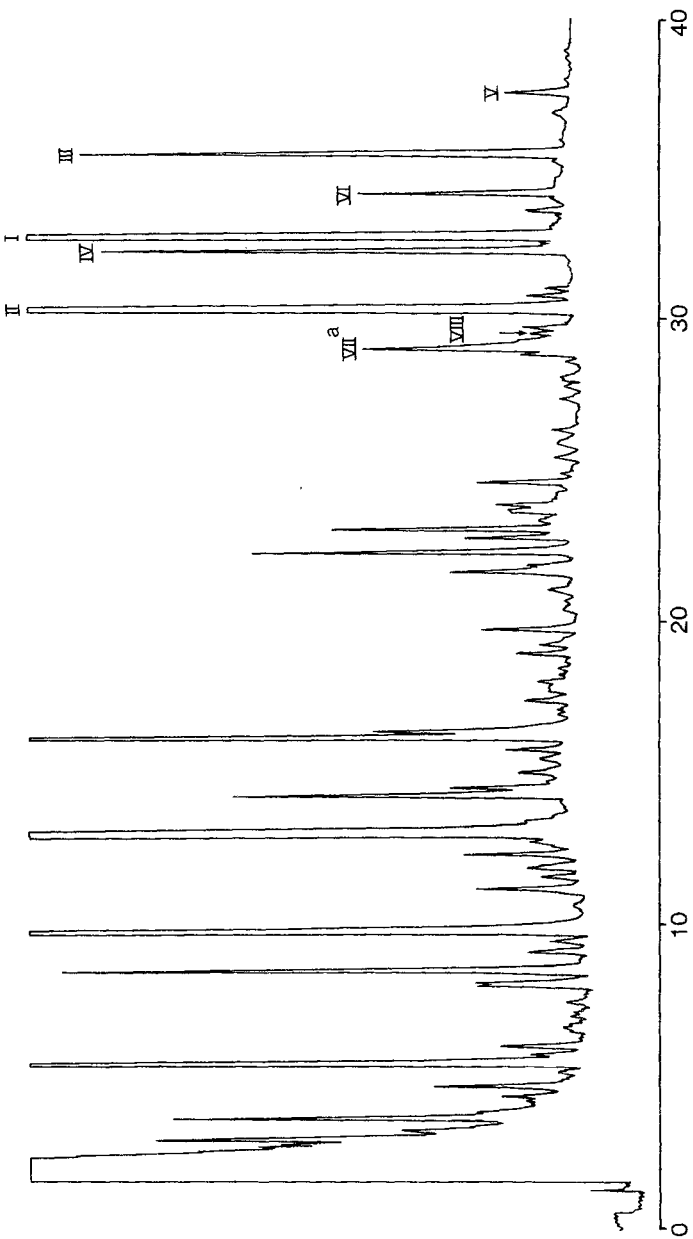


FIG. 2. Gas–liquid chromatogram of the pentane extract of Porapak Q-captured frass volatiles (20 gh, column C: 70–200°C programed at 4°C/min). (a) Compound VII coeluted with peak at 28.8 min.

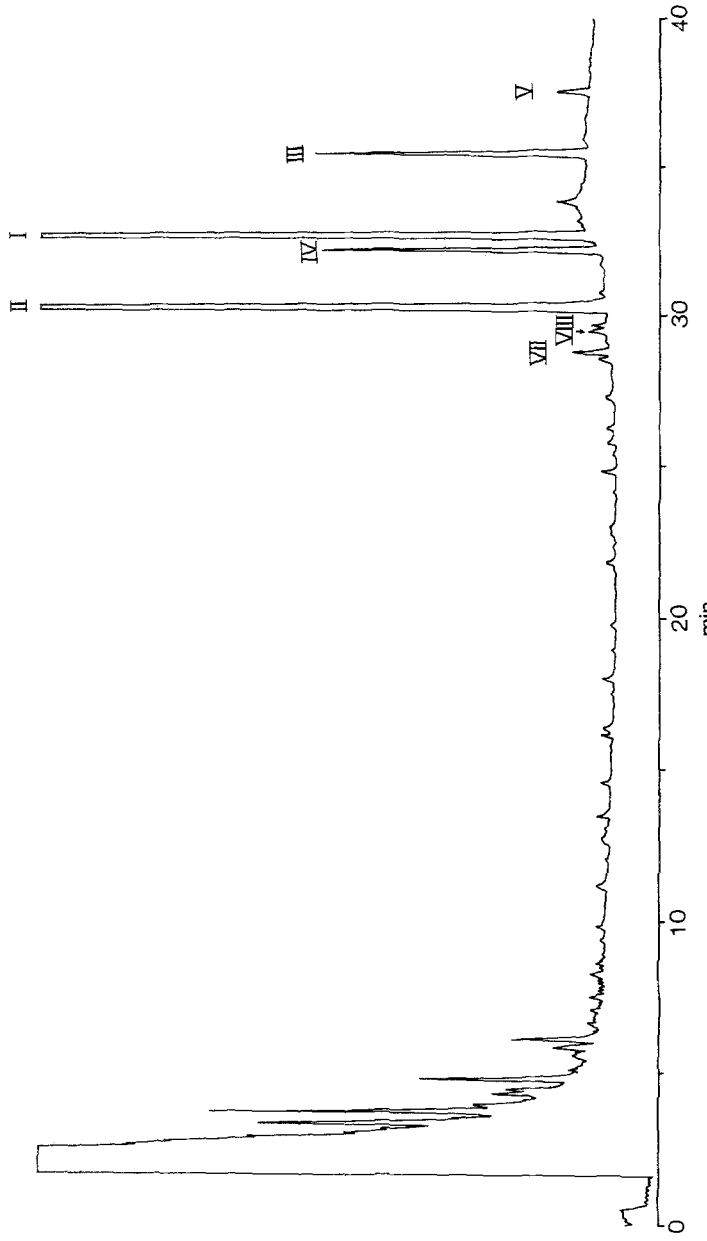


FIG. 3. Gas–liquid chromatogram of the pentane extract of Porapak Q-captured beetle volatiles (7450 bh, column C: 70–200°C programed at 4°C/min).

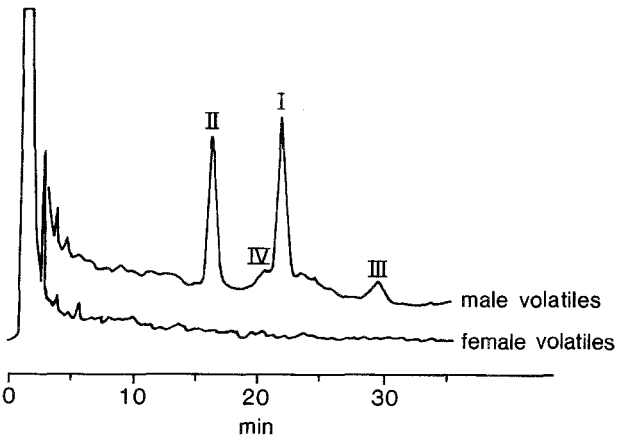


FIG. 4. Gas–liquid chromatogram of the pentane extract of Porapak Q-captured volatiles from separated males and females (column A: 60–160°C programed at 4°C/min).

the activity of fractions 8 and 9. This experiment demonstrated that aggregation was dependent upon synergism between two or three compounds and that II (fraction 8) is one of the synergists since its recombination with I and IV (fraction 9) resulted in the recovery of total activity.

Only fractions 9 (92.6% II), 10 (91.9% II), and 12 (60.7% I, 4.4% IV) from the HPLC fractionation of frass volatiles were individually attractive (Table 1). The mixture of these three fractions was more attractive than the individual components and was equal in activity to the mixture of all 39 fractions. However, unfractionated frass volatiles were more attractive than the recombined mixtures, indicating that a loss of activity had occurred during HPLC fractionation. Attractive components could have been lost during concentration of the pentane extract under a nitrogen stream or during passage of the extract through the silica column. Although the mixture of fractions 9, 10, and 12 contained other compounds in addition to I, II, and IV, the presence of these three compounds in the attractive mixture suggested that they were components of the aggregation pheromone.

Compounds I, II, and IV were singled out as the potential pheromones from frass and beetle volatiles by bioassays on HPLC fractions. However, the presence of other compounds in the HPLC fractions and the inability to separate I and IV by HPLC prevented us from determining the exact composition of the aggregation pheromone. The resolution of this problem was achieved by comparing the activities of pure (>99%) natural compounds and pure synthetic compounds. Natural products of requisite purity were obtained by the isolation procedures described in Methods and Materials.

TABLE 1. RESPONSE OF *C. ferrugineus* TO HPLC FRACTIONS OF BEETLE AND FRASS VOLATILES IN A TWO-CHOICE, PITFALL OLFACTOMER

Stimulus[a]	Dose	% Response[b] ($\bar{X}$ ± SE) Stimulus	Control
HPLC fractions of beetle volatiles, N = 10 replicates			
Fr.8c	15,000 bh	30.7 ± 2.0	24.0 ± 2.0 NS
Fr. 9c	15,000 bh	44.0 ± 2.3	14.0 ± 2.3**
Fr. 8 + 9c	15,000 bh	70.0 ± 3.3	12.7 ± 3.3**
Beetle volatiles c	15,000 bh	74.0 ± 2.5	9.3 ± 2.5**
HPLC fractions of frass volatiles, N = 6 replicates			
Fr. 9d	12.8 gh	61.1 ± 5.43	18.9 ± 5.43*
Fr. 10d	12.8 gh	64.4 ± 2.85	30.0 ± 2.85**
Fr. 12d	12.8 gh	77.8 ± 2.53	11.1 ± 2.53***
Fr. 9, 10 and 12d	12.8 gh	82.2 ± 1.91	14.4 ± 1.91***
Frass Volatiles d	12.8 gh	93.3 ± 2.04	5.6 ± 2.04***

[a]All tests followed by the same letter were performed in the same 2-hr session.
[b]Significant response (t test) to experimental stimulus indicated by: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS = not significant.

Syntheses of I and (*S*)-II were devised and carried out (Wong, 1982) to yield compounds which were chromatographically and spectrally identical to the natural products. The responses of *C. ferrugineus* to the synthetic compounds were identical to the responses for the natural compounds (Table 2). Compound I elicited a dose-dependent response over a 1000-fold range of doses starting from threshold at 20 ng. A similar response was observed for II, except that response reached a plateau above 200 ng. Although mixtures of I and II also elicited threshold responses at 20 ng (of each compound), responses were much higher than those observed for the individual compounds, thus confirming synergism between I and II. The 20-ng threshold dose for pure I and II is similar to the quantities found in the threshold doses of beetle volatiles (21.0:17.7 ng, II:I) and frass volatiles (14.0:22.7 ng, II:I) and thus supports our hypothesis that I and II constitute the aggregation pheromone. The maximum responses observed for the binary mixtures of I and II (Table 2) at 200 ng (of each compound, equivalent to 10.9 gh) were only slightly less than the response reported for unfractionated frass volatiles (Figure 1). The small difference may be due to compounds which, as mentioned previously, may be lost during HPLC fractionation. We have not attempted to identify these potential additional attractants in frass volatiles.

Surprisingly, beetle volatiles (Figure 1 and Table 1) appeared to be less

attractive than the pure binary mixture (200 ng is equivalent to 10,336 bh). This apparent discrepancy may be accounted for by variations in the responsiveness of the beetles. Although bioassay beetles (30–90 days old) were always chosen from vigorous cultures maintained under identical conditions, we have observed that the response to a particular stimulus may decrease sharply within a short period of time. For example, the threshold value for pure I and II changed from 20 ng to 200 ng over two bioassays performed on successive days. Decreased responsiveness of the bioassay beetles may also account for the apparent inactivity of II (fraction 8) from beetle volatiles, as shown in Table 1. No cause has yet been determined for this phenomenon.

We also prepared racemic II, (*R*)-II (Wong, 1982), and racemic III (J. Millar[2], unpublished result) to determine whether they were attractive to *C. ferrugineus*. Racemic II elicited a response similar to that for (*S*)-II, while (*R*)-II was inactive. A mixture of racemic II with I (1 : 1) elicited a synergistic

TABLE 2. RESPONSE OF *C. ferrugineus* TO NATURAL AND SYNTHETIC PHEROMONES IN A TWO-CHOICE, PITFALL OLFACTOMETER, $N = 12$ REPLICATES

Stimulus[a]	Dose (ng)	% Response[b] ($\bar{X}$ ± SE)	
		Stimulus	Control
Natural Ic	2000	75.9 ± 2.29	18.8 ± 2.29***
	200	62.3 ± 2.50	19.4 ± 2.50***
	20	51.2 ± 1.91	11.6 ± 1.91***
Synthetic Ic	2000	75.3 ± 1.97	18.0 ± 1.97***
	200	65.0 ± 2.96	17.5 ± 2.96***
	20	48.3 ± 2.51	28.7 ± 2.51*
Natural IId	200	65.3 ± 1.72	8.52 ± 1.72***
	20	52.3 ± 2.65	21.6 ± 2.65**
Synthetic (*S*)-IId	2000	63.6 ± 2.62	27.3 ± 2.62**
	200	61.6 ± 1.96	18.1 ± 1.96***
	20	53.8 ± 3.58	24.0 ± 3.58*
Natural I + IIe	2000 : 2000	85.7 ± 1.49	10.9 ± 1.49***
	200 : 200	87.8 ± 2.45	8.72 ± 2.45***
	20 : 20	61.5 ± 2.46	20.7 ± 2.46***
Synthetic I + (*S*)-IIe	2000 : 2000	84.0 ± 2.04	9.47 ± 2.04***
	200 : 200	88.4 ± 1.13	5.81 ± 1.13***
	20 : 20	63.1 ± 2.68	15.6 ± 2.68***
Natural IV	200	49.2 ± 3.53	29.7 ± 3.53 NS
	20	29.0 ± 2.06	28.5 ± 2.06 NS

[a]All tests followed by the same letter were performed in the same 2-hr session.
[b]Significant response (*t* test) to stimulus indicated by: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS = not significant.

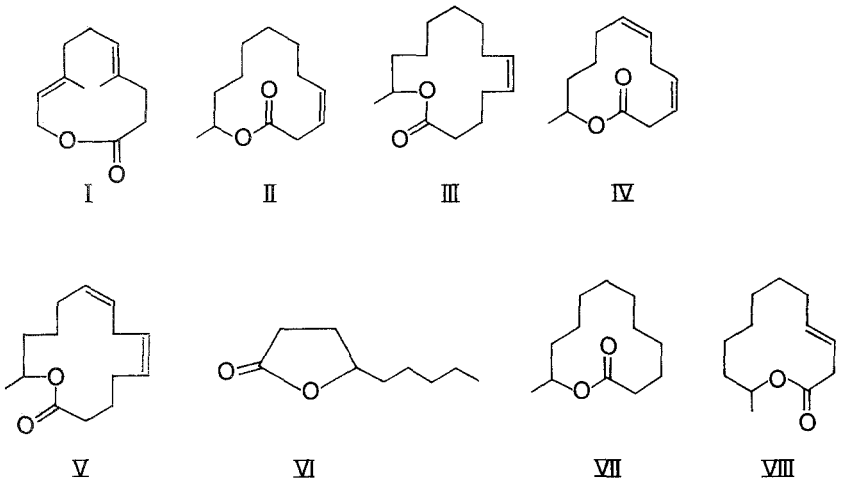


FIG. 5. Structures of macrolides isolated from *C. ferrugineus* frass volatiles. The double-bond positions and geometries in V and VIII are not confirmed.

response, identical to that for a mixture of I and (*S*)-II, while (*R*)-II did not behave synergistically with I. Therefore, (*R*)-II is completely inactive and does not inhibit the activity of (*S*)-II. Synthetic racemic II is also inactive and does not enhance the activity of a mixture of I and II.

Chemistry. Data from bioassays indicated that I and II were the aggregation pheromones. Therefore, structural elucidation and chemical syntheses were centered on these compounds. Some of the other constituents of frass and beetle volatiles (III–VIII) were also identified. The four major compounds (I–IV) were identified by spectroscopic methods and chemical tests. Minor constituents (V–VIII) were identified by GLC-MS, GLC retention times, and chemical transformations to known compounds. Figure 5 shows the proposed structures for I–VIII.

Approximately 200 μg of pure I (>99% by GLC on column C, 70° C (2 min) to 180° C at 4° C/min) was isolated from 5.38×10^4 gh of frass volatiles.

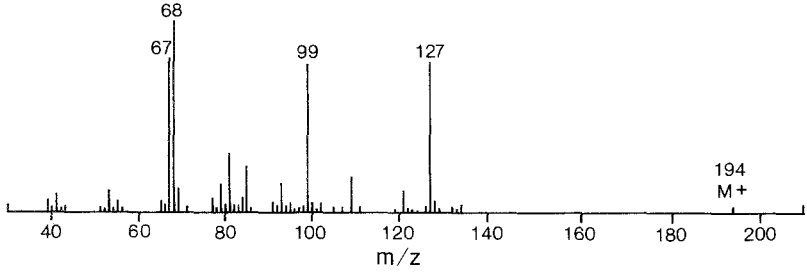


FIG. 6. Unit-resolution mass spectrum of I.

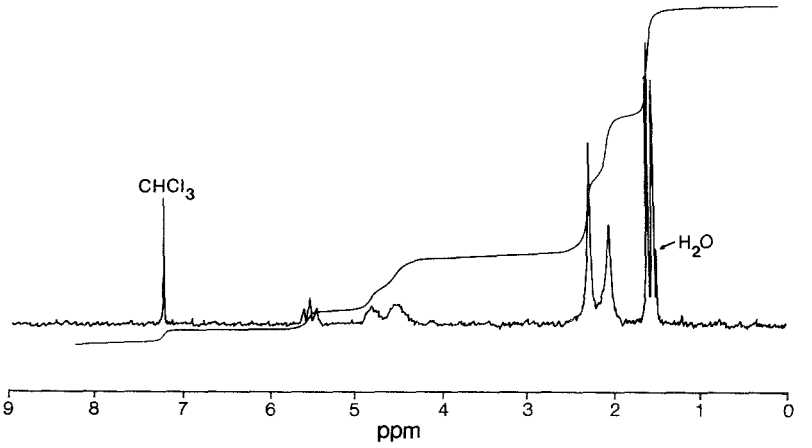
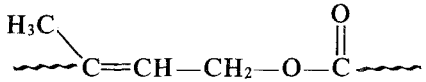


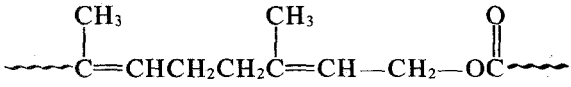
FIG. 7. PMR spectrum (100 MHz) of I recorded on 200 μg of sample in $CDCl_3$.

The high-resolution and unit-resolution mass spectra of I (Figure 6) indicated a molecular ion of $C_{12}H_{18}O_2$ (measured mass, 194.1291; calculated for $C_{12}H_{18}O_2$, 194.1307). Strong peaks at m/z 195 (M+H) and m/z 212 (M+NH_4) in the chemical-ionization mass spectrum confirmed that m/z 194 was the molecular ion. The presence of an ester function in I was indicated by a strong absorption at 1730 cm^{-1} (C=O stretch of ester) and 1132 cm^{-1} (C—O stretch of ester). Component I also contained three sites of unsaturation in addition to the ester function. The PMR spectrum (Figure 7) contained evidence of two trisubstituted double bonds. Signals for vinyl methyl doublets at δ 1.57 (J = 0.9 Hz) and δ 1.64 (J = 1.2 Hz) were separately coupled to the broad multiplet at δ 4.82 and the triplet at δ 5.54, respectively, as shown by decoupling experiments. Thus, irradiation at the frequency of the vinyl proton (δ 5.54) caused the signal for the vinyl methyl doublet at δ 1.64 to collapse to a singlet, but did not affect the signal at δ 1.57. The multiplet at δ 4.56 (2H) was also coupled to the vinyl proton signal at δ 5.54. This information indicates that the vinyl methyl groups are on separate double bonds. The chemical shift of the δ 4.56 signal is appropriate for an allylic methylene deshielded by an ester oxygen. These data led to the following partial structure:



The position of the second trisubstituted olefin was determined by irradiation at the frequency of the four-proton signal at δ 2.09 which resulted in simplification of the δ 4.82 vinyl proton signal. The chemical shift of the δ 2.09 signal is identical to the shifts observed for two methylenes between two

olefins. Therefore, the trisubstituted olefins were joined together by two methylene groups. However, the PMR data did not provide sufficient information to determine the orientation of the second trisubstituted olefin. Since other coleopterous insects utilize terpene precursors for pheromone synthesis (Brand et al., 1979), we favored a terpenoid arrangement of the double bonds:



The remaining four protons (δ 2.32) must be on carbons that connect the two ends of the chain to form an 11-membered lactone. The ring accounts for the remaining site of unsaturation.

The CMR spectrum of I, obtained on a 1000-μg sample isolated from 4.0×10^5 gh of frass volatiles, contains signals which support the proposed structure. Resonances at 142.8 ppm and 132.8 ppm are appropriate for the quaternary carbons of the double bonds, while those appearing at 126.4 ppm and 122.3 ppm are appropriate for the monoprotonated vinyl carbons. The signal at 61.5 ppm can be readily assigned to the allylic carbon adjacent to the ester oxygen. Three signals were observed in the methylene region at 38.5, 33.5, and 24.9 ppm. Since our proposed structure possesses four methylenes, in addition to the one next to the ester oxygen, two of the methylenes must share the same resonance. The remaining signals at 15.3 ppm and 14.7 ppm belong to two vinyl methyl carbons on double bonds with *E* geometry. The resonance for the carbonyl carbon was not observed, probably due to the small size of the sample. Final confirmation of the proposed structure of I was achieved via chemical synthesis of I. The synthetic compound, prepared by two routes (Wong, 1982), was identical to the natural product in all respects. The CMR spectrum of synthetic I contained all the signals reported above and a signal at 174.2 ppm for the carbonyl carbon.

Approximately 750 μg of pure II (>99% by GLC analysis on column C) was isolated from 4.04×10^5 gh of frass volatiles. The high-resolution and unit-resolution mass spectra (Figure 8) for II indicated a molecular ion of $C_{12}H_{20}O_2$ (measured mass, 196.1449; calculated for $C_{12}H_{20}O_2$, 196.1463). This formula was supported by the chemical-ionization mass spectrum which contained a prominent peak at m/z 214 ($M+NH_4$). Although the molecular weights of I and II only differ by two mass units, the mass spectra were quite different. A peak at m/z 181 (M-15) was indicative of a methyl branch in the molecule. Clusters of peaks separated by 14 mass units suggested the presence of a hydrocarbon chain. The IR spectrum of II contained a strong absorption at 1730 cm^{-1} and was devoid of a hydroxyl absorption which suggested that both oxygens were in an ester or lactone function. Two coupled, single proton resonances in the PMR spectrum (Figure 9) at δ 5.62 and δ 5.50 ppm (J = 9.0

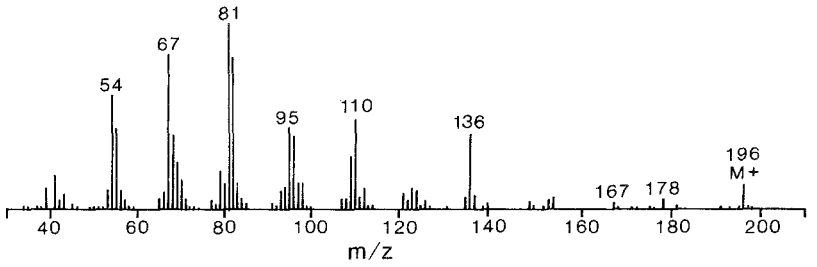


FIG. 8. Unit-resolution mass spectrum of II.

Hz) indicated the presence of a single disubstituted double bond with *Z* geometry. The assignment of *Z* geometry was supported by the absence of a strong absorption at 970 cm^{-1} in the IR spectrum. Since the olefin and the ester account for only two sites of unsaturation, II must contain one ring. The presence of resonances due to 10 methylene hydrogens (δ 1.3–1.7 ppm) is indicative of a hydrocarbon chain within the molecule. However, the absence of any signal at a higher field than the methyl doublet (δ 1.26) suggests that the methylenes are not involved in an open-chain structure terminated by a methyl group.

This information led us to consider a macrolide structure for II. Since a single methyl doublet was observed, a 12-membered ring was suspected. The chemical shifts of the methyl doublet and the signal of the methine proton (δ 5.04 ppm) to which it was coupled indicated that the methyl group was on the carbon directly adjacent to the oxygen atom. A number of macrolides bearing methyl groups adjacent to the oxygen atom exhibit similar proton chemical shifts in their PMR spectra (Kaiser and Lamparsky, 1978). The position of the double bond was established by the presence of two proton resonances at δ 3.14 and 3.01 ppm which are coupled to the signal of one of the vinyl protons ($J = 8.7$ Hz and $J = 7.7$ Hz). The chemical shifts of these signals are appropriate for an allylic methylene group adjacent to an ester carbonyl. Therefore II was considered to be a β,γ-unsaturated lactone.

The CMR spectrum of II (δ 20.2, 25.0, 25.8, 26.1, 27.8, 31.4, 34.4, 78.0, 122, and 135 ppm) also supports the proposed structure. The two signals at 122 and 135 ppm can be assigned to the vinyl carbons. The resonance at 78.0 ppm can be assigned to the carbon directly adjacent to the ester oxygen. The remaining signals can be assigned to the methyl carbon (20.2 ppm) and the seven methylene carbons. Since only six signals are unaccounted for, two of the methylene carbons must possess the same resonance frequency. The structure proposed for II accommodates all the spectral data which have been obtained. Partial confirmation of the proposed structure was obtained by catalytic hydrogenation of II. Hydrogenation yielded a compound which had

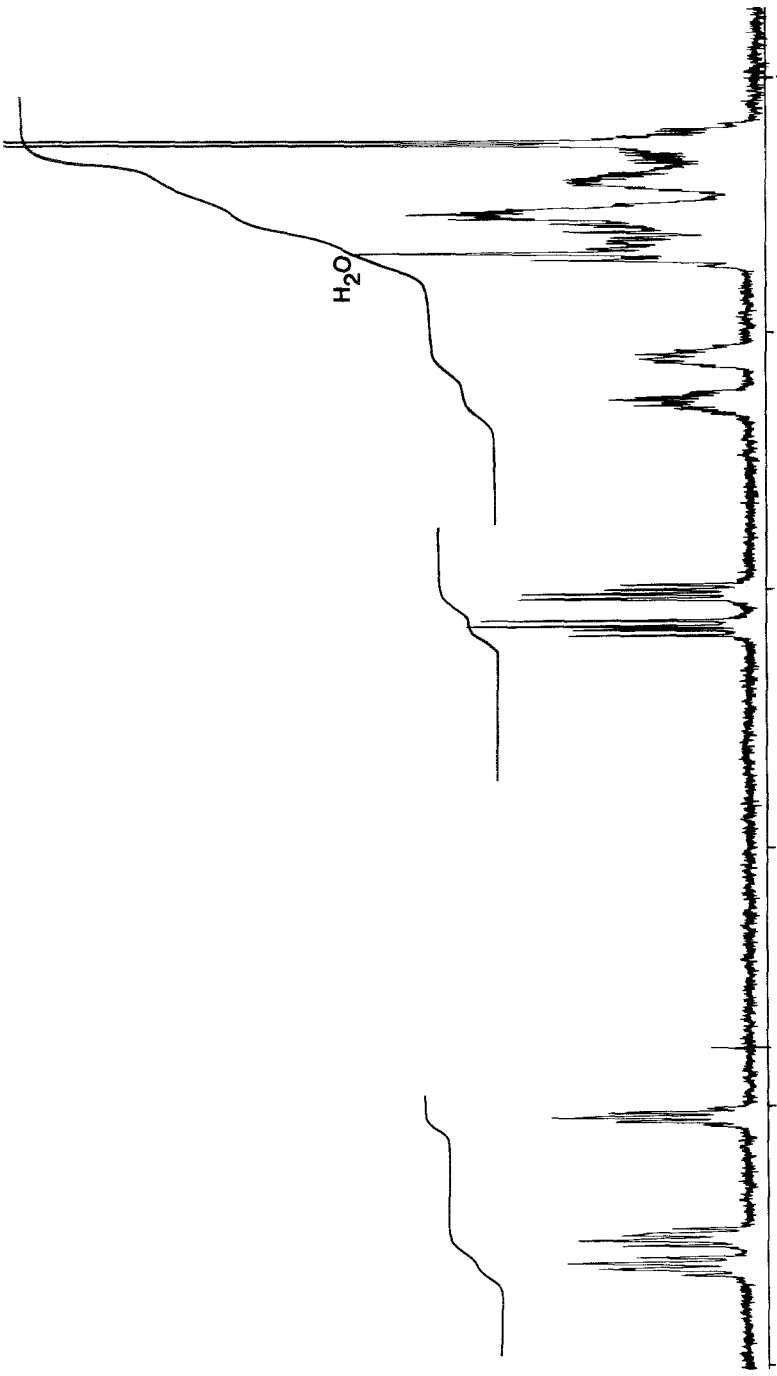


FIG. 9. PMR spectrum (400 MHz) of II recorded on 750 μg of sample in $CDCl_3$.

a molecular weight of 198 and a fragmentation pattern in the mass spectrum which as identical to that of 11-dodecanolide (Vesonder et al., 1971).

Compound II contains one chiral center and therefore could occur as the (*R*)- or (*S*)-enantiomer or some combination thereof. The determination of the absoute configuration of II was carried out by addition of the chiral NMR shift reagent, $Eu(tfc)_3$, to $CDCl_3$ solutions of II. In all of the spectra recorded on samples containing $Eu(tfc)_3$, the samples were irradiated at the resonance of the methine proton to simplify the methyl proton signal to a singlet. The addition of 20 mol% $Eu(tfc)_3$ to racemic II resulted in two methyl singlets separated by 5.4 Hz. Line-broadening offset the increased shift difference obtained by the addition of shift reagent above 20 mol %. When 20 mol % $Eu(tfc)_3$ was added separately to natural II and the synthetic (*S*)-enantiomer, only a single methyl singlet was observed in each spectrum. The addition of racemic II to the samples of natural II and synthetic (*S*)-II containing $Eu(tfc)_3$ both resulted in the enhancement of the high-field methyl singlet. Therefore, the natural product appears to be comprised of only the (*S*)-enantiomer. However, a small amount of the (*R*)-isomer ($<5\%$) would not be detectable by this method. The small quantity of natural material that was isolated precluded the determination of optical rotation. Confirmation of the structure of II was obtained by the synthesis of the racemic II and the (*S*)-isomer. The natural product and the synthetic products were chromatographically and spectrally identical (Wong, 1982).

Compound III, [(*Z*)-5-tetradecen-5-olide; 200 μg isolated from 4.04×10^5 gh of frass volatiles; >99% by GLC analysis on column C] was found to have an exact mass of 224.1779 by high-resolution mass spectroscopy. This molecular weight indicated a formula of $C_{14}H_{24}O_2$ (calculated mass, 224.1776) and revealed three sites of unsaturation. The IR spectrum of III was similar to the spectrum of II and contained a strong absorption at 1725 cm^{-1}. The fragmentation pattern for III in the electron impact (EI) mass spectrum is also similar to the fragmentation pattern for II. These similarities suggest that III is a homolog of II.

The 400-MHz PMR spectrum of III provided sufficient information to deduce the structure. Signals were observed at δ ppm (number of hydrogens, multiplicity: d = doublet, m = multiplet): 1.12 (1,m), 1.22 (3,d, $J = 6.1$ Hz) 1.24–1.66 (10,m), 1.72 (1,m), 1.78–1.95 (2,m), 2.12–2.26 (2,m), 2.30 (1,m), 2.41 (1,m), 4.98 (1,m), 5.30 (1,m), and 5.38 (1,m). Many similarities between the PMR spectra of II and III were apparent. The methyl signal (δ 1.22 ppm, d) was shown to be coupled to the methine proton signal at δ 4.98, indicating the presence of a methyl group on the carbon adjacent to the ester oxygen. The two vinyl proton signals at δ 5.30 and δ 5.38 ppm ($J = 10.5$ Hz) suggested the presence of a *Z*-disubstituted double bond. The presence of a saturated hydrocarbon chain was indicated by the 10-proton signal in the region between δ 1.24 and δ 1.66 ppm. Based on these spectroscopic data, III was

proposed to be an unsaturated 14-membered lactone. The position of the double bond was tentatively determined by decoupling experiments. Irradiation at the frequency of one of the protons α to the carbonyl (δ 2.41 ppm) resulted in simplification of a signal centered around δ 1.82 ppm. Presumably the proton signal at δ 1.82 ppm could be assigned as the resonance of one of the protons β to the carbonyl. When the sample was irradiated at the frequency of the β proton signal, the expected simplification of the α protons was observed (loss of 9.0-Hz coupling) along with simplification of signals due to a pair of allylic protons at δ 2.30 and δ 2.19 ppm (overlap with α proton). The direct coupling of the allylic proton signal and the vinyl proton signal was demonstrated by irradiation at the frequency of the signal at δ 2.30 ppm. This set of decoupling experiments revealed that the double bond was δ to the carbonyl.

Catalytic hydrogenation of III produced a single compound with a molecular weight of 226. The fragmentation pattern of the hydrogenated material was identical to the fragmentation pattern for the mass spectrum of 13-tetradecanolide which is a macrolide constituent of a *Galbanum* absolute (Kaiser and Lamparsky, 1978). These results confirmed the carbon skeleton proposed for III. Confirmation of the position of the double bond was carried out by the synthesis of racemic III (J. Millar[2], unpublished result), which was chromatographically and spectrally identical to isolated III.

The mass spectrum of IV (Figure 10) revealed a molecular ion at 194 which is identical to the molecular weight of I. However, IV, which elutes before I on the SP-1000 column, has a different fragmentation pattern in its EI mass spectrum. Catalytic hydrogenation of IV yielded a compound with a molecular weight of 198 and a retention time of GLC identical to that for 11-dodecanolide. The fragmentation patterns for reduced IV and 11-dodecanolide were identical. These experiments showed that IV has the same carbon skeleton as II, but has one more site of unsaturation. A 400-MHz PMR spectrum of IV (Figure 11) showed, as expected, a signal due to a methyl doublet (δ 1.23 ppm) coupled to the resonance for a single-proton multiplet at

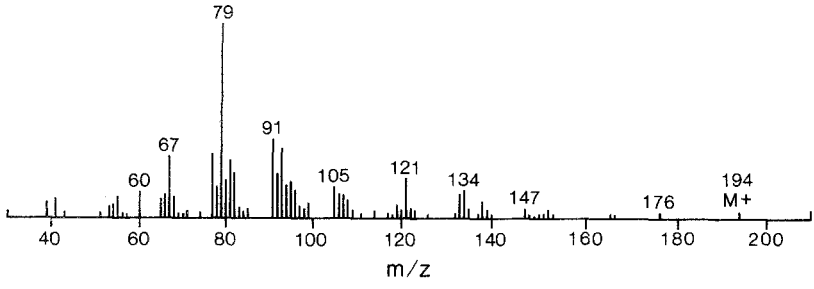


FIG. 10. Unit-resolution mass spectrum of IV.

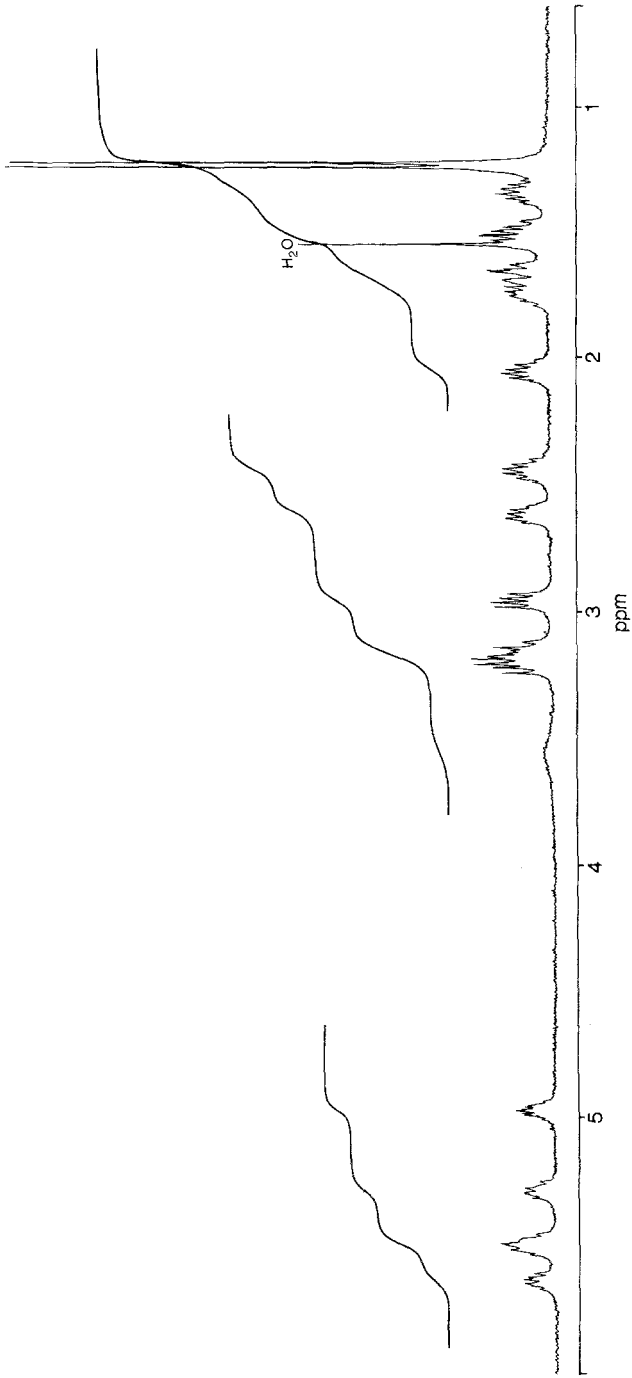


FIG. 11. PMR spectrum (400 MHz) of IV recorded on 100 μg of sample in $CDCl_3$.

δ 4.96 ppm (J = 6.3 Hz). Signals for four vinyl protons at δ 5.30 (1,m, J = 9.0 Hz), δ 5.50 (2,m), and δ 5.64 (1,m, J = 9.0 Hz) ppm indicated that two double bonds were present. The chemical shifts (<6 ppm) of the vinyl hydrogen resonances indicated that the double bonds were not conjugated with each other or with the carbonyl group. The coupling constants for the vinyl protons indicated a *Z,Z* geometry. Since only six protons were observed in the allylic proton region at δ 2.05 (1,m), δ 2.45 (1,m), δ 2.61 (1,m), δ 2.93 (1,m), and δ 3.18 (2,m) ppm, it was clear that IV possesses a 1,4-diene system. The position of the 1,4-diene system was determined by the presence of signals due to two coupled protons at δ 2.93 and δ 3.18 ppm (J = 14 Hz). These signals are virtually identical in chemical shift and splitting pattern to the signals due to the methylene protons in II which are in between the carbonyl and the double bond. Based on this information, IV is proposed to be (*Z, Z*)-3,6-dodecadien-11-olide (Figure 5).

The EI mass spectrum of V (Figure 12) closely resembles that of IV except that the molecular ion is at 222. Hydrogenation of V yielded a compound with a molecular ion at 226 and a GLC retention time and fragmentation pattern which was identical to that of 13-tetradecanolide. Therefore V must be a homolog of IV. Since the quantity of natural material available was insufficient for PMR, the position of the double bonds could not be determined. However, if the analogy between II and IV holds true for III and V, then V could be the 5,8-dienolide (Figure 5). Beetle volatiles typically contain approximately 0.6% of V.

The structure of VI was identified on the basis of its GLC retention time and characteristic mass spectrum. An intense peak at m/z 85 indicated that the compound could be a γ-lactone. Thus cleavage at the alkyl side chain results in the lactone moiety bearing virtually all the charge. The spectrum of VI was identical to the spectrum of 4-nonanolide (Jennings and Shibamoto, 1980). Authentic 4-nonanolide (ICN pharmaceuticals) and VI coeluted from column B. Usually VI accounts for only 0.3% of beetle volatiles.

A small quantity of VII was detected in frass and beetle volatiles (0.6%) by GLC-MS analysis. The natural material had a retention time (30 m × 0.32

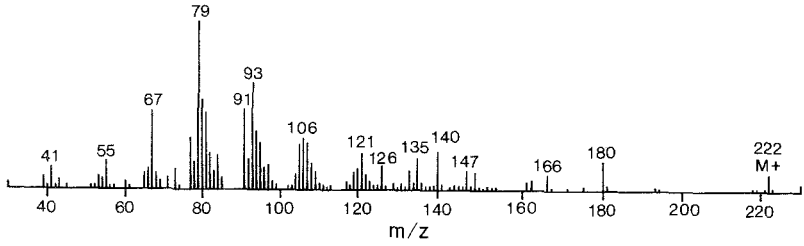


FIG. 12. Unit-resolution mass spectrum of V.

mm open-tubular column coated with Carboxwax 20 M) and fragmentation pattern which was identical to that of authentic 11-dodecanolide.

A compound which eluted just before II is suspected to be the *E* isomer (VIII) based on the identity of mass spectral fragmentation patterns for the two compounds. Isomers in which the unsaturation is at different positions would be expected to have different fragmentation patterns.

The identification of I and II as the aggregation pheromone for *C. ferrugineus* in this report is the first example of macrolide insect pheromones. We propose the names ferrulactone I and ferrulactone II for compounds I and II, respectively. Ferrulactone I has a novel, terpene-derived, macrolide structure. Other macrolide natural products have been identified from a variety of organism including insects. The metasternal gland secretion of *Phoracantha synonyma* (Newman) contains several macrolides (Moore and Brown, 1976) which are structurally similar to the compounds isolated from *C. ferrugineus*. Ten-, 12-, and 14-membered lactones, each bearing a methyl group adjacent to the alkyl oxygen of the ester, were identified. The 12-membered lactone which Moore and Brown (1976) discovered, (*Z*)-5-dodecen-11-olide, differs from II only in the position of the double bond. They also report the identification of (*Z*)-5-tetradecen-13-olide (III) and 5,8-tetradecadien-13-olide which may be the same as V. The macrolides produced by *P. synonyma* have not been implicated as pheromones.

A 14-membered lactone, 13-(1-methylpropyl)tridecanolide, has been isolated from the total extract of the argentine ant, *Iridomyrmex humilis* (Cavill *et al.*, 1979). The biological function of this macrolide has not yet been determined.

Recifeiolide, (8*E*, 11*R*)-8-dodecen-11-olide, is a fungal metabolite which was isolated from the growth medium of *Cephalosporium recifei* (Vesonder et al., 1971). The only differences between recifeiolide and II are the position and geometry of the double bond and the absolute configuration of the chiral center. A number of studies have been directed toward the synthesis of recifeiolide (Gerlach et al., 1976; Utimoto et al., 1977; Trost and Verhoeven, 1978; Shreiber, 1980).

Saturated 13-, 14,- 15-, and 16-membered lactones which have a methyl side chain next to the ester alkyl oxygen have been isolated from a *Galbanum* absolute (Kaiser and Lamparsky, 1978).

Nonan-4-olide is a constituent of wheat germ oil volatiles (Nara et al., 1981). Thus, the presence of nonan-4-olide in frass volatiles and not beetle volatiles may be due to the presence of wheat germ in frass. The role of nonan-4-olide as a pheromone of *C. ferrugineus* has not been determined.

Out experiments have led us to the discovery of two pheromones, ferrulactones I and II, which are strongly attractive to *C. ferrugineus* in our laboratory bioassay. Prior to field tests of the pheromones in grain storage

TABLE 3. RESPONSE OF *C. ferrugineus* TO DIFFERENT RATIOS OF SYNTHETIC PHEROMONES IN A TWO-CHOICE, PITFALL OLFACTOMETER, $N = 6$ REPLICATES

Stimulus[a] and ratio	Dose (ng)	% Response[b] ($\bar{X} \pm$ SE)	
		Stimulus	Control
I:(*R*,*S*)-II (9:1)	4500:500	75.0 ± 4.1	17.0 ± 3.8**
	450:50	85.1 ± 3.7	14.9 ± 2.5***
	45:5	80.0 ± 5.2	13.3 ± 1.6***
	4.5:0.5	66.3 ± 9.7	33.7 ± 8.4*
	0.45:0.05	42.5 ± 7.0	46.0 ± 12.3 NS
I:(*R*,*S*)-II (3:1)	1500:500	77.8 ± 4.5	18.9 ± 4.8**
	150:50	78.7 ± 1.4	20.2 ± 2.0***
	15:5	59.8 ± 2.0	31.5 ± 6.4*
	1.5:0.5	51.1 ± 4.0	30.0 ± 4.6 NS
	0.15:0.05	44.3 ± 3.9	27.3 ± 2.2 NS

[a]All tests were performed in the same 2-hr session.
[b]Significant response (*t* test) to stimulus indicated by: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS = not significant.

areas, we tested different ratios of the two components to determine the optimum ratio (Table 3). The more easily prepared racemic II was used instead of (*S*)-II since the (*R*)-enantiomer was not inhibitory. The results showed that the 9:1 mixture of I:(*R*,*S*)-II had a lower threshold level [4.75 ng, based on (*S*)-enantiomer only] than the 3:1 mixture (17.5 ng) or the 1:1 mixture (40 ng, Table 2). Therefore, the 9:1 mixture was utilized for field trials.

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THE ROLE OF IRIDOID GLYCOSIDES IN HOST-PLANT SPECIFICITY OF CHECKERSPOT BUTTERFLIES

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Abstract—The potential role of iridoid glycosides as feeding stimulants for *Euphydryas chalcedona* larvae was examined in three laboratory experiments. The first experiment examined larval behavior in choice tests between an artificial diet with no additives (AD) and an artificial diet with the iridoid glycoside, catalpol, added (AD + I) in one group; and AD and AD plus a crude extract from which the iridoid glycoside catalpol was crystallized (AD + Ex) in the second group. The larvae were found more often on AD + I or AD + Ex. The second experiment quantified larval consumption of artificial diets when given a choice of AD or AD + I, and AD or AD + Ex, and showed that larvae ate significantly more AD + I or AD + Ex than AD. The third experiment compared growth and survival on six diets: AD; AD + I; artificial diet with dried, ground up *Scrophularia californica* leaves (AD + S); artificial diet with dried, ground up *Plantago lanceolata* leaves (AD + P); *S. californica* leaves (S); and *P. lanceolata* leaves (P). Growth was best on *S. californica* leaves, and survival was highest on *S. californica* and *P. lanceolata* leaves. There were no differences in growth rate or survival between AD and AD + I. Thus, iridoid glycosides serve as feeding attractants and stimulants for larvae of *Euphydryas chalcedona* and are suggested as the basis of radiation in butterflies of the genus *Euphydryas*.

Key Words—Iridoid glycoside, catalpol, Scrophulariaceae, *Euphydryas*, checkerspot, host-plant specificity, Lepidoptera, Nymphalidae, coevolution, insect–plant interaction, chemical ecology.

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INTRODUCTION

Host-plant specificity in insects has been described as closely tied to the secondary chemistry of the plants utilized (e.g., Vershaffelt, 1911; Dethier, 1941, 1954; Fraenkel, 1959, 1969; Brower and Brower, 1964; Ehrlich and Raven, 1964; Feeny, 1975). More specifically, in some cases certain compounds typical of host-plants have been shown to be feeding or oviposition stimulants to insects specializing on those plants (e.g., Dethier, 1941; David and Gardner, 1966a,b; Schoonhoven, 1972; Ma and Kubo, 1977; Stanton, 1979). The Lepidoptera in particular have been used in efforts to pinpoint the chemical factors responsible for producing specificity in feeding habits. Although mistakes do occur (e.g., Straatman, 1962; Sevastopolo, 1964; Chew, 1977), female butterflies are generally quite discriminating about where they will lay their eggs (e.g., Chew, 1977; Tabashnik, 1981; Wiklund, 1981; Rausher, 1982; Singer, 1982), and this discriminatory ability is important in ensuring the survival of offspring, as most newly hatched larvae cannot go far in search of food. Larval feeding preferences, however, may be more catholic; larvae may exhaust the food supply and in later instars have to search for additional resources. The ranges of plants utilized by larvae, particularly later instars, may be much larger than those acceptable to ovipositing females, although females may become less discriminating the longer they are prevented from ovipositing (e.g., Singer, 1981). Thus, larvae may respond to a more general range of stimuli than ovipositing females.

In North America, butterflies of the genus *Euphydryas* Scudder (Nymphalidae) utilize plants in four families: Scrophulariaceae, Plantaginaceae, Caprifoliaceae, and Oleaceae (Clark, 1927; Klots, 1958; Masters, 1969; Bowe, 1972; Tietz, 1972; Ehrlich et al., 1975) and in one instance the Labiatae (D. Wiernasz, personal communication). These plant families have in common the presence of a group of plant secondary compounds known as iridoid glycosides (Table 6) (Kooiman, 1972; Jensen et al., 1975). A variety of iridoid glycosides occur in *Euphydryas* food plants, and no one single iridoid is characteristic of all the host-plants (Jirawongse, 1964; Kooiman, 1972; Jensen et al., 1975). However, all of the plant genera fed on by *Euphydryas* that have been tested contain iridoid glycosides (Table 6).

Feeding behavior in another lepidopteran, *Ceratomia catalpae* (Sphingidae) (the catalpa sphinx) was shown by Nayar and Fraenkel (1963) to be elicited by an artificial diet containing a mixture of "catalposides" (i.e., iridoid glycosides) isolated from its food plant, *Catalpa bignonioides* (Bignoniaceae). In contrast, Bernays and de Luca (1981) found that another iridoid glycoside, ipolamiide [isolated from *Stachytarpheta mutabilis* (Verbenaceae)] was a feeding deterrent for three generalist insects: *Spodoptera littoralis* (Lepidoptera: Noctuidae), *Schistocerca gregaria*, and *Locusta migratoria* (both

Orthoptera: Acrididae). Thus iridoid glycosides seem to deter generalist insects, protecting the plants containing them against all but a few specialist species which have circumvented this defense.

Different *Euphydryas* species feed on different plants and have different feeding strategies: *E. gilletii*, for example, is monophagous on *Lonicera involucrata* (Caprifoliaceae) (Williams et al., 1983), while in *E. editha*, different populations use different host-plants, some populations being confined to a single plant species and others using multiple hosts (Ehrlich et al., 1975; Singer and Ehrlich, 1979). Strong circumstantial evidence, such as *Euphydryas* larvae feeding on "non-host" plants which contain iridoid glycosides (Bowers, 1981, and unpublished) coupled with the ubiquity of these compounds in the host-plants, suggested that iridoid glycosides might provide the chemical basis for host-plant specificity in the genus *Euphydryas*.

To determine the role of iridoid glycosides in *Euphydryas* foodplant specialization, I undertook a series of experiments using artificial diet, into which I could incorporate plant material or iridoid glycosides. The first experiment examined the behavioral response of larvae to artificial diets with or without iridoid glycosides; and the second quantified larval consumption of artificial diets with and without iridoid glycosides. The third experiment compared the growth and survival of larvae reared on fresh leaves and on artificial diets with a variety of additives.

METHODS AND MATERIALS

Butterflies and Plants. Larvae of *Euphydryas chalcedona* (Doubleday) were reared from the eggs of females from two populations. Larvae of this species diapause in the fourth instar and thus all experiments were conducted on prediapause larvae. Caterpillars used in the behavior and consumption tests were from Echo Lake, El Dorado County, California, where the foodplant is *Penstemon newberryi* Gray (Scrophulariaceae) (D. Murphy, personal observation). Those used for measuring growth and survival on different diets were taken from Jasper Ridge Biological Preserve, San Mateo County, California, where the food plants are two members of the Scrophulariaceae, *Diplacus aurantiacus* (Curtis) Jeps. and *Scrophularia californica* Cham. & Schlecht.

Scrophularia californica, *P. newberryi*, and *Plantago lanceolata* L. (Plantaginaceae) were used for rearing larvae and incorporation into the artificial diets. *Scrophularia californica* and *P. newberryi* were collected from the native populations, potted, and maintained in a greenhouse. *Plantago lanceolata* leaves were collected on the Stanford campus.

All larvae were reared in plastic Petri dishes with a piece of damp paper

towel taped to the lid to prevent desiccation. The food was placed in the center of the dish. The Petri dishes were kept in an environmental chamber with 16 hr of light and 8 hr of dark, and a day temperature of 25°C and a night temperature of 15°C.

Extraction of the Iridoid Glycoside, Catalpol. Catalpol was extracted from fresh *P. lanceolata* leaves using the charcoal adsorption method of Trim and Hill (1952). This involved a water extraction of the plant material followed by charcoal adsorption of the iridoid glycosides. The adsorbed iridoid glycoside was eluted using 50:50 EtOH–H_2O, and the eluate was concentrated by evaporation. The catalpol crystallized out of the resulting liquor and was purified by recrystallization three times. Thin-layer chromatography showed the compound to be catalpol: the single spot had an R_f of 0.3, and a brown color reaction with H_2SO_4 in MeOH (Wieffering, 1966; Bobbitt and Segebarth, 1969), and an orange color reaction with a *p*-anisidine phosphate reagent (Kooiman, 1967).

Artificial Diet. The artificial diets were made using a slight modification of the recipe of Lincoln et al. (1982) (Table 1). To this basic diet (total dry

TABLE 1. COMPONENTS OF BASIC ARTIFICIAL DIET[a]

Ingredient	Amount
Starch	3.00 g
Sucrose	4.95 g
Wheat germ	2.40 g
Wesson salts	1.38 g
Vandersandt, vitamin mix	2.67 g
Brewer's yeast	0.60 g
Choline chloride	0.135 g
Methyl parabenzoate	0.21 g
Cholesterol	0.09 g
Casein	7.29 g
Ascorbic acid	0.90 g
Tetracycline	0.15 g
Agar	4.00 g
Safflower oil	0.67 ml
Formaldehyde	0.30 ml
KOH	0.72 ml
H_2O	149.00 ml

[a]From Lincoln et al., 1982. Experimental diets contained one of the following in addition to the ingredients above: 1.00 g dried plant material, 0.05 g catalpol, 0.02 g catalpol, or 1 ml crude plant extract.

weight 28.4 g) could be added one of the following: 1 g dried plant material (40.0 mg plant/g diet), 0.02 g of the iridoid glycoside catalpol (0.70 mg catalpol/g diet), 0.05 g catalpol (1.76 mg catalpol/g diet), 1 ml of the crude extract from which the catalpol was crystallized, or nothing. Although relatively little quantitative work has been done on iridoid plant constituents, the amount of iridoid glycoside added to the diet is within the range found in 1 g of plant material (e.g., Trim, 1952; Bobbitt and Segebarth, 1969; Takino et al., 1980). Thus the amount of catalpol found in artificial diets with catalpol corresponds to the amount in artificial diets plus leaf material. The amount of catalpol in the crude extract was not quantified.

The diet was stored until use in covered plastic boxes in the refrigerator, at 3–5°C, and fed to larvae in chunks about 1 × 2 × 10 mm. Larvae in all experiments were given fresh food every two or three days.

Choice Test—Behavior. Larvae in this experiment hatched from eggs obtained from eight Echo Lake females. The eggs from females ovipositing on one day were combined, and all replicates were begun on the same day. Two groups, each containing five replicates of ten larvae, were used. One group was offered a choice of artificial diet with catalpol or artificial diet with no additive; while the second group was offered a choice of artificial diet with the crude extract or artificial diet with no additive. The diets were placed about 2 cm apart in the dish and the dishes oriented randomly to control for position effects (see e.g., Chew, 1980).

Twice a day, once in the morning between 0930 and 1100, and once in the afternoon, between 1500 and 1630, the positions of the larvae were noted as follows: number on AD, number on AD + I or AD + Ex, and number off diet. This was continued for 30 days, when the larvae began to enter diapause.

Choice Test—Consumption. Preliminary experiments had indicated that for prediapause larvae only the third instars ate enough food to be detectable on a weight basis and that field-collected larvae did not initially treat artificial diet with catalpol added as food. If, however, field-collected larvae were fed on artificial diet plus ground *P. newberryi* leaves (the natural host plant), which they ate, the larvae then accepted AD + I or AD + Ex as food. So the experiment was designed using field-collected third instar *E chalcedona* larvae which had fed on AD + *P. newberryi* for two days. One hundred thirty larvae were used in the experiment, five groups of 13 larvae were offered a choice of AD or AD + I and five groups of 13 larvae were offered a choice of AD or AD + Ex.

The larvae were given weighed pieces of diet and allowed to feed for 48 hr (trial 1), the diet collected, fresh diet given, and the larvae allowed to feed for another 48 hr (trial 2). Consumption during each trial was determined on a dry weight basis using the gravimetric techniques of Waldbauer (1968).

While in the refrigerator during the course of the experiment, artificial diets gradually dried out, so a separate regression was used to calculate the

appropriate wet weight-to-dry weight conversion factor, for a particular day of the experiment, for each of the three diets: AD, AD+ I, and AD + Ex. The equations for the lines used to calculate the conversion factors were: AD: $y = 0.2014 - 0.00121x + 0.00005x^2$; AD + I: $y = 0.1838 - 0.0064x + 0.0006x^2$; AD + Ex: $y = 0.1137 + 0.00437x$.

Estimates of the amount of diet eaten in each of the ten groups of larvae included some negative values. These negative numbers reflect the small amounts eaten of the artificial diet with no iridoid glycosides (AD), as well as the necessity of using a wet weight-to-dry weight conversion method. They may also be a function of nonhomogeneity of the diet. The amounts eaten of AD + I and AD + Ex were never negative. To correct for the few negative numbers, the amounts eaten were adjusted by adding 4.0 mg to each (the lowest "negative" amount eaten was −4.00 mg). All statistics and the figures (Figures 3 and 4) reflect these recalculated values.

Growth Rate and Survival. Euphydryas chalcedona eggs were obtained from 12 Jasper Ridge females over a period of one week. Batches of eggs from all females ovipositing on a given day were pooled, so that larvae were not from a single female. Each day that at least 150 larvae hatched, a new replicate of the experiment was set up, for a total of six replicates of six treatments, with 25 larvae in each treatment.

Groups of 25 larvae were reared on one of six diets: (1) S, *Scrophularia californica* leaves; (2) P, *Plantago lanceolata* leaves; (3) AD + S, artificial diet with 1 g dried, ground *S. californica* leaves; (4) AD + P, artificial diet with 1 g dried ground *P. lanceolata* leaves; (5) AD + I, artificial diet with 0.02 g catalpol; (6) AD, artificial diet with nothing added.

This experiment compared the larval growth rate and survival on the various diets, in particular to see how AD + I compared with the others. Every five days, for 25 days, the larvae in each replicate of each treatment were weighed as a group, and the number of surviving larvae was counted. Using these numbers, the mean weight per larva for each replicate of each treatment was calculated.

RESULTS

Choice Experiment—Behavior. When given a choice of AD versus AD + I or AD versus AD + Ex, the pooled results (AM and PM combined) showed that larvae were found significantly more often on AD + I and AD + Ex (Figures 1 and 2). When the AM and PM observations are considered separately, the differences are significant in each case except the PM observations of larvae offered AD + I (Figure 1).

Within a few days after hatching, the larvae constructed a web on one side of the dish and left this only to feed. This resulted in the larvae spending most of their time off the diet (see legends, Figures 1 and 2).

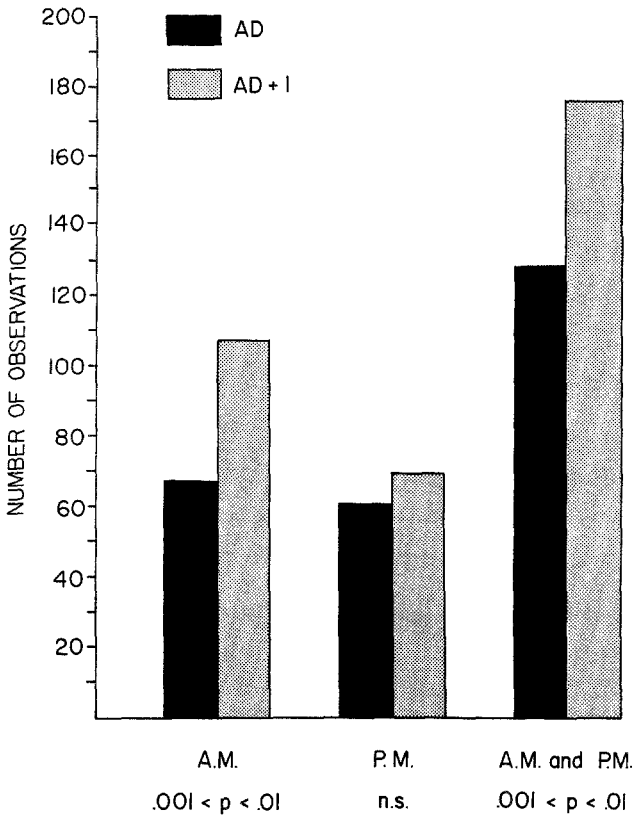

FIG. 1. Behavior of larvae when offered a choice of AD or AD + I, showing the number of larvae observed on each diet. The number of larvae not on either diet was as follows: AM, 750/924 = 81.2%; PM, 784/914 = 85.8%.

Mortality of larvae was high and almost equal in the two groups: group 1 (offered a choice of AD or AD + I), 60%; group 2 (offered a choice of AD or AD + Ex), 62%.

Choice Experiment—Consumption. The results show that much more of the two diets containing iridoid glycosides (AD + I and AD + Ex) were eaten than the artificial diet with nothing added (AD) (Figures 3 and 4). Two-way analyses of variance comparing the amounts eaten of AD and AD + I, and AD and AD + Ex for each of the two trials showed that in every case, more of the artificial diets containing iridoid glycosides (AD + I and AD + Ex) were eaten (Tables 2 and 3, Figure 3 and 4). There were no significant differences among the five dishes of larvae in each trial ($P > 0.10$ in each case).

Growth Rate and Survival. Growth (as measured by weight gain) was greater on *Scrophularia* leaves than on any of the other foods (Table 4, Figure

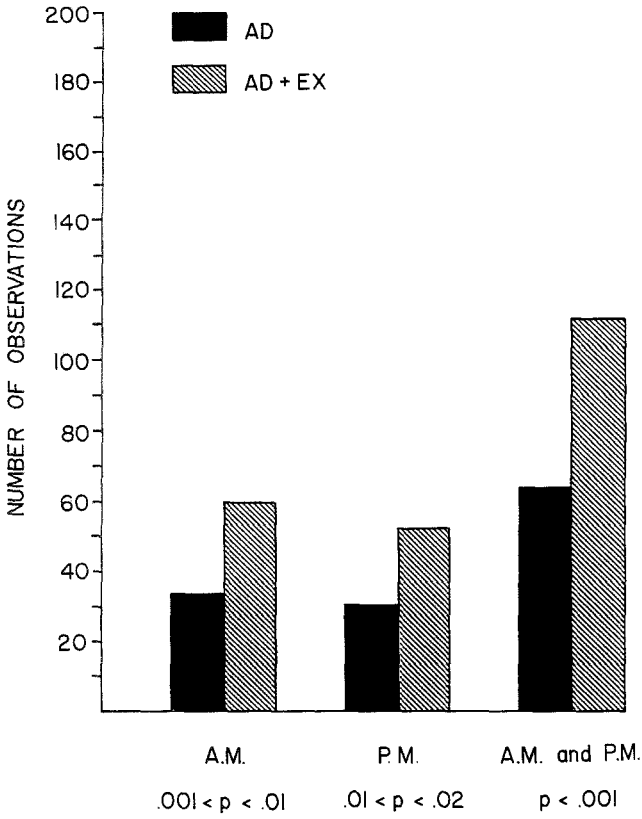

FIG. 2. Behavior of larvae when offered a choice of AD or AD + Ex, showing the number of larvae observed on each diet. The number of larvae not on either diet was as follows: AM, 917/1007 = 90.9%; PM, 920/1002 = 91.8%.

5), while there were no differences between AD and AD + I. Although there were no significant differences in mean larval weight among treatments P, AD + S, AD + P, AD + I, and AD, the relative rankings of these means did change. In particular, the ranking of the larvae fed AD moved from sixth to second. This, as well as the relatively high weight of the AD + I-fed larvae, is due to several of the larvae in these groups bypassing diapause and molting to fifth instar. Larvae in the other treatments, however, were eating less by day 25, preparatory to entering diapause. For all other days, larvae fed AD + I were relatively low in weight, similar to those fed AD.

Survival of larvae was significantly higher on S and P than on AD + I and AD on all days (Table 5, Figure 6), although survival was never

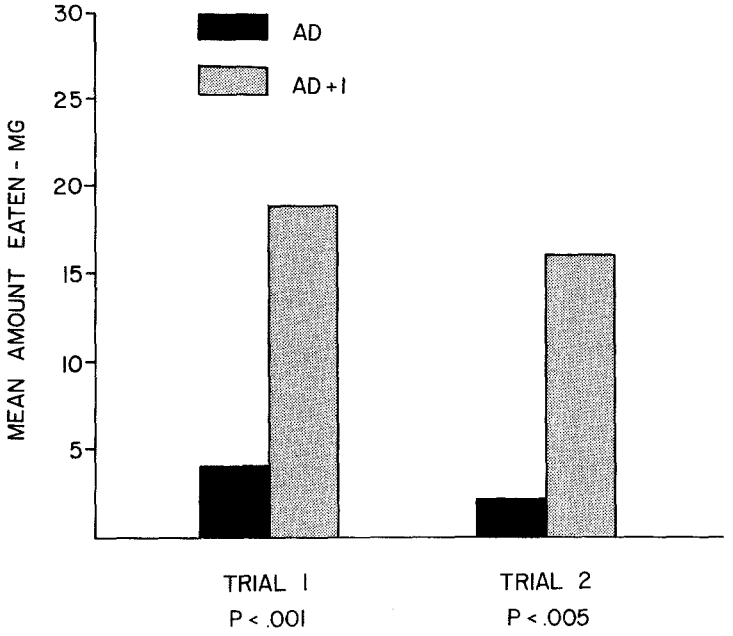

FIG. 3. Consumption by larvae when offered a choice of AD and AD + I: mean amounts eaten in two 48-hr trials.

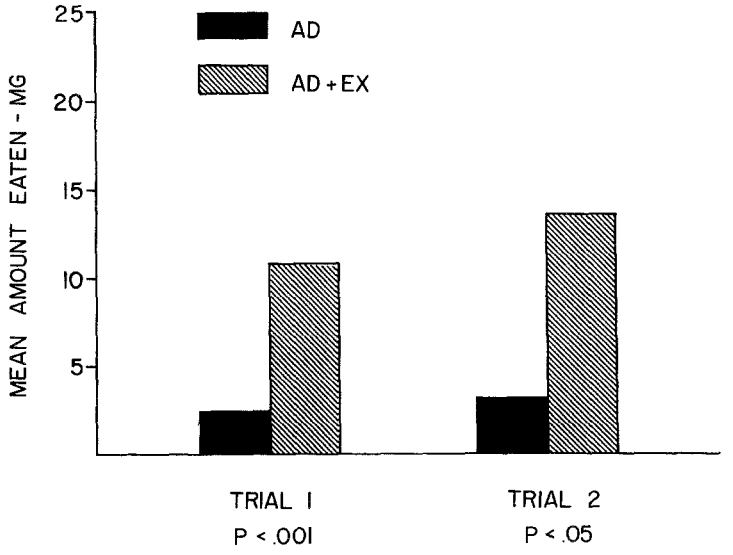

FIG. 4. Consumption by larvae when offered a choice of AD and AD + Ex: mean amounts eaten in two 48-hr trials.

TABLE 2. TWO-WAY ANALYSIS OF VARIANCE COMPARING AMOUNTS EATEN OF AD AND AD + I IN 2 TRIALS, USING 5 DISHES OF 10 LARVAE

Source	Sum of squares	*df*	Mean square	F	*P*
A. Trial 1					
Diet	552.05	1	552.05	111.38	<0.001
Dish	2.71	4	0.68	0.14	>0.10
Error	19.38	4	4.96		
B. Trial 2					
Diet	481.64	1	481.64	569.31	<0.001
Dish	5.14	4	1.29	1.52	>0.10
Error	3.38	4	0.85		

significantly different among larvae fed on S, P, and AD + S, nor among larvae fed on AD + P, AD + I, and AD (Table 5, Figure 6).

DISCUSSION

Bowers (1981) suggested that iridoid glycosides might play a role in determining patterns of hostplant utilization in *Euphydryas*. A literature survey of the genera of food plants of *Euphydryas* butterflies (refs. in Bowers, 1981) showed that they all contained iridoid glycosides, except *Diplacus* and *Besseya*, both in the Scrophulariaceae, which had not been tested (see Table 6). Laboratory tests however, revealed that *Diplacus aurantiacus* and *Besseya alpina* do indeed contain iridoid glycosides (Bowers, unpublished) as

TABLE 3. TWO-WAY ANALYSIS OF VARIANCE COMPARING AMOUNTS EATEN OF AD AND AD + EX IN 2 TRIALS, USING 5 DISHES OF 10 LARVAE

Source	Sum of squares	*df*	Mean square	F	*P*
A. Trial 1					
Diet	176.40	1	176.40	32.15	<0.005
Dish	13.91	4	3.48	0.63	>0.10
Error	21.95	4	5.49		
B. Trial 2					
Diet	201.60	1	201.60	7.92	<0.05
Dish	161.95	4	40.49	1.59	>0.10
Error	101.77	4	25.44		

TABLE 4. COMPARISON OF MEAN WEIGHT PER LARVA (6 REPLICATES PER MEAN) OF LARVAE REARED ON SIX DIFFERENT DIETS (EXPERIMENT 3), OVER 25 DAYS[a]

Day	Mean weight per larva (mg) on various diets					
5	S	P	AD + S	AD + I	AD + P	AD
	1.63	1.41	.894	.799	.649	.619
10	S	AD + S	P	AD + I	AD	AD + P
	6.22	3.87	3.65	2.31	2.19	2.09
15	S	P	AD + S	AD + I	AD	AD + P
	16.49	7.07	5.70	4.26	4.13	3.77
20	S	P	AD + S	AD	AD + P	AD + I
	22.48	12.67	10.38	8.47	7.84	7.70
25	S	AD	AD + I	P	AD + S	AD + P
	26.84	15.22	14.07	12.12	10.97	9.07

[a]According to the Student-Newman-Keuls range test (Sokal and Rohlf, 1969) those means connected by a line are not significantly different at the 1% level.

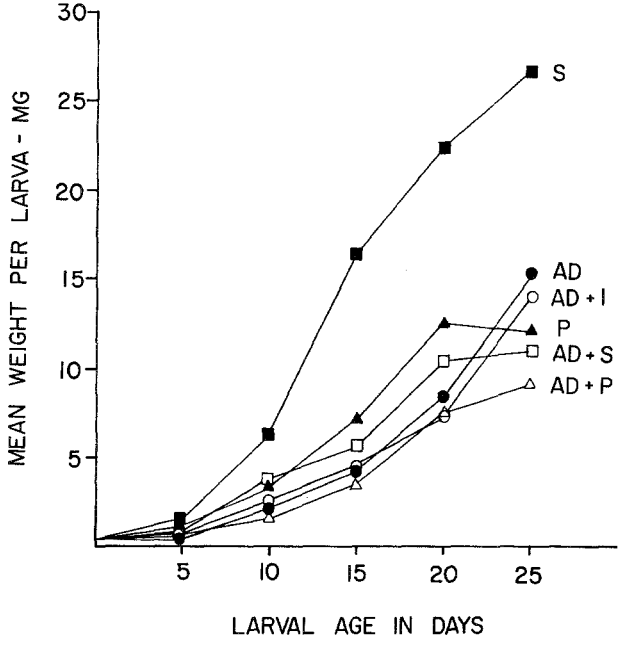

FIG. 5. Mean weight per larva of larvae fed on 6 diets: S, P, AD + S, AD + P, AD + I, and AD; over the 25 days of the experiment.

TABLE 5. COMPARISON OF MEAN SURVIVAL (6 REPLICATES PER MEAN) OF LARVAE REARED ON SIX DIFFERENT DIETS (EXPERIMENT 3), OVER 25 DAYS[a]

Day	Mean Survival (out of 25) of Larvae on Various Diets					
5	S	P	AD + S	AD + P	AD + I	AD
	22.7	22.2	21.0	17.7	14.7	12.5
10	S	P	AD + S	AD + P	AD + I	AD
	22.2	21.5	17.0	12.7	8.3	7.2
15	S	P	AD + S	AD + P	AD + I	AD
	21.0	19.2	15.2	10.7	6.0	5.7
20	S	P	AD + S	AD + P	AD + I	AD
	17.5	17.3	14.3	9.5	5.5	4.8
25	S	P	AD + S	AD + P	AD + I	AD
	16.2	15.0	12.8	8.0	4.5	3.8

[a]According to the Student-Newman-Keuls range test (Sokal and Rohlf, 1969) those means connected by a line are not significantly different at the 1% level.

determined by the Wieffering field test (Wieffering, 1966) and thin-layer chromatography. In addition, *E. chalcedona* larvae will feed and develop on *Aucuba japonica* (Cornaceae), a nonnative shrub which contains the iridoid glycoside, aucubin. Circumstantial evidence thus supports the hypothesis that iridoid glycosides are feeding cues for *Euphydryas* larvae.

The results of the behavior and consumption experiments show that *E. chalcedona* larvae were attracted to and ate much more of the two artificial diets containing iridoid glycosides (AD + I and AD + Ex) than the artificial diet with no additives (AD) (Figures 1–4).

The growth rate and survival experiment showed that larvae fed on artificial diet containing the iridoid glycoside catalpol had no better survival or growth than larvae fed artificial diet with nothing added (Figure 5 and 6). This suggests that larvae were not eating much of either of these diets. Several factors may have contributed to this result: first, the iridoid glycoside used in the AD + I was catalpol, which is only a minor constituent (if present at all) of various *Scrophularia* species (Kooiman, 1972). *Scrophularia californica* is a normal food plant of the *E. chalcedona* used in this experiment. Second, the amount of catalpol used was 0.02 g/28.4 g diet (dry weight), while experiments 1 and 2 used 0.05 g catalpol/28.4 g diet; 0.02 g may be too low a concentration to stimulate much feeding. Third, although catalpol is a larval feeding

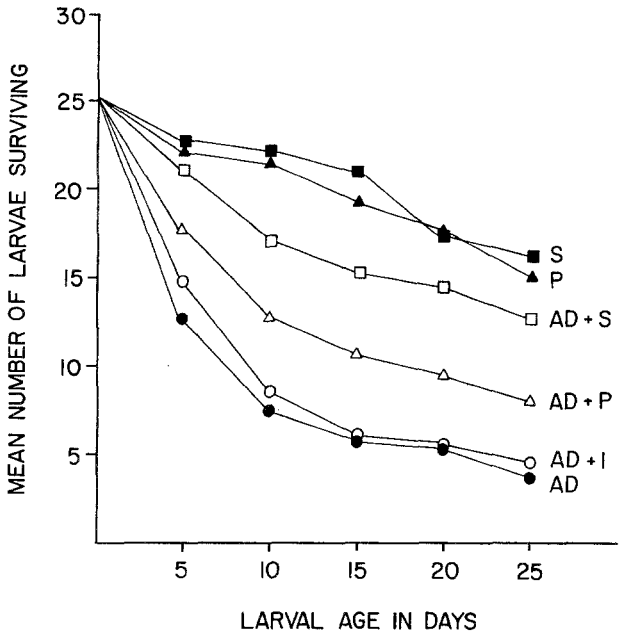

FIG. 6. Mean survival of larvae fed on 6 diets: S, P, AD + S, AD + P, AD + I, and AD; over the 25 days of the experiment.

attractant and stimulant for *E. chalcedona* as shown by the behavior and consumption experiments, other components may be involved as well, which were not present in the AD + I.

The possibility that the demonstrated larval preference in the consumption experiment for iridoid glycoside-containing diets was a conditioned response (Jermy et al., 1968), due to their feeding on an iridoid glycoside-containing food (host plant and artificial diet containing host plant), is belied by the behavior of the newly hatched larvae in the behavior experiment. The newly hatched larvae were given two diets to choose from, one containing iridoid glycosides (AD + I or AD + Ex) and the other not (AD). For the first 4 days, larvae were about equally divided between the two diets, but after this time, larvae clearly preferred the iridoid glycoside-containing diets (Figures 1 and 2). Thus unconditioned larvae also preferred the iridoid glycoside-containing diets.

The AD + I contained only catalpol in addition to the standard dietary components (Table 1); thus, catalpol is a feeding attractant and stimulant for larvae of *E. chalcedona*. The crude extract used to make the AD + Ex was from *Plantago lanceolata*, not a normal host plant of the *E. chalcedona* population that provided these larvae, and both the extract and the plant

TABLE 6. OCCURRENCE OF IRIDOID GLYCOSIDES IN FOOD PLANTS OF NORTH AMERICAN *Euphydryas*[a]

Butterfly	Food-plant genus	Family	Presence of iridoids	Reference
E. phaeton	*Chelone*	Scrophulariaceae	+	Kooiman, 1970
	Aureolaria	Scrophulariaceae	+	Kooiman, 1970
	Penstemon	Scrophulariaceae	+	Kooiman, 1970
	Plantago	Plantaginaceae	+	Duff et al., 1965; Bobbitt and Segebarth, 1969
	Lonicera	Caprifoliaceae	+	Bobbitt and Segebarth, 1969; Souzu and Mitsuhashi, 1969
	Viburnum	Caprifoliaceae	+	Jensen et al., 1975
	Fraxinus	Oleaceae	+	Jensen et al., 1975
E. chalcedona	*Castilleja*	Scrophulariaceae	+	Kooiman, 1970
	Diplacus	Scrophulariaceae	+	Bowers, unpublished
	Pedicularis	Scrophulariaceae	+	Kooiman, 1970
	Penstemon	Scrophulariaceae	+	Kooiman, 1970
	Scrophularia	Scrophulariaceae	+	Kooiman, 1970
	Symphoricarpus	Caprifoliaceae	+	Jensen et al., 1975
E. anicia	*Besseya*	Scrophulariaceae	+	Bowers, unpublished
	Castilleja	Scrophulariaceae	+	Kooiman, 1970
	Penstemon	Scrophulariaceae	+	Kooiman, 1970
	Symphoricarpus	Caprifoliaceae	+	Jensen et al., 1975
E. colon	*Penstemon*	Scrophulariaceae	+	Kooiman, 1970
	Symphoricarpus	Caprifoliaceae	+	Jensen et al., 1975
E. editha	*Castilleja*	Scrophulariaceae	+	Kooiman, 1970
	Collinsia	Scrophulariaceae	+	Kooiman, 1970
	Orthocarpus	Scrophulariaceae	+	Kooiman, 1970
	Pedicularis	Scrophulariaceae	+	Kooiman, 1970
	Penstemon	Scrophulariaceae	+	Kooiman, 1970
	Plantago	Plantaginaceae	+	Kooiman, 1970
	Lonicera	Caprifoliaceae	+	Bobbitt and Segebarth, 1969; Souzu and Mitsuhashi, 1969
E. gilletii	*Lonicera*	Caprifoliaceae	+	Bobbitt and Segebarth, 1969; Souzu and Mitsuhashi, 1969

[a]Parts of this table, in a slightly different form, were published in a previous article: Bowers, 1981.

contain an additional iridoid glycoside, aucubin, not found in the AD + I (Kooiman, 1972, and refs. therein; Bowers, unpublished), as well as other unknown, noniridoid components. Thus, despite containing constituents of a non-host plant, the crude extract was still very attractive to larvae.

Dethier (1947, 1954, 1973) and others (e.g., Schoonhoven, 1972, and refs. therein) have emphasized the importance of olfaction in a larva's initial assessment of the suitability of a plant as food. Crystalline catalpol is not volatile and had no odor detectable to me; the crude extract, however, did contain volatile components and had a distinctive odor. Despite these and the other differences in the two diets, the amounts eaten of AD + I and AD + Ex as a function of the amount of AD eaten were not significantly different in trial 1 or 2 or the two trials combined.

The importance of odor as a key discriminant factor may help explain why third instar larvae in the consumption experiment would not initially accept AD + I as food—there was no appropriate odor to initiate their tasting a food presented in the unusual form of a chunk of artificial diet. However, after eating AD + *P. newberryi*, which provided the correct olfactory cues, a chunk of artificial diet was perceived as food and so tasted; the gustatory cues were correct, thus the larvae would feed.

The extensive work that has been done on the biology of the six North American species of *Euphydryas* (*E. chalcedona, E. editha, E. anicia, E. colon, E. gillettii,* and *E. phaeton*) (Ehrlich et al., 1975; Cullenward et al., 1979; Bowers, 1980, 1981; Brown and Ehrlich, 1981; Morrison et al., 1983; Williams et al., 1983) suggests that while iridoid glycosides may be the basis of host plant specialization in this group, other factors are certainly involved, such as ecological factors and other plant secondary compounds. For example, in *E. editha* two populations have the same two potential food plants present; however, in the Jasper Ridge population females oviposit on *Plantago erecta*, while the Edgewood Road population uses *Orthocarpus densiflorus* Benth. (Scrophulariaceae) (Singer, 1971). Singer (1971) suggested that this difference was due to differences in food plant quality.

In a more recent paper, Singer (1982) illustrated different specificities of individual females from different populations, suggesting a genetic component to female oviposition preference in *E. editha*. In a *Colias* species, Tabashnik et al. (1981) found intrapopulation variation in oviposition choice among females and suggested that these differences were genetically based. Rausher (1982) reared larvae of *E. editha* from two populations on their own and the other's host plants. He found that larvae from each population survived better and grew faster on their own host plant than larvae from the other population [although both groups of larvae did better overall on one of the plants, *Collinsia* (Scrophulariaceae)], suggesting that larvae from a particular population are genetically adapted to their own normal food plant.

Other secondary chemicals as well as iridoid glycosides may play an

important role in determining patterns of host plant specificity. *Diplacus aurantiacus*, for example, which has a digestibility-reducing phenolic resin covering its leaves (Lincoln, 1980; Lincoln et al., 1982), is used as a host plant by some populations of *E. chalcedona*, while individuals from other populations will die if they try to eat those leaves (N. Johnson, personal communication). Some species in the genus *Pedicularis* (Scrophulariaceae), a genus which is fed on by several *E. editha* populations, contain alkaloids (e.g., Lutfullin et al., 1965; Abdusamatov and Yusanov, 1971) which may require special adaptations in individuals from populations feeding on those plants.

The experiments described above show that one iridoid glycoside, catalpol, acts as a feeding stimulant and attractant to *E. chalcedona*. The ubiquity of these compounds among the *Euphydryas* host plants, coupled with the results from these experiments, suggest a general role for iridoid glycosides as the basic feeding attractant/stimulant for the larvae and, I would suggest, oviposition stimulant for females.

Thus, the evolution of the ability of *Euphydryas* species to utilize plants containing iridoid glycosides, and in fact to use these compounds as larval feeding stimulants (and probably adult female oviposition stimulants) may have enabled them to radiate onto a variety of plant families containing those compounds. The related European genera *Euphydryas, Mellicta,* and *Melitaea* also feed primarily, although not exclusively, on plants containing iridoid glycosides, such as *Plantago* (Plantaginaceae), *Scabiosa* (Dipsacaceae), *Gentiana* (Gentianaceae), *Lonicera* (Caprifoliaceae), and a variety of scrophulariaceous plants such as *Linaria, Veronica, Melampyrum*, and *Antirrhinum* (Jensen et al., 1975; Higgins and Riley, 1980). Thus, although many factors influence patterns of host-plant utilization in *Euphydryas*, iridoid glycosides seem to play a fundamental role in determining host-plant specificity in these species.

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ENVIRONMENTAL STUDIES ON NATURAL HALOGEN COMPOUNDS. I. Estimation of Biomass of the Acorn Worm *Ptychodera flava* Eschscholtz (Hemichordata: Enteropneusta) and Excretion Rate of Metabolites at Kattore Bay, Kohama Island, Okinawa

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Abstract—In order to study the environmental significance of the acorn worm *Ptychodera flava*, which excretes a copious amount of halogenated metabolites, the biomass and the excretion rate were estimated at Kattore Bay, Kohama Island, Okinawa. The habitat, which extends over 1 km^2, could be divided into two areas: zone A, 3.0×10^5 m^2, density 95.6/m^2; and zone B, 7.2×10^5 m^2, density 48.8/m^2, according to the densities. The total population was estimated to be $6.4 \times 10^7 \pm 2.0 \times 10^7$ individuals or 93.0 ± 28.9 tons. These worms daily excrete about 480 tons of fecal sand which was estimated to contain 43 kg of the material extractable with organic solvents. The material contained halogenated metabolites which showed antimicrobial activity.

Key Words—Acorn worm, *Ptychodera flava*, fecal sand, halogenated metabolites, antimicrobial activity, environmental study.

INTRODUCTION

Recent advances in marine natural products chemistry have revealed a wealth of halogen-containing compounds as constituents of various marine organisms (Scheuer, 1973, 1978a,b, 1980, 1981, 1982; Baker and Murphy, 1976, 1981; Faulkner and Fenical, 1977). They are represented by diverse structures: phenols, terpenes, lipids, nitrogenous compounds, and so on. Many of them exhibit antimicrobial or toxic properties (Faulkner, 1978; Glombitza, 1979;

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Hashimoto, 1979). Their ecological and physiological roles, however, are little understood. Some of them structurally resemble some notorious organochlorine compounds, and their metabolic products of environmental concerns. If such natural products are excreted in large quantities, their impact to the environment could not be overlooked. It is our view that studies on such metabolites are extremely important for better understanding and assessment of the environmental problems that have generally been regarded as being inherent in anthropogenic substances. We therefore initiated a study to evaluate the environmental significance of halogenated metabolites produced by marine organisms, particularly by acorn worms.

The acorn worms are benthic animals living usually in sandy flats of inshore waters. Since they feed on adhering organic matter, they ingest and excrete large quantities of sand. The animals are better known by an offensive odor which is said to be reminiscent of iodoform (Hyman, 1959). In our previous studies we have identified the compounds responsible for the odor and many related halogenated phenols and indoles (Figure 1) (Higa and Scheuer, 1975, 1976, 1977; Higa et al., 1980). These compounds are antimicrobial. Some of them are known to have molluscicidal (Steiner, 1971) and plant growth-regulating activities (Harper and Wain, 1969).

The acorn worm *Ptychodera flava* (Nishikawa, 1977) is widely distributed in the tropical Indo-Pacific area and often forms a large and dense habitat on a sandy intertidal flat. The species contains many of the compounds shown in Figure 1. The halogenated metabolites are constantly excreted through the mucus and fecal sand castings (Ashworth and Cormier, 1967) to the environment. The amount of such metabolic excretion on a habitat may be estimated by determining the biomass and the discharging rate of the fecal sand and by analyzing the sand for halogenated constituents. As a first step toward the evaluation of environmental significance of *P. flava* in tropical inshore waters, we now describe our preliminary results of the surveys made at Kattore Bay, Kohama Island in October 1981 and March 1982.

METHODS AND MATERIALS

Survey of the Habitat and Sampling of P. flava. The area of the habitat was surveyed by using a sextant, a three-arm protractor, and a map (1/5000) of Kohama Island, published by National Bureau of Geographic Survey. A boundary between the inhabited and uninhabited region was determined at low tide by observing the characteristic sand castings on emerged areas and by digging for the worms at intervals of 5–10 m in submerged areas. The points of the boundary were taken with a sextant at intervals of 50 m and plotted on the map by using a three-arm protractor. The habitat thus obtained on the map was traced on graph paper to determine the area. The habitat could be divided into two zones according to density (Figure 2).

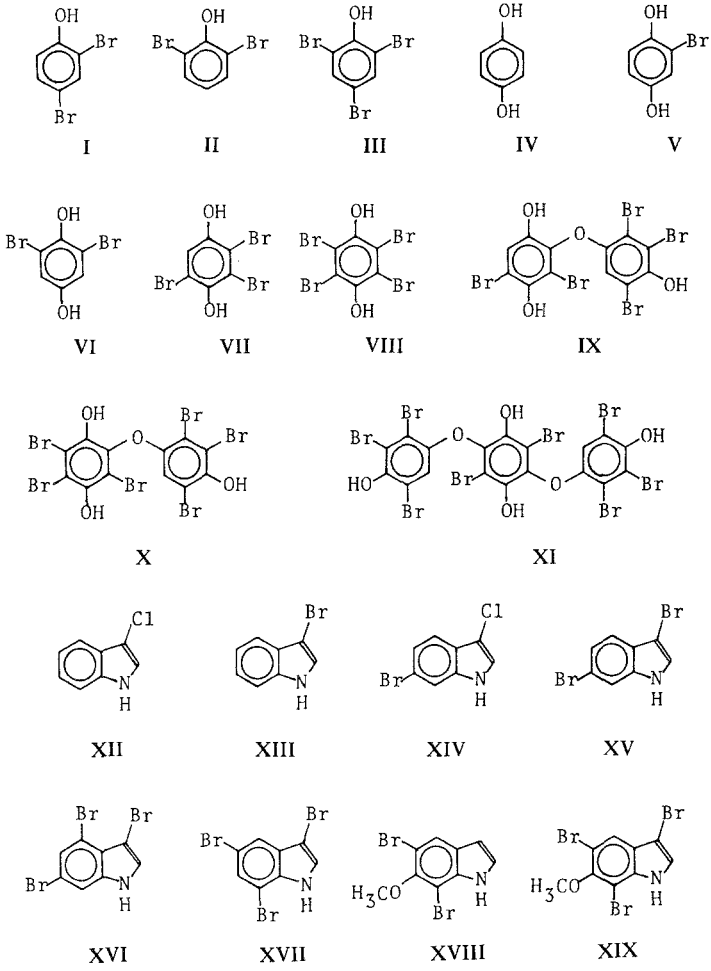

FIG. 1. Halogenated metabolites of the acorn worms, *Ptychodera flava* (III, VII-XV, XVII–XIX), *Glossobalanus* sp. (III–VI, XIII, XV), *Balanoglossus carnosus* (I–III, VII, XVI), *B. misakiensis* (II, VI), and *B. biminiensis* (II). The present population of *P. flava* has been shown to contain the compounds III, VII, XI–XV, XVII, and XIX (Higa et al., 1980).

In order to determine average population densities, the worms were collected and counted by using a 50 × 50-cm quadrate at 12 (3 m^2) and 28 (7 m^2) randomly selected sites in zones A and B, respectively.

Composition Analysis of P. flava. A wet sample of *P. flava* with ingested sand was weighed, freeze-dried to complete dryness, and weighed again. After adding a small amount of ethanol, the sample was burned to determine the weight proportion of the animal tissue and the ingested sand. Addition of

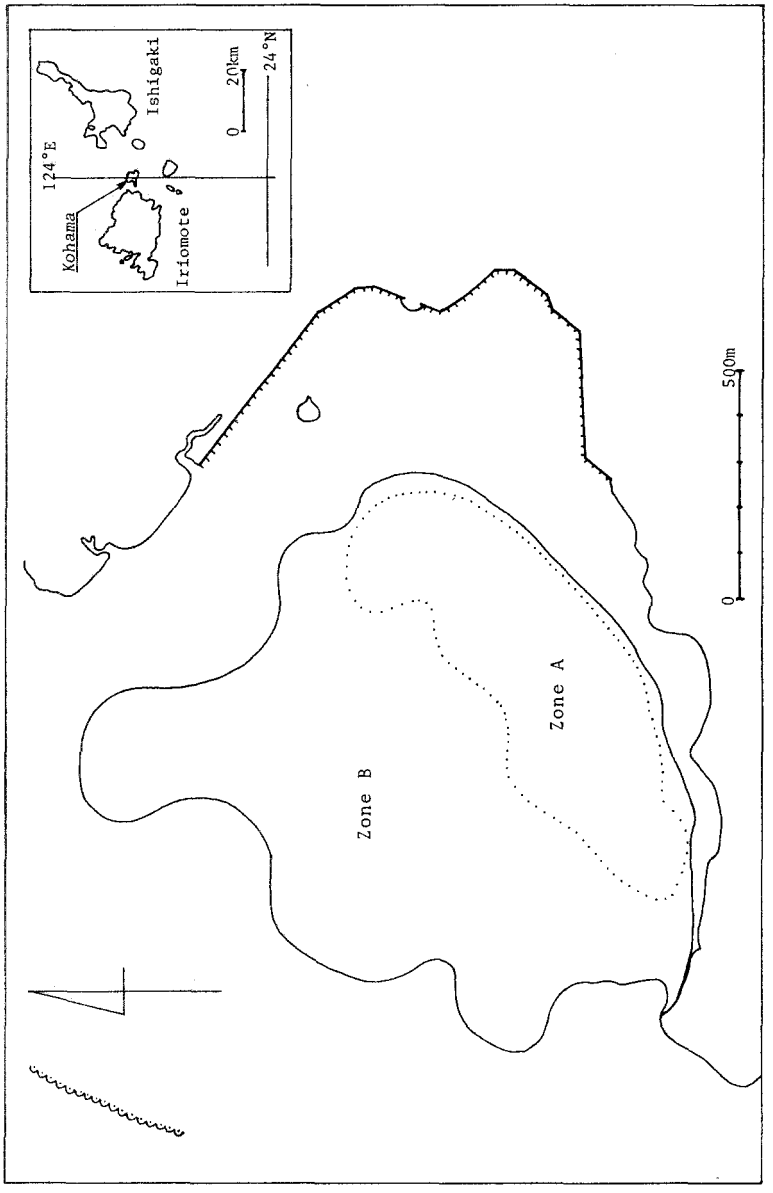

FIG. 2. The habitat of *Ptychodera flava* at Kattore Bay, Kohama Island, Okinawa.

ethanol and burning was repeated until the tissue was thoroughly charred. A portion of the well-blended sample was taken in a porcelain crucible, weighed, heated in a muffle at 550° for 3 hr, cooled in a desiccator, and measured the weight.

Collection and Extraction of Fecal Sand. Since the sand castings easily break off in water, the castings present on the surface at an ebb tide are those accumulated from the time of the emergence of the surface. Thus, the fecal sand newly cast in a given period of time was collected by using a 50 × 50-cm quadrate and a spatula at 40 (10 m^2) each randomly selected sites in zones A and B. The collection was started after 4 hr from the beginning of the emergence in zone A and after 3 hr in zone B. Each collection was done in 1 hr. Thus, the times allowed for the fecal sand accumulation could be taken, on the average, as 4.5 hr for zone A and 3.5 hr for zone B.

A sample of wet fecal sand was thoroughly extracted with acetone. The sand was air-dried and weighed. The acetone extracts were concentrated and successively extracted with hexane, ethyl acetate, and *n*-butanol.

Detection of Halogenated Compounds and Antibacterial Tests. Each of the above extracts was subjected to the Beilstein test for halogen compounds. Antibacterial tests were performed by the standard agar-plate technique using strains of *E. coli*, *S. aureus*, and *B. subtilis*.

RESULTS AND DISCUSSION

Area of Habitat, Density, and Total Population. Figure 2 shows the habitat of *P. flava* at Kattore Bay, Kohama Island. As shown in Table 1 the area of the habitat was approximately 1 km^2. Zone A is the habitat which completely emerges at ebb tide and where more than 50 sand castings per square meter can easily be recognized by surface observation; Zone B is that showing less than 50 castings per square meter and all submerged habitats up to a depth of about 50 cm at the ebb tide on March 9, 1982. The habitat extending beyond that depth was not included in the present surveys.

Numbers of the worms collected in a quadrat (0.25 m^2) varied from 15 to 30 individuals in zone A and from 4 to 23 in zone B. The mean values were 23.9 (SD = 5.0) for zone A and 12.2 (SD = 4.8) for zone B. A total of 629 individuals which weighed 920 g were collected from the 40 sites encompassing the entire habitat. Average wet weight of an individual was therefore determined to be 1.46 g including ingested sand. Thus, the densities per square meter were calculated as shown in Table 1. Using these values, the total population in the entire habitat was estimated to be 6.4×10^7 (SD = 2.0×10^7) individuals or 93.0 (SD = 28.9) tons as shown in Table 1.

Composition of P. flava. Freeze-drying of 920 g of the wet animals with ingested sand gave 440 g (47.8%) of dry material which was composed of sand,

TABLE 1. AREAS OF HABITAT, DENSITIES, POPULATIONS, AND FECAL SAND EXCRETION RATES OF *P. flava* AT KATTORE BAY, KOHAMA ISLAND, OKINAWA

	Zone A	Zone B	Total
Area (m^2)	299,100	719,700	1,018,800
Density			
Number (m^{-2})	95.6	48.8	
(SD)	(20.0)	(19.2)	
Wet wt (g/m^2)	139.6	71.2	
(SD)	(29.2)	(28.0)	
Population			
Number	2.86×10^7	3.52×10^7	6.38×10^7
(SD)	(0.60×10^7)	(1.38×10^7)	(1.98×10^7)
Wet wt (ton)	41.8	51.2	93.0
(SD)	(8.7)	(20.2)	(28.9)
Sand excretion rate			
Dry wt ($g/m^2/hr$)	27.8	16.3	
Dry wt ($g/m^2/d$)	667	391	
Amount of daily excretion			
Dry wt (ton/d)	199	281	480

salt, tissue, and organic extractable material. The weight fractions of inorganic and organic material were determined, by combustion experiment, as 388 g (42.2%) and 52 g (5.6%), respectively.

Excretion Rate of Fecal Sand. The weights of the sand collected from 10 m^2 each in zones A and B were 1700 g (dry wt: 1250 g) and 760 g (dry wt: 570 g), respectively. Using these data, hourly excretion rates were calculated as shown in Table 1. Our observations indicated that some worms are actively discharging sand while some are resting on any sites of the habitat at any given moment and that the excretion rate at a given locale of the habitat appears to be relatively constant throughout 24 hr regardless of the tide. On this assumption, daily excretion rates were calculated and the amount of daily discharge of the sand for the entire habitat was estimated (Table 1). The estimated amount of the daily discharge (480 tons) is five times the wet weight of the whole population of the worms. It also amounts to 12 times the sand contained in their bodies, since 42.2% of the body weight is ingested sand.

Extraction of Fecal Sand and the Antimicrobial Test. Extraction of 11.5 kg (dry wt) of the fecal sand furnished 0.46 g of hexane-, 0.28 g of ethyl acetate-, and 0.29 g of *n*-butanol-soluble material. Thus, the total daily excretion of the material soluble in these solvents amounts to 19.2, 11.7, and 12.1 kg, respectively, or altogether 43 kg in the entire habitat. The hexane-soluble material showed the same characteristic odor of the organism. All the extracts

showed positive Beilstein tests for halogen compounds. The ethyl acetate-soluble fraction was highly active against *E. coli*, *S. aureus*, and *B. subtilis*, while other fractions were inactive. Thus, it is likely that the ethyl acetate extract contains most of the halogenated constituents of the fecal sand, which we have not yet identified. Nevertheless, we estimate that more than 4 tons of the ethyl acetate-soluble materials is annually discharged in the bay and a substantial amount of that is, no doubt, halogen-containing compounds.

Examination of an extract of *P. flava* by thin-layer chromatography indicated the presence of a number of unidentified halogen compounds other than those listed in Figure 1. Judging from the structures and biosynthetic outcome of some of the identified compounds, one of the most likely compounds to be present is a dibenzo-*p*-dioxin derivative. Chlorinated dioxins, e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are a class of extremely toxic substances and are an environmental hazard (Cattabeni et al., 1978). We are continuing work to evaluate the environmental significance of natural halogen-containing compounds, including dioxin derivatives, which may be present in *P. flava*.

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IDENTIFICATION OF TWO COMPONENTS OF THE SEX PHEROMONE OF THE MOTH, *Epiphyas postvittana* (LEPIDOPTERA, TORTRICIDAE)

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Abstract—Two compounds, (*E*)-11-tetradecen-1-yl acetate and (*E,E*)-9,11-tetradecadien-1-yl acetate, have been identified in extracts of females of the lightbrown apple moth, *Epiphyas postvittana* (Walker). The two compounds are active as a coalitive pair and are present in extracts of females in a ratio of about 20:1.

Key Words—*Epiphyas postvittana*, Lepidoptera, Fortricidae, lightbrown apple moth, sex pheromone, (*E*)-11-tetradecen-1-yl acetate, (*E,E*)-9,11-tetradecadien-1-yl acetate.

INTRODUCTION

The lightbrown apple moth, *Epiphyas postvittana* (Walker), is a pest of fruit trees and of grape vines in southeastern Australia. The insect has a wide range of host plants (Danthanarayana, 1975), and it is a common inhabitant of surburban gardens. It has become established as a pest in New Zealand and is also present in southwest England (Baker, 1968) and in Hawaii (Zimmerman, 1978). The presence of a pheromone in the female has been established and behavioral studies have been conducted (Bartell and Lawrence, 1977, and references therein). This paper describes the isolation and identification of two components, (*E*)-11-tetradecen-1-yl acetate (I) and (*E,E*)-9,11--tetradecadien-1-yl acetate (II), which are shown to be responsible for the activity of extracts of females in eliciting sexual behavior in males.

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METHODS AND MATERIALS

The insects were from a laboratory strain [defined as the Bartell strain (Geier and Springett, 1976)] which has been maintained for several years. Virgin female moths were collected after emergence and kept under a 14.5 hr:9.5 hr light–dark regime for three days. The last three or four segments of the abdomen were cut off and accumulated and stored under ether.

The pheromone was extracted by the following procedure. About 6000 tips plus the ether solvent were placed in a blender with sufficient pentane to cover the tips by 2–3 cm, and mashed for about 1 min. The slurry was poured into a flask and ethanol (100 ml) added. After addition of NaOH (30 g) in water (50 ml), the mixture was heated under reflux for several hours. The hydrolysate was then continuously extracted with pentane for 10 hr. The pentane extract (ca. 80 ml) was stirred with acetic anhydride (3 ml) and ether (10 ml) which contained one drop of concentrated H_2SO_4. After 30 hr, the solution was washed with water and then dried over Na_2SO_4. The dry solution, which was active on bioassay, was concentrated to about 5 ml by slow distillation of the solvent through a short fractionating column. This extract was sufficiently free of fatty acids to enable direct isolation of the active components by preparative GLC. Two further batches of tips were treated in the same way. In all about 27,000 female moths were used.

The bioassay was that described by Bartell and Shorey (1968) as later modified by Bartell and Lawrence (1973). The stimulus was presented for 20 sec in the stream of air. The numbers of males which responded by performing the wing fanning behavior were counted and averaged for each treatment.

Gas chromatographic analyses and collections were conducted on a Varian Aerograph model 1200 instrument which had been fitted with an annular splitter (Brownlee and Silverstein, 1968). Samples were collected in glass capillaries cooled with solid CO_2. The helium gas flow was about 20 ml/min. All columns were made of stainless steel (2 m × 2.3 mm) and contained the stationary phase at 5% on Gas-chrom Z, 80/100 mesh, unless otherwise stated. Retention index measurements were made under the same conditions with the splitter usually removed. Calculations of Kovats' indices were carried out by computer (Bellas, 1975).

Separations were first conducted on an OV-1 column using a temperature program of 6° or 8°/min from 80° to 200°. Initially the acetates of saturated alcohols containing 12–18 carbon atoms were coinjected as markers but, as these proved almost coincident with the homologous series of normal hydrocarbons already present from the insect, in later runs the hydrocarbons were used as internal markers.

The method of Beroza and Bierl (1967) was followed for ozonolysis experiments using spectrograde CS_2 as the solvent and the phase OV-101, 10% on Gas-chrom Q at 190° for gas chromatography. The samples for ozonolysis

were collected from a Carbowax 20 M–TPA column at 160°. The retention times of the two compounds were: I, 5.06 min; and II, 9.45 min.

Mass spectra were determined on an AEI MS902 instrument. The sample was placed on the ceramic probe as a solution in hexane and the solvent allowed to evaporate before insertion of the probe into the source of the mass spectrometer.

Electroantennogram (EAG) data were measured following the method of Roelofs and Comeau (1971). Each tube contained 10 μg of the test chemical. Both excised antennae and whole insect mounts were utilized. (*E*)-5-Tetradecenyl acetate was the reference substance. The data from each run of test samples were normalized by dividing each response by the mean of the responses for all of the test samples in that run.

Measurements of the amounts of (*E*)-11-tetradecen-1-yl acetate (I) and of (*E,E*)-9,11-tetradecadien-1-yl acetate (II) were made on an extract prepared by maceration of 100 tips in cyclohexane. After filtration of the supernatant liquid, the volume was adjusted to 100 μl by blowing down with a stream of dry N_2. A standard solution containing 7.86 ng/μl of I and 9.36 ng/μl of II in cyclohexane was prepared. The instruments employed were a Varian Aerograph model 1400 gas chromatograph which was connected through a jet separator to a VG 70-70 mass spectrometer. The glass column (3 m × 2 mm) contained Carbowax 20 M as stationary phase. The carrier gas was helium at 30 ml/min and the oven temperature was 180°. Under these conditons I and II had retention times of 5.65 min and 9.76 min, respectively. The mass spectrometer was operated in the selected ion monitoring mode. The ions selected were m/z 194 ($[M\text{-}60]^+$) for I and m/z 252 (M^+) for II. Three injections of the standard solution and four of the extract were made for each component, and the height of the peak at the appropriate retention time was measured. The amounts in the extract and the ratio of the two components were calculated and the standard deviations estimated according to the method in Bennett and Franklin (1954).

Field tests were conducted in an apple orchard near Canberra. Materials were dispensed from rubber septa in sticky traps. Each treatment was replicated five times and catches were removed at regular intervals over 35 days. Survey traps were prepared containing 100 μg of lure at a ratio of I to II of 10:1. Trapping was conducted in various parts of Australia where *E. postvittana* was known or suspected to occur.

Isomerization of (E,E)-*9,11-Tetradecadien-1-ol.* The mixture of 9,11-tetradecadien-1-yl acetates as received from Dr. Tamaki contained about 80% of 9,11-diene acetates of which some 82% was the *Z,E* isomer and 18% the *E,E* isomer. The oil (0.64 g) was dissolved in ethanol (20 ml) and a solution of NaOH (1 g) in water (10 ml) was added. The flask was heated under reflux for 1 hr. The solution was cooled and poured into water. The mixture was extracted twice with ether-hexane (1:1) and the combined extracts washed

with water until the washings were neutral. The solution was dried over Na_2SO_4 and the solvent removed in vacuo to leave the mixed alcohols as a pale yellow oil.

The mixture of alcohols was dissolved in light petroleum (bp 40–60°, 30 ml) and 16 drops of an iodine solution (0.05 M) added. The solution was placed into a glass tube which was opaque to UV light of wavelength below 308 nm. The tube was irradiated for 4 min by a Philips HPK 125-watt lamp. The solution was cooled, washed with a dilute $Na_2S_2O_3$ solution (25 ml) and water (2×), and dried over Na_2SO_4. The solvent was removed in vacuo. The ratio of *Z,E* to *E,E* isomers was found to be 21:79.

Hexane (4 ml) was added to dissolve the oil and the solution was stored in the freezing compartment of a refrigerator overnight when crystals separated. The supernatant liquid was removed with a glass pipet containing a plug of glass wool. The crystals remaining were recrystallized a further three times following the same procedure, except that the cooling was for shorter periods. The purified (*E,E*)-9,11-tetradecadien-1-ol was a liquid at room temperature.

The alcohol was treated overnight with pyridine–acetic anhydride to afford 197 mg of (*E,E*)-9,11-tetradecadien-1-yl acetate (30% yield: 37% based on the diene content of the starting mixture). The product contained 97% dienes of which less than 0.4% was the *Z,E* isomer.

The isomer ratios were determined by gas chromatography at 170° on a column (2 m × 2.3 mm) containing 10% HIEFF-10BP. The resolution was about 900 plates/m. The retention time for the *E,E* isomer was 17.9 min and for the *Z,E* isomer 16.2 min.

RESULTS

Maceration of abdominal tips in ether, pentane, or cyclohexane afforded crude extracts which were active on bioassay. Hydrolysis of an active tip extract with aqueous ethanol containing NaOH afforded an inactive product. Activity was restored by reaction with acetic anhydride in ether–H_2SO_4. The biological activity of the extract was destroyed by the action of ozone and much reduced after treatment with one drop of a solution of bromine in pentane. These results suggested that the active substance was an unsaturated acetate of the type previously identified from other Tortricidae.

Collections of fractions from the OV-1 column before and after nominated markers showed that the fraction responsible for the activity followed dodecyl acetate (or hexadecane) and preceded hexadecyl acetate (or eicosane). Separation at tetradecyl acetate (or octadecane) afforded two fractions without activity. If these two fractions were combined, the activity returned. The presence of two or more components was indicated. Further fractionation showed that the active components eluted between heptadecane

and octadecane (fraction A) and between octadecane and nonadecane (fraction B).

Fraction A. The collection from OV-101 was injected onto a Carbowax 20 M column at 150°. There was one major component following the tetradecyl acetate which was present as a marker. This component was active on bioassay when tested together with fraction B. The mass spectrum of this component collected from the injection of about 100 female equivalents showed an ion at m/z 194 consistent with the loss of the elements of acetic acid from the acetate of a monounsaturated C_{14} alcohol. The retention index of this active component on EGSS-X at 145° (2354) was the same as that of (*E*)-11-tetradecen-1-yl acetate (I) which proved to be active on bioassay when presented with fraction B. A combination of the (*Z*)-isomer with fraction B yielded no response on bioassay. GLC comparisons showed that the product of ozonolysis of the moth component and that of I were identical. Products corresponding to other positional isomers were not observed. The moth component was coincident with I on Carbowax 20 M and gave similar retention indices on Carbowax 20 M–TPA at 160° (2136 against 2135), ECNSS-M at 160° (2314 against 2312), and 10% PEGA at 170° (2247 against 2246).

The *Z* isomer of I had a retention index 12 greater than the *E* isomer on the Carbowax 20 M–TPA column and 19 greater on the PEGA column. Chromatography of the insect-derived material on the latter column failed to show the presence of any of the *Z* isomer (detection limit about 0.5%).

Fraction B. The identification of the active component of fraction A as an unsaturated acetate left open the chemical nature of the active component of fraction B. That this component was also an acetate was concluded from examination of a batch of tips which had been allowed to stand under methanol in a freezer for many months. A pentane extract showed no activity on bioassay but activity could be restored by acetylation of the mixture. Any acetates originally present in the extract would have been converted into alcohols through transesterification with methanol. The inactive extract was separated by GLC on OV-1 into two fractions, one eluting between tetradecane and octadecane and which would contain any C14 alcohols, and the other eluting between octadecane and nonadecane and which would be expected to contain the active component of fraction B if this had been unaffected by transesterfication. The first fraction proved to be active after acetylation but the second fraction was inactive either alone or in the presence of I.

Gas chromatographic separation of fraction B on Carbowax 20 M followed by bioassay located an active zone with a relative retention time between 1.69 and 2.07 that of I. The two peaks of this zone were very small. Fraction B was fractionated on Carbowax 20 M–TPA at 160°, and EAG of

the fractions showed that there was an EAG active zone centerd at a retention index of 2325 ± 6. Under the same column conditions, I had an index of 2131 and its *Z* isomer 2143. The difference of 180–190 is comparable to that between (*Z*)-8-dodecen-1-ol (2005) and (*E,E*)-8,10-dodecadien-1-ol (2183) on the same column.

GLC of fraction B on 10% PEGA at 170° and collection of a small component of retention time 18.2 min and retention index 2456 afforded a fraction which was active on bioassay when presented with 10 ng of I.

Ozonolysis of the insect-derived material gave the same product (retention time 3.13 min) as did ozonolysis of (*E,E*)-9,11-tetradecadien-1-yl acetate and of (*Z*)-9-tetradecen-1-yl acetate.

(*E,E*)-9,11-Tetradecadien-1-yl acetate had a retention index of 2457 while that of the *Z,E* isomer was 2425. The synthetic *E,E* isomer was active, but the *Z,I* isomer had no significant activity when bioassayed together with I. The other two isomers *Z,Z* and *E,Z*, were unavailable for comparison. Tamaki et al. (1973) have shown that the four isomers are well separated on the PEGA phase with the order of elution being *Z,E*, *E,Z*, *Z,Z*, and *E,E* (Tamaki, personal communication). That some of the *Z,Z* isomer, if present, could have been collected in the bioassay sample cannot be excluded, but there was no evidence for the presence of any isomer other than the *E,E* from the SIM studies where the *Z,E* and *E,E* isomers were well separated on the Carbowax 20 M column employed.

A comparison using bioassay was made between the female tip extract and a mixture of synthetic I and II at the ratio of 25:1 which, in other tests, had been found to be in the midrange of ratios eliciting maximal levels of sexual response (Bellas and Bartell, 1982). The tip extract was diluted to provide a range of samples containing 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} female equivalents per 10 μl. The mixture of synthetic acetates was diluted to provide a series which contained 10^{-19}–10^{-12} g I/10 μl. A GLC determination of the quantity of I in the female tip extract enabled the plotting of both dose–response curves onto the same dose ordinate. Statistical analysis of the results showed that the median response dose (RD_{50}) for the tip extract (4.93×10^{-11} g, upper and lower 1% fiducial limits: 8.07×10^{-11} g, 3.02×10^{-11} g) was not significantly different from that for the synthetic mixture (2.84×10^{-11} g, upper and lower 1% fiducial limits: 5.26×10^{-11} g, 1.52×10^{-11} g). A test for parallelism of the regressions yielded no significant difference between the slopes, and the median response doses derived from the fit of the common slope were RD_{50}(extract) = 4.92×10^{-11} g and RD_{50}(synthetic) = 2.84×10^{-11} g.

Gas chromatography of the 100 female tip extract and measurement of components in the effluent by selected ion monitoring showed that the amounts present per female tip in the extract were: I, 13.3 ± 0.7 ng; II, 0.65 ± 0.04 ng. The ratio of the two components (I to II) was 20.4:1.8.

TABLE 1. FIELD CAPTURES ON STICKY TRAPS BAITED WITH VARIOUS RATIOS OF TWO SYNTHETIC COMPONENTS[a]

Quantities (μg)			
I	II	Ratio	Mean number of males per trap
100	0	I alone	1.0 ef
100	100	1:1	11.0 bcde
100	50	2:1	12.0 abcd
100	20	5:1	7.2 cdef
100	10	10:1	20.8 ab
100	7	14:1	14.8 abcd
100	5	20:1	30.6 ab
100	3	33:1	28.6 ab
100	2	50:1	22.6 ab
50	100	1:2	2.6 def
0	100	II alone	0 f

[a]Data were analyzed by the Duncan multiple-range test on transformed data ($\sqrt{x + 1}$). Captures followed by the same letter are not significantly different at the 5% level.

The results of the field tests are presented in Table 1. Greater numbers of male moths were captured over the range 50:1 to 10:1 while on 5 of the 7 collection days the traps containing the 20:1 lure had the greatest number of captures. In a comparison between virgin females (two per trap) and synthetic lures in field traps, where fresh females were placed in the traps every second day, the female-baited traps caught 15.2 males per trap over 4 traps and the synthetic lures (2 of 20:1 and 2 of 33:1) 18.4 males per trap.

In survey trapping, males of *E. postvittana* were captured over the entire range of the moth in eastern Australia, from southern Queensland to Tasmania, as well as in Western Australia where *E. postvittana* is an introduced species. In South Australia there is a population of moths which is to some extent isolated from the main areas of the range. Although moths identified on morphological grounds as *E. postvittana* were present in this area, no moths were captured in traps baited with the standard lure.

The results of an electroantennogram survey of a series of unsaturated C_{14} acetates are shown in Figure 1. Activity associated with the 11 double bond clearly stands out, but no other double bond position is as active. The synthetic compound II is also included in the results shown in the figure. It is no more active than the 9-monounsaturated acetate, and for this species the electroantennogram results do not give a clear indication of the position of the second double bond. In contrast, both I and II have high activity in single-cell electrophysiological studies (Rumbo, personal communication).

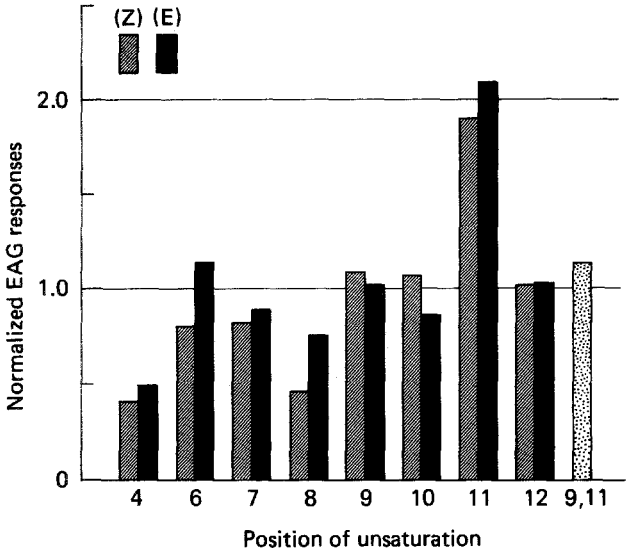

FIG. 1. Normalized electroantennogram responses of male *E. postvittana*, Bartell strain, to a series of unsaturated C_{14} acetates. The data represent the means of three runs on each of three insects.

DISCUSSION

The foregoing results indicate that the compounds present in the female glands are (*E*)-11-tetradecenyl acetate(I) and (*E,E*)-9,11-tetradecadienyl acetate in a ratio of about 20:1. When presented to the males in a ratio of 25:1, the two compounds produce the full range of sexual responses and seem to fully account for the activity of the female extracts under bioassay conditions. This does not necessarily exclude the possbility that other components may be present which mediate orientative and other sexually specific responses in the field.

Neither of the two compounds is active alone in the bioassay, and neither has significant activity by itself in the field. Such coalitive[2] properties are not unusual, having been reported for many species (Tamaki, 1977, and references therin). A wide range of combinations spanning that found in the extracts from females effects substantial field trap captures. Other work (Bellas and Bartell, 1982) has shown that for the Bartell strain there is an optimum ratio of about 24:1.

Two hypotheses have been suggested about the nature of the active

[2]Coalitive is used to describe components which are active only when present together, that is, components that work in coalition (Hewlett, 1969).

compounds of the subfamily Tortricinae. The first is that the chain length of the acetates, alcohols, or aldehydes is 14 and the second is that the pheromone always contains (*Z*)-11-tetradecenyl acetate even if only as a minor component (Descoins and Frerot, 1979). While our findings do not disprove the first hypothesis, the second is not supported. *E. Postvittana* is not unique, since *Platynota idaeulis* was shown to contain none of the *Z* compound (Hill et al., 1974). The recent report of the presence of 12-carbon acetates in *Sparganothis directana* disproves the first hypothesis (Bjostad et al., 1980).

The conjugated diene has not previously been reported as a component of any pheromone. The *Z,E* isomer is present in some of the noctuid genus *Spodoptera* (Tamaki, 1977). Conjugated dienes have been reported from some other families of Lepidoptera, from Bombycidae, Cossidae, Noctuidae, Lasiocampidae, and Pyralidae. Among the Tortricidae, conjugated dienes had been reported from members of the subfamily Olethreutinae, e.g., *Cydia pomonella* (Roelofs et al., 1971), *Lobesia botrana* (Buser et al., 1974), and *Hedya ochroleucana* (Descoins and Frerot, 1979), where each of the dienes had a 12-carbon chain. In the Tortricinae, *Sparganothis directana* contained, among other components, both (*Z*)- and (*E*)-9,11-dodecadien-1-yl acetates (Bjostad et al., 1980) and *Amorbia cuneana* has the (*E,E*)- and (*E,Z*)-10,12-tetradecadien-1-yl acetates (McDonough et al., 1982).

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VOLATILE COMPOUNDS FROM THE RED DEER (*Cervus elaphus*) Secretion from the Tail Gland

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Abstract—The major volatile, organic compounds secreted by the tail gland of the red deer (*Cervus elaphus*) have been identified as phenol, *m*-cresol, cyclohexanecarboxylic acid, benzoic acid, phenylacetic acid, ethylphenol, dimethyl sulfone, *o*-cresol and 3-phenylpropanoic acid. The secretion from ten dead and three live animals was investigated, those from the live ones over a period of time. There were variations in the relative amounts of the identified compounds, but no correlation of the variations with time of sampling was possible.

Key Words—Red deer, *Cervus elaphus*, tail secretion, phenol, *m*-cresol, cyclohexanecarboxylic acid, benzoic acid, phenylacetic acid, 3-phenylpropanoic acid, tail gland, pheromones.

INTRODUCTION

The chemical communication of some of the species of the deer family (*Cervidae*) has been investigated. The North American black-tailed deer (*Odocoileus hemionus columbianus*) and white-tailed deer (*Odocoileus virginianus*) have been examined from both a chemical and biological viewpoint (Brownlee et al., 1969, Müller-Schwarze, 1980), as has the reindeer (*Rangifer tarandus*, Brundin et al., 1978, Andersson et al., 1975, 1979; Müller-Schwarze et al., 1979). The chemical composition of the odoriferous secretion of the musk deer (*Moschus moschiferus*, Ruzicka, 1926a, b) and the moose (*Alces a. americanus*, Bubenik et al., 1980) have also been reported. There are, however, no reports on the chemical communication of the European red deer

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(*Cervus elaphus*), one of the most important members of the deer family in northern Europe.

The Norwegian State Game Research Institute has a program for the study of the red deer ecology which is centered around a research station of 75 km^2 area. On the station there is a 200,000-m^2 enclosure with 5–10 red deer living under seminatural conditions. The program also requires that hunters deliver desired samples of killed animals. This offers a unique opportunity of studying the red deer from all aspects under wild or semiwild circumstances.

As part of a study of the chemical communication of deer (Espmark 1981), we have studied the volatile lipophilic compounds secreted by the red deer. On the underside of the tail is often found a strong-smelling tarlike mixture which is not present on the hindquarters. The tail itself has a liver-tissue-like substance under the skin. This paper reports the results from the investigation of the secretion of the tail region.

METHODS AND MATERIALS

The samples were obtained from ten dead and three live animals. From the dead animals, the skin and hair from the tail were extracted separately with dichloromethane. The liver-tissue-like material from underneath the skin of the tail was carefully separated from the rest of the tail and then extracted with dichloromethane. The animals from Songli Research Station (63° 20′N, 09° 33′E) were anesthetized (Etorphin M-99®) and the ventral side of the tails washed with dichloromethane (Merck) on fine glass wool. The glass wool was then extracted with dichloromethane. The extracts were concentrated by evaporating solvent with dry nitrogen gas and distilling the residue (Büchi GKR-50, 0.5 torr, 20–120° C, CO_2-ice as coolant). The distilled samples were analyzed by GC-MS (Hewlett Packard 5985A, 25 m SP2100 fused silica capillary column, 0.20 mm ID, leading directly into the MS; carrier gas He; inlet pressure 0.5 atm; temperature program 70° C 2 min, 5° C/min to 250° C; ionization chamber temperature 200° C, ionization energy (EI) 70 eV; for CI methane was used as reaction gas, ionization energy 200 eV).

Portions of each sample were methylated (diazomethane) and silylated (chlorotrimethylsilane–1,1,1,3,3,3,-hexamethyldisilazane–pyridine 1:3:9). In addition to the above samples, one consisting of hair from the back of a female animal and one of fat from the hind leg of a male were investigated. The fat sample was distilled without extraction. In addition to these, one blank (pure glass wool) was taken through the work-up and analysis procedure. The compounds were identified by comparing the mass spectra (from EI and CI ionization) and retention times (if necessary by coinjection) of untreated, silylated, and methylated samples with those of authentic compounds. The relative estimates (Table 2) were made by comparison with synthetic mixtures.

RESULTS AND DISCUSSION

The total ion chromatogram of a typical sample after methylation is shown in Figure 1. The identified compounds are marked 1–28, corresponding to the numbers in Table 1. Of these compounds 10, 12, and 14–28 were introduced during the work-up, compounds 8 and 9 were present in the hair sample, and compound 8 in the fat sample. Compounds 1–7, 11, and 13 were found in the tail samples only. These compounds (cyclohexanecarboxylic acid, dimethyl sulfone, benzoic acid, phenol, phenylacetic acid, *o*-cresol, *m*-cresol, 3-phenylpropanoic acid, and ethylphenol) were found both on the surface of the tail and in extracts of the tail gland tissue.

The latter also contained several other unidentified components which were not found on the tail surface. Deer lactone (*cis*-4-hydroxydodec-6-enoic acid lactone, Brownlee et al., 1969), a social pheromone from black-tailed deer was not detected from red deer, either by direct inspection of the mass spectra or by mass fragmentogram. A preliminary investigation of the urine showed it to contain ca. 30 components with retention times of 5–20 min on the GC (under the applied conditions). The compounds in Table 2 were present in small amounts. However, the major hydrophobic components, e.g., dihydrocoumarin, were not found in the tail secretion, only small amounts of the major hydrophilic one (dimethyl sulfone). If the compounds in Table 2 originated from the urine, one would have expected the opposite relationship. Therefore we are confident that compounds 1, 3–7, 11, and 13 originated in the tail.

We assume that compounds of interest in chemical communication will have a relatively high vapor pressure and therefore appear early in the chromatogram. Figure 1 further shows that the compounds eluted at higher temperatures were mainly fatty acids and alkanes introduced during the work-up procedure. The investigation has therefore concentrated on the substances eluted during the first 20 min (below 150° C) in the chromatogram. The relative amounts of the six most quantitatively important substances in that region for different animals and at different times of the year are given in Table 2. A synthetic mixture of these compounds has an odor similar to that of the animal secretion.

All six compounds have been found earlier in mammalian secretions: *m*-cresol in secretion of the bontebok (*Damaliscus dorcas dorcas*, Burger et al., 1977) and African elephant (*Loxodonta africana*, Adams et al., 1978); phenol in swine urine (Sakata et al., 1977) and in secretion from African elephant (Adams et al., 1978); phenylacetic acid in secretions from lion (*Panthera lion*, Albone et al., 1977), swine (Sakata et al., 1977), mongolian gerbil (*Meriones unguiculatus*, Thiessen et al., 1974), fox (*Vulpes vulpes*, Albone, 1977), and sheep (Martin, 1973); benzoic acid in swine urine (Sakata

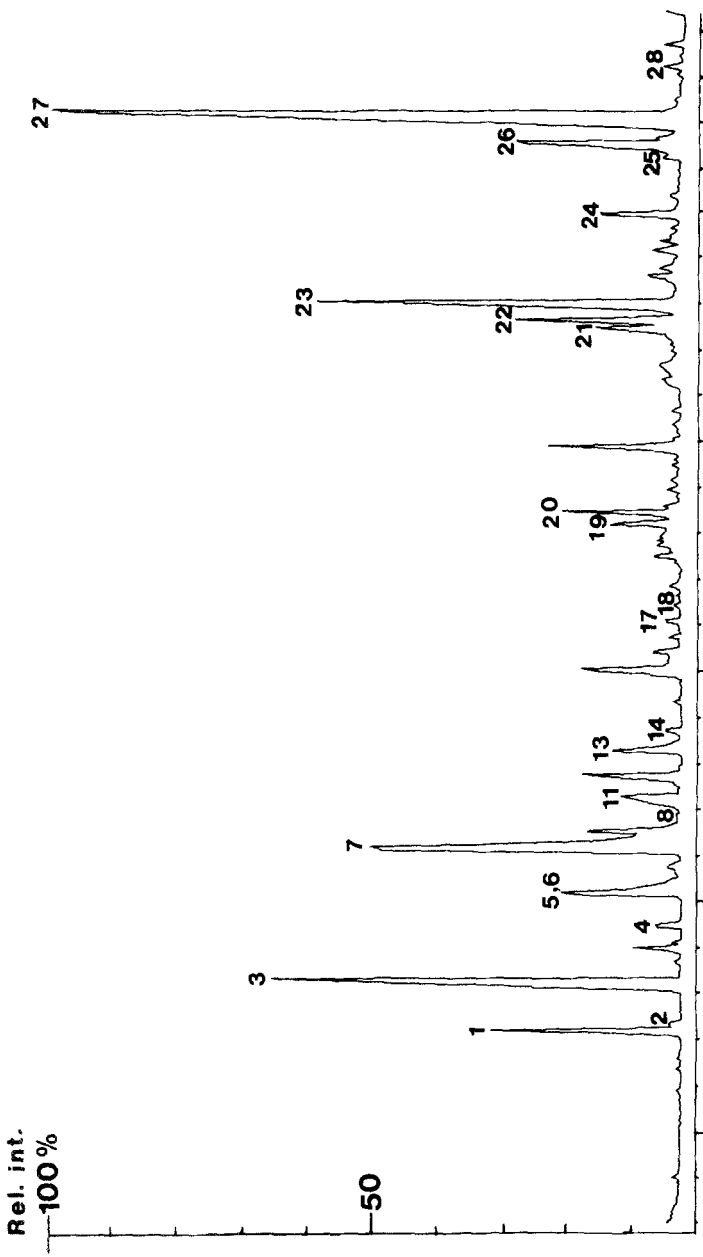

FIG. 1. Total ion chromatogram of the methylated sample (CH_2N_2) of the tail secretion of animal 5 (Table 2). Numbers refer to substance numbers in Table 1. All acids were present as free acids before methylation and as the methyl esters in the chromatogram.

TABLE 1. SUBSTANCES IDENTIFIED FROM RED DEER (*Cervus elaphus*)

No.	Substance	Tail secretion	Substance found in		
			Hair[a]	Fat[a]	Blank
1	Cyclohexanecarboxylic acid	Yes	No	No	No
2	Dimethyl sulfone	Yes	No	No	No
3	Benzoic acid	Yes	No	No	No
4	Phenol	Yes	No	No	No
5	Phenylacetic acid	Yes	No	No	No
6	*o*-Cresol	Yes	No	No	No
7	*m*-Cresol	Yes	No	No	No
8	Benzothiazol	Yes	Yes	Yes	No
9	Dodecane[b]	Yes	Yes	No	No
10	Nonanoic acid[b]	Yes	Yes	No	Yes
11	3-Phenylpropanoic acid	Yes	No	No	No
12	Tridecane[b]	Yes	Yes	No	Yes
13	Ethylphenol[b]	Yes	No	No	No
14	Decanoic acid[b]	Yes	Yes	No	Yes
15	1-Methylnaphthalene	Yes	Yes	No	Yes
16	2-Methylnaphthalene	Yes	Yes	No	Yes
17	Tetradecane[b]	Yes	Yes	Yes	Yes
18	Undecanoic acid[b]	Yes	Yes	No	Yes
19	2,6-Di-*tert*-butyl-4-methylphenol	Yes	Yes	Yes	Yes
20	Dodecanoic acid[b]	Yes	Yes	No	Yes
21	Heptadecane[b]	Yes			Yes
22	Tetradecenoic acid[b]	Yes			Yes
23	Tetradecanoic acid[b]	Yes			Yes
24	Pentadecanoic acid[b]	Yes			Yes
25	Nonadecane[b]	Yes			Yes
26	Hexadecenoic acid[b]	Yes			Yes
27	Hexadecanoic acid[b]	Yes			Yes
28	Hepatadecanoic acid[b]	Yes			Yes

[a]GC-MS runs stopped before emergence of substances 21–28.
[b]Structure determined from MS and comparison of retention times with those of authentic samples. Other isomers were not available for comparison.

et al., 1977), and sheep urine (Martin, 1973); 3-phenylpropionic acid in swine urine (Sakata et al., 1977), fox (Albone et al., 1974), and in the vaginal secretion of monkeys (Keverne, 1976); and finally cyclohexanecarboxylic acid in cattle urine (Seumitsue, 1971). However, the combination of all six compounds has not been reported from one species.

From Table 2 it is clear that there are large variations in the compositions of the secretions, both between animals and during the year for each individual. The different sampling techniques may have caused variations

TABLE 2. RELATIVE AMOUNTS OF SIX MAJOR COMPONENTS SECRETED BY TAIL GLAND OF RED DEER (*Cervus elaphus*)

Animal no.	Status of sample[a]	Sex of animal	Date of sampling	Amount of destilled secretion (mg)	Phenol	*m*-Cresol	Cyclohexanecarboxylic acid	Benzoic acid	Phenylacetic acid	3-Phenyl propanoic acid
1	a	F	1/14/80	42	1	13	8	75	2	1
2	a	F	3/7/80	18	1	10	6	83		
3	a	F	8/26/80	28	3	20	23	49	4	1
4	b	F	9/30/80	19	1	21	13	59	3	3
5	b	F	10/2/81	17	2	50	8	36	1	3
6	b	F	10/5/81	22	1	16	24	55	1	3
7	b	F	10/25/79	51	2	31	17	43	4	3
8	b	F	11/8/80	22	2	15	8	70	3	2
9	b	M	9/30/80	3	6	18	14	28	14	20
10	b	M	10/5/81	3	10	66	1	16	1	6
5	c	F	10/2/81	8	2	50	8	36	1	3
11	d	F	9/18/80	7	3	40	10	35	5	7
11	d	F	10/8/80	12	6	48	32	12	2	
11	d	F	10/22/80	9	6	54	5	23	4	8
12	d	F	8/18/80	13	4	37	12	18	23	6
12	d	F	9/18/80	13	1	32	10	55	1	1
12	d	F	10/22/80	6	5	34	15	24	12	10
13	d	F	9/18/80	16	3	41	21	32	2	1
13	d	F	10/22/80	7	2	57	14	24	3	

[a]Killed calf, hair of tail extracted; (b) killed adult, hair of tail extracted; (c) killed adult, tail gland tissue extracted; (d) live adult, hair of tail washed with CH_2Cl_2.

between the killed and live animal groups but not within each group. The observed variations therefore represent real variations in the animal secretion.

The secretion of benzoic acid and phenylacetic acid from sheep has been reported to depend on the feeding state of the animals, benzoic acid decreasing and phenylacetic acid increasing during fasting (Martin, 1973). However, the three live red deer (animals 11, 12, and 13) all had practically unlimited food supply. The feeding state of the animals is therefore unlikely to be the cause of the variations in the tail secretion.

There is a difference between male and female animals in that the male's secretion is only one tenth as much as the female's. The male secretion also contains three compounds with molecular weight 136, tentatively identified as monoterpenes, which are present only as traces in the female secretions. Thus there are both quantitative and qualitative differences in the secretions between males and females.

However, we have not been able to see any pattern in the variations within each group. There are obviously such variations, both from animal to animal, and for each animal during the year (animals 11, 12, and 13), but is has not been possible to correlate them with, e.g., the rutting season. The significance of these variations may appear when the various substances are tested for biological activity. An introductory experiment indicated that the synthetic mixture of compounds 1, 3, 4, 5, 7, and 11 induced flehmen behavior in male red deer.

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CARDENOLIDE SEQUESTRATION BY THE DOGBANE TIGER MOTH (*Cycnia tenera*; Arctiidae)

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Abstract—*Cycnia tenera* adults, reared as larvae on *Asclepias humistrata*, had 10 times higher cardenolide concentrations, and contained 15 times more total cardenolide, than did moths reared on *A. tuberosa*. Thin-layer chromatography confirmed that each individual cardenolide visualized in the adult moths reared on the former host plant corresponds to one present in the plant, thus demonstrating that the insects' cardenolides are indeed derived from the larval food. Adult weights were significantly greater when the larvae had been fed upon the higher cardenolide plant species, *A. humistrata*. Similar results for other milkweed-feeding insects have been interpreted by some authors as evidence against a metabolic cost of handling cardenolides. However, such interpretations confound cardenolide differences among milkweed species with other differences in plant primary and secondary chemistry that affect insect growth and development. While the cooccurrence in *C. tenera* of other noxious chemicals (e.g., alkaloids) is not precluded, cardenolides sequestered from larval host plants have probably contributed to the evolution of visual and auditory aposematism in this species. As the eggs are laid in large clutches and larvae are gregarious, such aposematism may have evolved via kin selection.

Key Words—*Cycnia tenera*, Arctiidae, Lepidoptera, *Asclepias*, milkweeds, cardenolides, cardiac glycosides, allelochemics, plant–insect interactions, plant secondary chemistry, chemical ecology, chemical defense, kin selection.

INTRODUCTION

Immature stages of monarch butterflies (*Danaus plexippus* L.) (Parsons, 1965; Reichstein et al., 1968; Brower, 1969) and milkweed bugs (Hemiptera:

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Lygaeidae) (e.g., Scudder and Duffey, 1972; Isman et al., 1977a,b) are well-known for their ability to sequester plant defense chemicals known as cardenolides (cardiac glycosides) from their milkweed host plants (Asclepiadaceae). Moreover, laboratory studies have shown that these stored chemicals protect the insects from some vertebrate predators (Brower and Brower, 1964; Brower, 1969; Rothschild and Kellett, 1972). Several other insect species feed at least occasionally on milkweed plants (Wilbur, 1976; Price and Willson, 1979), and some of these have been assayed for the presence or absence of cardenolides (e.g., Duffey and Scudder, 1972; Rothschild and Reichstein, 1976). However, for relatively few species has it been experimentally demonstrated that the cardenolides found in wild and/or lab-reared insects are indeed derived from the host plants (allochthonous) rather than synthesized by the animals de novo (autochthonous), as occurs, for example, in some chrysomelid beetles (Pasteels and Daloze, 1977; Daloze and Pasteels, 1979).

One may distinguish these two possibilities by simultaneous rearings of insects on cardenolide-rich and cardenolide-poor diets (e.g., Brower et al., 1967; Rothschild et al., 1978). Differences in the cardenolide contents of insects reared on such foods may then be attributed to these dietary differences. This approach was taken in the present study of the dogbane tiger moth, *Cycnia tenera* Huebner (Arctiidae), a species previously reported not to sequester host-plant cardenolides (Rothschild et al., 1970). Since a related species (*C. inopinatus*), which is also a specialist feeder on asclepiadaceous and apocynaceous plants (Tietz, 1972; Nishio, 1980), has since been found to store these chemicals (Nishio, 1980), a reevaluation of *C. tenera* seemed desirable. Among other arctiids, two species (*Arctia caja* and *Euchaetias antica*) are known to sequester host-plant cardenolides (see Rothschild and Reichstein, 1976). While three others (*Euchaetias egle, Digama aganais, D. sinuosa*) were reportedly devoid of cardenolides, it remains unclear whether this was due to storage inability or perhaps to feeding on milkweeds low or lacking in these compounds. Sufficient intraspecific and interspecific diversity of milkweed cardenolide content exists (e.g., Roeske et al., 1976; Nelson et al., 1981; Seiber et al., 1982), so that it is possible for an herbivore to be a milkweed specialist without being a cardenolide specialist (see also Rothschild et al., 1970).

Cycnia tenera females lay clutches of 50–100 eggs (personal observations), and larvae feed gregariously on their host plants (we have frequently seen groups of 5–7 larvae on a single *A. humistrata* leaf). If the female has mated only once prior to oviposition, these larvae will be full siblings, whereas if she has mated more than once, they will be at least half-siblings. This high degree of relatedness permits the evolution of unpalatability and aposematism via kin selection (Hamilton, 1964; Harvey et al., 1982; Brower, 1983). Thus, if cardenolides are, in fact, sequestered by the larvae and retained into

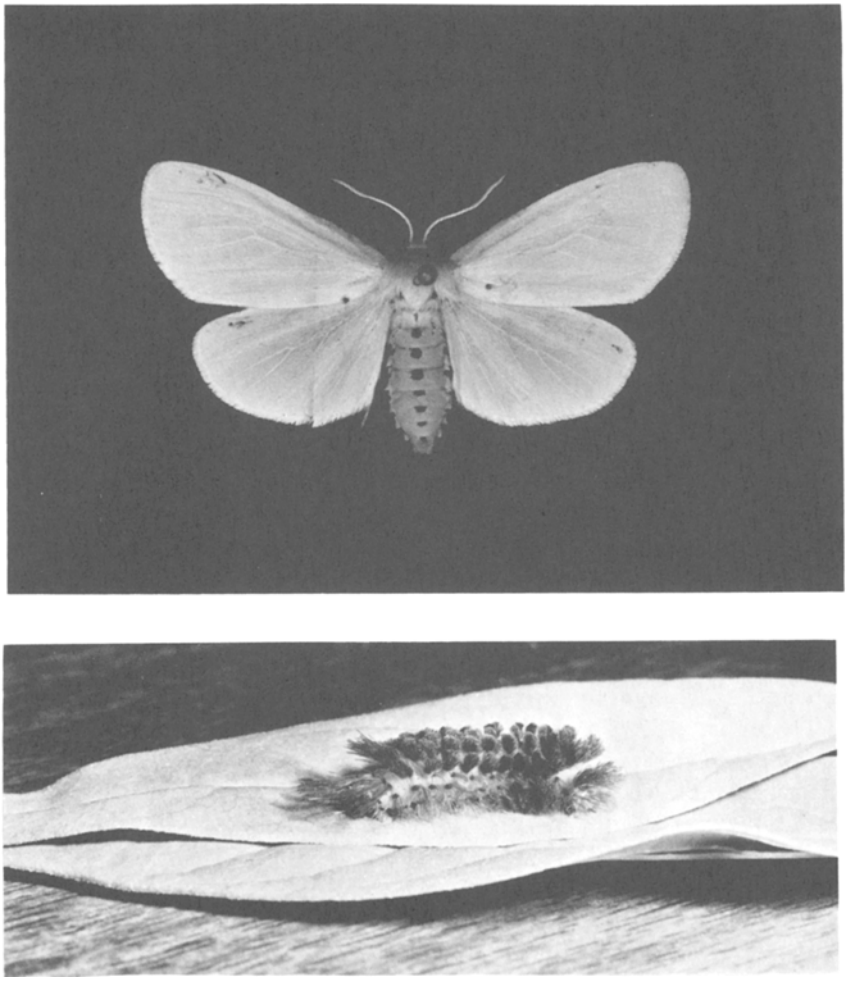

FIG. 1. An adult male *Cycnia tenera* and a late-instar larva. The taxonomy of this genus has been disputed. Forbes (1962) considered *C. tenera* as distinct from *C. inopinatus*, while Kimball (1965) merged both species under the latter name. More recent treatments (e.g., Hodges, 1982) maintain separate status for the two species. Voucher specimens of larvae and adults used in this study have been deposited in the Florida State Collection of Arthropods, Doyle Conner Building, Gainesville, Florida. (Photos by L.P. Brower.)

adulthood (both stages being aposematic; Figure 1), then they may contribute importantly to the chemical defense and evolution of this species.

METHODS AND MATERIALS

In north-central Florida, *Cycnia tenera* larvae are commonly found on *Asclepias humistrata*, a milkweed species having a relatively high cardenolide concentration in its leaves (Nishio, 1980; Cohen and Brower, 1982; cf. Roeske et al., 1976). A clutch of 54 *C. tenera* eggs was collected on May 12, 1981, from an *A. humistrata* plant, growing wild approximately 3/Km west of Gainesville (Alachua County), Florida. The eggs were brought to the laboratory and, upon hatching, each larva was placed in an individual 250-cc closed (vented) plastic container and reared to eclosion at 23 ± 1° C. Of these, 7 were reared through to adulthood on a total diet of *A. humistrata* leaves, while 12 fed only on *A. tuberosa*, a local species known to contain only very slight amounts of cardenolide (Roeske et al., 1976; Cohen, unpublished data). All adult moths were killed by freezing between 12 and 24 hr after eclosion and remained frozen until chemical analysis in March 1982.

Prior to analysis, the moths were dried at 60° C for 16 hr in a forced-draft oven. Dry weights were determined to the nearest 0.1 mg using a Mettler AK 160 electronic balance. Fat was then removed from each insect by petroleum ether extraction of the dried material for 30 min in a 35° C shaker bath (methods in Walford and Brower, in preparation). Fat extraction removes only negligible amounts of milkweed cardenolide from the insect material (C. Nelson and L.P. Brower, unpublished data; see also Nishio, 1980).

Cardenolides were extracted from the fat-free material and concentrations determined (as microgram equivalents of digitoxin) using spectrophotometric procedures described in Brower et al. (1972, 1975). A lead-acetate clean-up procedure (Brower et al., 1982) was then used to remove interfering pigments and other noncardenolide compounds from the extract remaining after spectroassay. Thin-layer chromatography (TLC), employing an ethyl acetate–methanol solvent system (97:3 by volume; see Brower et al., 1982), was used to visualize the cardenolides present in each host plant species and in two randomly selected moths that had developed on each species.

RESULTS

The mean dry and lean weights, cardenolide concentrations, and total cardenolide contents of *C. tenera* adults reared on *A. humistrata* and *A. tuberosa* are shown in Table 1. Two-way analyses of variance demonstrate that females reach heavier dry weights than do males, regardless of foodplant eaten ($F_{1,15} = 32.5, P < 0.001$), but that both sexes are heavier when reared on

TABLE 1. MEANS ($\bar{X}$) AND STANDARD DEVIATIONS (SD) FOR WEIGHTS AND CARDENOLIDE CONTENTS OF ADULT *Cycnia tenera* REARED ON THE MILKWEEDS, *Asclepias humistrata* AND *A. tuberosa*

			Dry weight (mg)		Lean weight (mg)		Weight of extracted fat (mg)		Cardenolide[a] concentration[b]		Total cardenolide[a] per moth	
Host plant	Sex	*N*	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
Asclepias	M	3	30.8	5.1	12.3	2.4	18.5	2.8	759.3	290.4	90.0	29.2
humistrata	F	4	44.4	4.4	20.8	6.4	23.6	4.0	645.4	174.9	135.2	61.8
Asclepias	M	8	17.2	3.8	7.5	2.4	9.7	2.7	98.1	90.5	7.6	7.8
tuberosa	F	4	29.2	5.9	15.8	5.6	13.4	7.0	52.7	37.1	8.2	6.6

[a]Microgram equivalents of digitoxin.
[b]Per 0.1 g lean, dry moth biomass.

A. humistrata than on *A. tuberosa* ($F_{1,15} = 40.9$, $P < 0.001$). The interaction of sex and food plant was not significant ($P > 0.50$). The results for lean weights are similar, with females heavier than males ($F_{1,15} = 16.2$, $P < 0.005$) and both sexes tending to be heavier on *A. humistrata* than on *A. tuberosa* ($F_{1,15} = 5.6$, $0.1 > P > 0.05$). Females contain slightly more fat than do males ($F_{1,15} = 4.6$, $0.1 > P > 0.05$), and both sexes store more fat when fed *A. humistrata* than when fed *A. tuberosa* ($F_{1,15} = 21.2$, $P < 0.001$). Overall, the fat content of the adult moths constitutes 45–60% of the total dry weight.

Clearly, both sexes sequestered higher concentrations and total amounts of cardenolide from *A. humistrata* than from *A. tuberosa* ($F_{1,15} = 76.4$ for concentration; $F_{1,15} = 50.0$ for total amount; $P < 0.0001$ for both tests). However, the sexes did not differ significantly either in concentration ($F_{1,15} = 1.2$, $P > 0.2$) or in total cardenolide content ($F_{1,15} = 2.4$, $P > 0.2$). No significant interaction effects were identified ($P > 0.2$). When both sexes are considered together, moths reared on *A. humistrata* had, on average, 10 times higher concentrations, and contained 15 times more total cardenolide, than moths reared on *A. tuberosa*.

Thin-layer chromatography (Figure 2) clearly confirms that sequestration of host-plant cardenolides occurred, since individuals reared on *A. humistrata* contain virtually every cardenolide visualized in their host plants. However, the chromatogram of *A. tuberosa*, a very low-cardenolide species, consists only of a faint band in the range of approximately $R_f = 0.1$–0.5. Much of this band is pink (i.e., noncardenolide interference) rather than blue (cardenolide), and it is possible that these interfering compounds may have contributed to the absorbances recorded for the moths reared on this species, thereby overestimating cardenolide concentrations. If this is so, then the true difference in cardenolide contents between moths reared on the two host plants may be even greater than reported above.

DISCUSSION

Our data demonstrate the sequestration of host-plant cardenolides by *Cycnia tenera* larvae and the subsequent retention of these chemicals into adulthood. Individuals reared on *Asclepias humistrata,* a cardenolides-rich milkweed species, attained much higher concentrations and total amounts of cardenolide than did those reared on a cardenolide-poor diet of *A. tuberosa*. Moreover, TLC analysis demonstrates a close correspondence between the individual cardenolides of *A. humistrata* and those of the moths reared thereon. If all of the cardenolide present in *C. tenera* were of autochthonous origin, moths reared on both milkweed species would be expected to contain similar amounts and kinds of cardenolide. Clearly, they do not. However, we cannot exclude the possibility that the small amount of cardenolide present in *A. tuberosa*-reared moths may, at least in part, be of autochthonous origin.

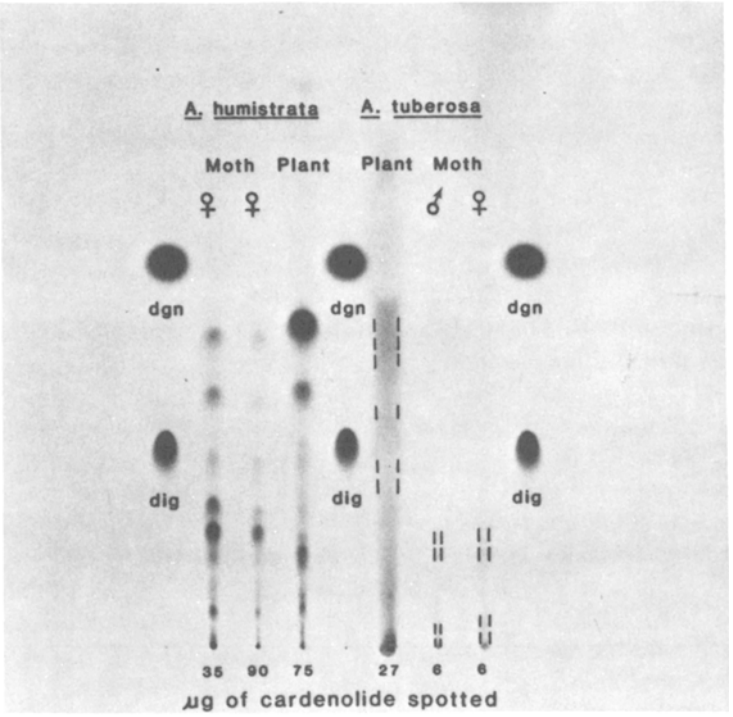

FIG. 2. TLC profiles of four adult *Cycnia tenera* reared on either *Asclepias humistrata* (2 ♀♀) or *A. tuberosa* (1 ♂, 1 ♀). For reference, a mixture of commercially procured digitoxin (dig; $R_f = 0.31$) and digitoxigenin (dgn; $R_f = 0.60$) was spotted in three channels. The amounts of cardenolide (μg equivalent to digitoxin) spotted in each channel are shown at the bottom of the plate. (These are calculated prior to lead-acetate clean-up, a procedure which sometimes reduces the amount actually spotted. Thus, the female in channel 3 has a lighter trace than that in channel 2, presumably having lost disproportionately more cardenolide during clean-up.) For *A. humistrata* there is a very close correspondence between the cardenolides present in the leaf tissue and those in the moths reared thereon. However, *A. tuberosa* has a very low cardenolide concentration and only faint spots are produced from the leaf and moth extracts. In the original color prints, all cardenolides appear as blue spots. However, much of the *A. tuberosa* leaf and moth chromatographs appear pink in the color original, except for faint blue cardenolide regions indicated by broken lines. No such interference occurs in the chromatographs of *A. humistrata*, the moths reared thereon, and the commercial standards.

Both sexes reached higher fat and lean weights when reared on the higher cardenolide species, *A. humistrata*. Similar results for *Danaus plexippus* (Erickson, 1973), *D. chrysippus* (Smith, 1978), and *Oncopeltus fasciatus* (Isman, 1977; Chaplin and Chaplin, 1981) have been interpreted as evidence against a metabolic cost of handling host-plant cardenolides (see also Blum, 1981). However, this inference is problematical because milkweed species differ in many ways other than in cardenolide content (e.g., nitrogen and water content, Erickson, 1973; leaf shape, growth form, texture and pubescence, Woodson, 1954). These differences contribute to the overall suitability and quality of these plants as insect food (Scriber and Slansky, 1981).

Asclepias tuberosa is a relatively poor food source due to low nitrogen and water content (Erickson, 1973) and is infrequently used by herbivores in north-central Florida (Cohen and Brower, unpublished observations) or elsewhere (e.g. Wilbur, 1976; Price and Willson, 1979). It is to be expected that such poor food-plant species will be under weaker selection for antiherbivore adaptations than will species of higher nutritive value and might therefore have relatively lower cardenolide contents. Hence, it is possible that herbivores develop more successfully on relatively high-cardenolide species, such as *A. humistrata*, not because there is no cost of handling these chemicals, but because the higher food quality is great enough to offset any such costs that might exist. Thus, a resolution of the problem of metabolic sequestration costs is not likely to come from studies of the comparative utilization of different milkweed species, but rather from studies of feeding on cardenolide-poor (or cardenolide-free) species to which purified cardenolides are added (Cohen, in preparation).

It is interesting that the extracted fat from *C. tenera* adults may constitute 45–60% of the total dry weight (Table 1). This is sharply higher than the 5–20% common for freshly eclosed danaid butterflies (Beall, 1948) and is similar to the levels found in migrating monarch butterflies just prior to overwintering in Mexico (Walford and Brower, in preparation). Moths of a related arctiid genus, *Euchaetias*, do not feed as adults (Forbes, 1960; Schroeder, 1977), and life-time energy stores must be accumulated by the larvae prior to pupation. However, unlike *Euchaetias*, adult *Cycnia* have well-developed probosces (Forbes, 1960) and presumably feed. Thus their apparently high fat storage remains enigmatic. While this result is not directly relevant to the issue of cardenolide defense, it does raise an intriguing question and it should prove interesting to compare larval fat storage between those arctiid species which have a feeding adult stage and those which do not.

Our results on cardenolide sequestration are in contrast to those of Rothschild et al. (1970), in which no cardenolides were found in *C. tenera* reared on *Asclepias syriaca*. That plant species, however, is extremely variable in cardenolide content (see Roeske et al., 1976), and certain strains are

reportedly devoid of cardenolides (Rothschild et al., 1975). It is thus possible that the particular plants fed to the larvae in the experiments of these authors were of insufficient cardenolide content for larval sequestration to occur. Alternatively, intraspecific geographic differences in sequestration abilities in the moths may be indicated. Such differences have been hypothesized for the various geographic races of the African queen butterfly, *Danaus chrysippus* (Rothschild, et al., 1975), but have not yet been definitively established for any species. A third possibility is that *C. tenera* is not adapted to sequester the particular array of cardenolides found in *A. syriaca*. Indeed, of six milkweed species studied by Price and Willson (1979) in central Illinois, *A. syriaca* was the one of two never utilized by *C. tenera* as a larval food plant. However, there is no evidence that this apparent rejection was determined in any way by the cardenolides of *A. syriaca*.

Cycnia tenera adults are conspicuously colored, with off-white wings and black-spotted, yellow abdomens (Figure 1). The bitter-tasting cardenolides they contain probably impart to them a noxious quality, as is true in the monarch butterfly (Brower, 1969; Brower and Moffitt, 1974). Moreover, they produce both audible and ultrasonic sounds (Fullard, 1977) which may serve to warn bats and other potential predators of the unpalatability of the moths, thus serving an aposematic function (e.g., Dunning and Roeder, 1965).

Larvae, too, are aposematic, with bright orange bodies and contrasting dark tufts of setae along the dorsum (Figure 1). Interestingly, the setae of a closely related species, *C. inopinatus*, are virtually devoid of cardenolides, while the underlying larval cuticle is rich in these compounds (Nishio, 1980). This suggests the potential operation of three separate lines of defense in larval *Cycnia*. The setae may have a sensory function, permitting the larvae to recognize the approach of a predator or parasite. Larvae respond to tactile stimulation by dropping to the ground and curling the body (unpublished observations). This behavior has the effect of exposing the dorsal setae maximally. Should the larva nevertheless be found by a vertebrate predator, a second, mechanical line of defense may come into play: naive predators may attempt to eat a larva, but release it unharmed when the mouth is irritated by the hairs. The setae may then become an aposematic signal, preventing further attacks by experienced predators.

The evolution of such sensory and mechanical defense is not problematic since larvae having such setae would presumably be more likely than those lacking them to survive an attack. If, however, these defenses should fail, the cardenolides present in the larval tissues would provide an unpalatable, and possibly emetic, experience (see, e.g., Brower, 1969), leading to later avoidance of further larvae encountered. Indeed, it is possible that the setae, once ingested, may irritate the gastrointestinal lining (Bisset et al., 1960; Frazer, 1965) and thereby facilitate absorption of cardenolides, i.e., an interaction of mechanical and chemical defenses! The larva would die in the

process, however, and thus its genes for cardenolide sequestration would not be propagated in the population. Kin selection (see Hamilton, 1964) would be required for the evolution of such chemical defense and seems plausible in this species, since eggs are laid in clutches and the larvae feed gregariously on their host plants. Thus, kin groups of at least half-siblings, and possibly full siblings, feed together on the same plant. If a predator were to sample and kill one or a few of these aposematic larvae before learning to avoid them, the remaining siblings would be spared and shared genes both for unpalatability and aposematism would continue to spread within the population.

Our work on cardenolides in *C. tenera* suggests that these compounds may provide at least a partial basis for an underlying unpalatability of both larvae and adults. However, this does not preclude the possibility of other chemical defenses. Parsons and Rothschild (in Rothschild et al., 1970) have noted the presence of histamine-like and/or acetylcholine-like substances in *C. tenera*, although the manner in which these compounds function in nature remains largely unexplored. Moreover, several species of arctiid moths (including *Cycnia mendica*; Rothschild, 1973) are known to sequester pyrrolizidine alkaloids (PAs) from larval hostplants (although these have not yet been identified from Asclepiadaceae) or, for those species with feeding adult stages, from decomposing leaves (Rothschild et al., 1979). Finally, it is possible that other noxious chemicals in these moths may be of autochthonous origin, rather than derived from plant sources (Rothschild et al., 1970, 1979). The relative contributions and possible interactions of cardenolides, biogenic amines, PAs, and/or other noxious substances in the defensive strategies of insects can only be determined through controlled-rearing schemes followed by ecologically relevant predation studies.

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STRUCTURAL SIGNIFICANCE OF THE GEMINAL-DIMETHYL GROUP OF (+)-*trans*-VERBENYL ACETATE, SEX PHEROMONE MIMIC OF THE AMERICAN COCKROACH[1]

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Abstract—The significance of the geminal dimethyl group at C-6 of (+)-*trans*-verbenyl acetate (Ib), a sex pheromone mimic of the American cockroach, was investigated. Our interest was first directed to eliminating either methyl group of the geminal dimethyl group. Consequently, alcohols (IIIa and IVa) possessing a monomethyl group at C-6 were successfully obtained. Acetate (IVb) and propionate (IVc) of alcohol (IVa) possessing only a C-9 methyl group induced sex pheromonal activity at the 0.5 (IVb) and 0.02 mg (IVc) dose levels, respectively, while acetate (IIIb) of a alcohol (IIIa) with only C-8 methyl group was inactive. Alcohol (Va), which has an ethyl group at the C-6 instead of C-9 methyl, was also synthesized and converted into its acetate (Vb), which showed no activity. From these results, the significance of the dimethyl group is discussed relative to spatial requirements of the molecule for the receptor space. The M/F ratio in EAG was also evaluated for the synthesized esters. The M/F ratio index represented well the potency of pheromonal activity.

Key Words—Sex pheromone mimics, American cockroach, Orthoptera, Blattidae, *Periplaneta americana*, [IR-(1α,2α,4α,5α,6β)]-4,6-dimethylbicyclo[3·1·1]heptan-2-yl propionate, EAG, M/F ratio index.

INTRODUCTION

Since we discovered (+)-*trans*-verbenyl acetate (Ib) as a sex pheromone mimic of the American cockroach (*Periplaneta americana* L.) (Nishino et al., 1977a),

[1]Studies on the sex pheromone mimic of the American cockroach, (+)-*trans*-verbenyl acetate. Part X. For Part IX, see Nishino et al., *Agric. Biol. Chem.* 46:2781 (1982).

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our continuing investigations for elucidating important chemical factors of Ib for sex pheromonal activity have revealed several of the factors (Nishino and Takayanagi, 1981a; Takayanagi and Nishino, 1982; Nishino et al., 1982). The role of the geminal dimethyl group at C-6 of Ib, however, remains unsolved so far. Hence, in the present work our effort is directed to elimination and elongation of a methyl of the dimethyl group.

Since it was revealed that (+)-verbanyl acetate (IIb) was more active than the original mimic Ib (Nishino and Takayanagi, 1981a), the basic skeleton of the synthesized alcohols followed (+)-verbanol (IIa). Elimination of each methyl from the dimethyl group of IIa was first carried out, and then, elongation of the significant methyl group (C-9 methyl) was performed.

In our previous electroantennogram (EAG) studies with the cockroach, the significance of male-to-female EAG response ratio (M/F ratio) was proved to be useful for a supplementary indicator to the conventional behavioral activity (Nishino et al., 1977b, 1980; Nishino and Takayanagi, 1981b). The M/F ratio index derived from the ratio was evaluated for the present esters related to the behavioral assay results.

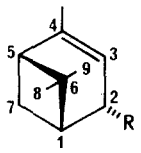
METHODS AND MATERIALS

Instrumentation

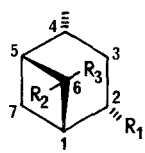
All melting points were uncorrected. Specific rotations were measured in methanol (compound XI) or benzene (other compounds) at 25° C with a Union automatic polarimeter PM-201. IR spectra were recorded on a JASCO IRA-1. [^{1}H]NMR (PMR) spectra were taken in methanol-d_4 (XI) or deuteriochloroform ($CDCl_3$) (others) and tetramethylsilane (TMS, internal standard) on a JEOL FX-270 (270 MHz) (IIIa), JEOL FX-90Q (90 MHz) (IVa and Va) or Hitachi R-24 (60 MHz) (others). Chemical shifts (δ ppm) were measured from the TMS Signal. Gas chromatography (GC) was performed with a Shimadzu 4BM-PF using a 2-m × 3-mm column packed with 3% OV-225 at 110° C, unless otherwise stated, and nitrogen gas (40 ml/min). Mass spectra (MS) were measured with a Shimadzu GCMS-7000 in which the column used was the same as in GC analysis. MS were also taken with a direct inlet system. For column chromatography, silica get 60 (Merck) was used. High-performance liquid chromatography (HPLC) was performed with a Shimadzu LC-3 equipped with a refractometer (Shodex RISE-11). A 10 × 250-mm column packed with Develosil 30-3 (Nomura Chemical Co. Ltd.) was used for HPLC separation.

Synthesis

Structures of the target analogs are illustrated in Figure 1 together with several analogs previously synthesized. Figure 2 shows synthetic routes to the



Ia: R=OH
Ib: R=OCOCH$_3$
Ic: R=OCOCH$_2$CH$_3$



IIa: R_1=OH, R_2=R_3=CH$_3$
IIb: R_1=OCOCH$_3$, R_2=R_3=CH$_3$
IIc: R_1=OCOCH$_2$CH$_3$, R_2=R_3=CH$_3$
IIIa: R_1=OH, R_2=CH$_3$, R_3=H
IIIb: R_1=OCOCH$_3$, R_2=CH$_3$, R_3=H
IVa: R_1=OH, R_2=H, R_3=CH$_3$
IVb: R_1=OCOCH$_3$, R_2=H, R_3=CH$_3$
IVc: R_1=OCOCH$_2$CH$_3$, R_2=H, R_3=CH$_3$
Va: R_1=OH, R_2=CH$_3$, R_3=CH$_2$CH$_3$
Vb: R_1=OCOCH$_3$, R_2=CH$_3$, R_3=CH$_2$CH$_3$

FIG. 1. Structures of known compounds I and II and synthesized compounds III-V.

present alcohols. Purity of all the analogs was proved to be almost 100% by GC and HPLC analyses.

Remote Oxidation of (+)-*Neoverbanol* (*VI*). (+)-Neoverbanol (VI) (Nishino and Takayanagi, 1979a) (1.9 g), lead tetraacetate (25 g), and iodine (3.6 g) was vigorously stirred in refluxing cyclohexane (400 ml) during irradiation with a 500-W tangsten lamp. After 3 hr the pink color of iodine disappeared. The resulting precipitates were filtered off and washed with *n*-hexane repeatedly. The combined filtrates were washed with saturated $NaHCO_3$, a mixture of water and pyridine (1 : 1), and water successively. The organic layer was dried ($MgSO_4$) and concentrated to give VII quantitatively (1.9 g) as a colorless oil which was used for the next step without further purification. Compound VII, $[\alpha]_D$ +29.2° (c = 0.67); IR(film): 1235, 1130 cm^{-1}; PMR: 0.86 [3H, doublet (d), J = 6 Hz, CH_3(10)], 1.29 [3H, singlet(s), CH_3(8)], 3.34 (1H, d, J = 8.5 Hz, CHaHO), 3.88 (1H, d, J = 8.5 Hz, CHH_bO), 4.52 [1H, double triplet (dt), J = 6.5, 2.5 Hz, H(2)]; MS: m/e 152 (M^+, $C_{10}H_{16}O$), 137 [$M^+-15(CH_3)$], 123, 109, 94, 82 [base peak (B^+)]; retention time (t_R, min) in GC: 5.4.

Lactone (*VIII*). To a vigorously stirred suspension of an aqueous solution (100 ml) of sodium metaperiodate (10 g) and a carbon tetrachloride solution (85 ml) of hydrated ruthenium dioxide (135 mg), VII (1.9 g) was added at room temperature. After 2 days, the organic layer was washed with water. Isopropyl alcohol (3 ml) was added to the organic solution, and the mixed solution was filtered. The filtrate was dried and concentrated to give VIII (1.8 g, 90%), mp 45.5–46° C (recrystallized from *n*-hexane); $[\alpha]_D$ +120.7° (c = 0.57);

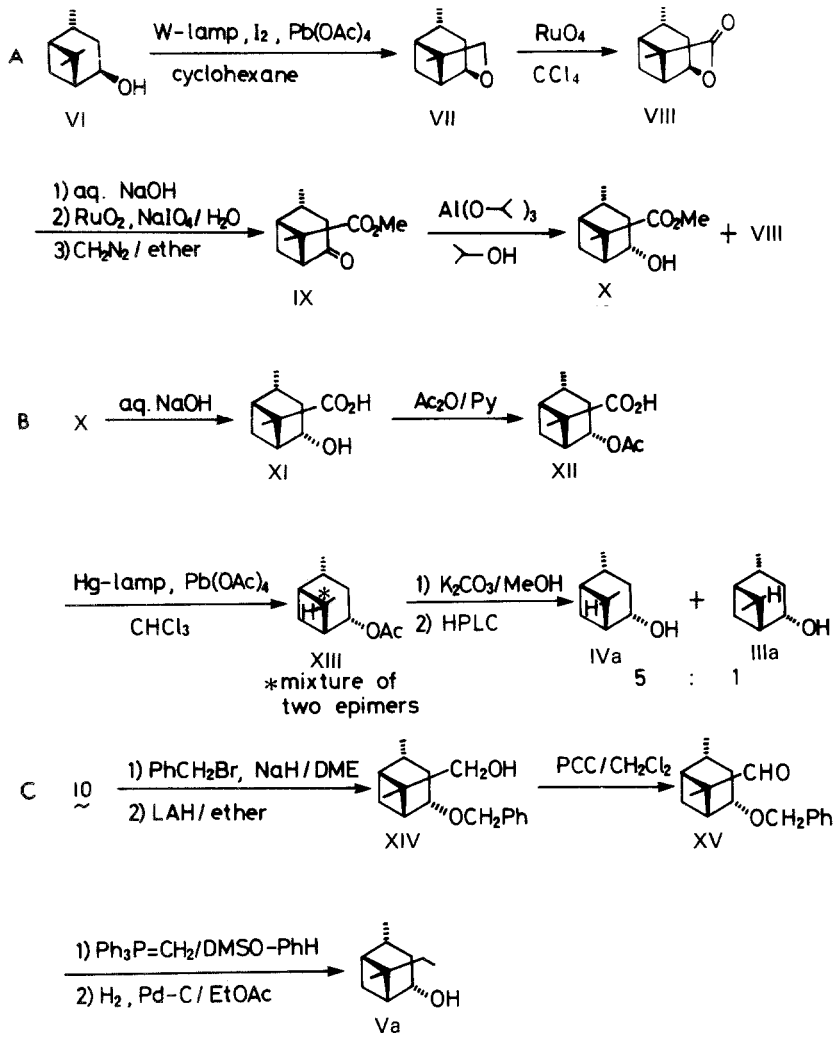


FIG. 2. Synthetic routes to the target alcohols, acids, and their esters.

IR(KBr): 1760 cm^{-1}; PMR: 0.93 [3H, d, J = 6 Hz, CH_3(10)], 1.37 [3H, s, CH_3(8)], 4.94 [1H, dt, J = 7, 2.5 Hz, H(2)]; MS: m/e 166 (M^+, $C_{10}H_{14}O_2$), 151 ($M^+ - 15$), 138 [$M^+ - 28$(CO)], 121, 110, 98 (B^+); t_R: 14.6 at 160°.

Keto Ester (IX). A mixture of VIII (1.3 g) and 1 N NaOH (7.9 ml) was stirred at 80° C for 2 hr. After neutralization of the solution with 0.1 N HCl monitoring with phenolphthalein, hydrated ruthenium dioxide (20 mg) was added. To the stirred suspension, 1.5-ml portions of sodium metaperiodate in water (100 mg/ml) were added until the yellow color of ruthenium tetraoxide

disappeared no longer. After addition of isopropyl alcohol (2 ml), the organic substances were extracted with ether. The ether layer was washed with water and brine, dried, anc concentrated to give a solid. Recrystallization of the solid from ether–acetone afforded keto acid (957 mg, 67%) as a colorless crystal, mp 120–120.5° C; $[\alpha]_D$ +34.2° (c = 1.02); IR(KBr): 3260, 1730-1680 cm^{-1}; PMR: 1.02 [3H, d, J = 6 Hz, CH_3(10)], 1.57 [3H, s, CH_3(8)], 10.10 [1H, broad singlet (bs), COOH]; MS: m/e 182 (M^+, $C_{10}H_{14}O_3$), 164 [$M^+ - 18(H_2O)$]. 154 ($M^+ - 28$), 149, 136 [$M^+ - 46(HCO_2H)$], 113, 109, 95 (B^+). The keto acid (450 mg) was esterified with diazomethane in dichloromethane at 0° C for 20 min. Silica gel (5 g) chromatography of the residue of the dichloromethane solution [n-hexane-ethyl acetate (4 : 1)] yielded IX (450 mg, 93%), $[\alpha]_D$ +61.8° (c = 0.99); IR(film): 1730, 1710, 1285, 1130 cm^{-1}; PMR: 1.03 [3H, d, J = 6 Hz, CH_3(10)], 1.57 [3H, s, CH_3(8)], 3.66 (3H, s, OCH_3); MS: m/e 196 (M^+, $C_{11}H_{16}O_3$), 168 ($M^+ - 28$), 136 [$M^+ - 60(HCO_2CH_3)$], 127, 109, 95 (B^+), t_R: 17.9 at 160° C.

Meerwein-Ponndorf Reduction of IX. By the usual Meerwein-Ponndorf reduction [aluminum foil (125 mg), isopropyl alcohol (2.5 ml) and mercuric chloride (3 mg)], IX (818 mg) gave a mixture of VIII and X. Silica gel chromatography [n-hexane-ethyl acetate (4 : 1)] of the mixture gave VIII (208 mg, 30%). The ratio of the eluting solvent was changed to 2 : 1 to give X (507 mg, 61%), $[\alpha]_D$ +3.5° (c = 1.20); IR(film): 3380, 1720, 1275 and 1130 cm^{-1}; PMR: 0.92 [3H, d, J = 6 Hz, CH_3(10)], 1.49 [3H, s, CH_3(8)], 3.64 (3H, s, OCH_3), 4.44 [1H, bt, J = 7 H_8, H(2)]; MS: m/e 198 (M^+, $C_{11}H_{18}O_3$), 183 ($M^+ - 15$), 180 ($M^+ - 18$), 166 [$M^+ - 32(CH_3OH)$], 149, 138 ($M^+ - 60$), 121, 97 (B^+), t_R: 11.5 at 160° C.

Saponification of X. The ester (X) (300 mg) was saponified with 1 N NaOH (3.0 ml) (refluxed for 15 hr) and acidified to give crude XI. Compound XI was purified by recrystallization from acetone; XI (222 mg, 80%), mp 174–176° C (in shield tube); $[\alpha]_D$ +17.4° (c = 1.00, methanol); IR(KBr): 3380, 2560, 1683 cm^{-1}; PMR (CD_3OD): 0.90 [3H, d, J = 6 Hz, CH_3(10)], 1.47 [3H, s, CH_3(8)], 4.41 [1H, bt, J = 6.5 Hz, H(2)]; MS: m/e 184 (M^+, $C_{10}H_{16}O_3$), 166 ($M^+ - 18$), 151 [$M^+ - 33$ (H_2O+CH_3)], 148, 138 ($M^+ - 46$), 121, 97 (B^+).

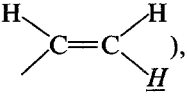
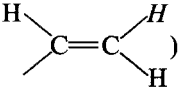
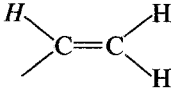
Alcohols IIIa and IVa. Compound XI (184 mg) was acetylated with acetic anhydride (0.4 ml) and pyridine (0.5 ml) (50° C for 2 hr) to give XII (193 mg), mp 122–123° C (from n-hexane); $[\alpha]_D$ +78.2° (c = 1.00); IR (KBr): 2580, 1730, 1690 cm^{-1}; PMR: 0.91 [1H, d, J = 6 Hz, CH_3(10)], 1.50 [3H, s, CH_3(8)], 1.90 (3H, s, $COCH_3$), 5.38 [1H, bt, J = 7 Hz, H(2)], 9.43 (1H, bs, COOH); MS: m/e 226 (M^+), 184 [$M^+ - 42$, ($H_2C{=}C{=}O$)], 166 ($M^+ - 60$), 151, 148, 121 (B^+), 98, 93, Anal. calcd. for $C_{12}H_{18}O_4$: C, 63.70; H, 8.02; 0, 28.28. Found: C, 63.82, H, 7.76; 0, 28.42. A mixture of XII (230 mg), lead tetraacetate (496 mg), isopropyl alcohol (0.15 ml) and chloroform (9 ml) was stirred in a pyrex round-bottom flask, while the reaction mixture was irradiated with a high-pressure Hg lamp (100-W). All of the reaction system was adjusted at 18–20° C

by flowing water. After 4 hr insoluble precipitates were filtered off and washed with *n*-hexane repeatedly. The combined filtrates were washed with 5% aq. $NaHSO_3$ and saturated $NaHCO_3$, and dried. The residue obtained by evaporation of the solvent was chromatographed over silica gel [10 g, *n*-pentane–ethyl acetate (10:1)] to afford a mixture of acetates of IIIa and IVa (XIII). Hydrolysis of XII [potassium carbonate (20 mg) in methanol (2 ml) at room temperature for 2 hr] gave a mixture of IIIa and IVa which was separated by repeating HPLC eluting with *n*-hexane-ethyl acetate (2:1). Compound IIIa (6.0 mg, 4.2% from XII), $[\alpha]_D$ +9.4° (c = 0.50); IR (film): 3285, 1132, 1030 cm^{-1}; PMR (270 MHz): 0.91 [3H, d, J = 6.6 Hz, CH_3(10)], 1.21 [3H, d, J = 6.9 Hz, CH_3(8)], 3.98 [1H, double double doublet (ddd), J = 7.0, 7.2, 1.6 Hz, H(2)]; MS: m/e 140 (M^+, $C_9H_{16}O$), 125 (M^+-15), 122 (M^+-18), 111, 107 (M^+-33), 93, 84, 81, 71 (B^+); t_R: 7.2. Compound IVa (32.3 mg, 23% from XII), mp 51.5–52° C (from petroleum ether); $[\alpha]_D$ +16.0° (c = 1.00); IR (KBr): 3230, 1020 cm^{-1}; PMR (90 MHz): 0.80 [3H, d, J = 6.8 Hz, Ch_3(9)], 0.91 [3H, d, J = 6.6 Hz, CH_3(10)], 4.04 [1H, ddd, J = 7.5, 7.5, 1.6 Hz, H(2)]; MS: m/e 140 (M^+, $C_9H_{16}O$), 125 (M^+-15), 122 (M^+-18), 111, 107 (M^+-33), 93, 84, 81, 71 (B^+); t_R: 8.5.

Compound XIV. To a stirred suspension of excess oil-free sodium hydride in dry 1,2-dimethoxyethane (DME) (1.5 ml), a solution of X (65 mg) in DME (1.5 ml) and benzyl bromide (112 mg) was added at room temperature under argon gas atmosphere and reacted for 3.5 hr. After the mixture was cooled to 0° C, saturated NH_4Cl (5 ml) and ether (15 ml) were added. The organic layer was separated from the aqueous layer which was extracted with ether. The combined ether solutions were washed with brine, dried, and concentrated to give crude benzyl ether which was purified by silica gel (2 g) chromatography [*n*-hexane–ethyl acetate (10:1)]. Benzyl ether (58.4 mg, 61%), $[\alpha]_D$ +23.1° (c = 0.82); IR (film): 3050, 3020, 1725, 1500, 1240, 1130, 735, 695 cm^{-1}; PMR: 0.91 [3H, d, J = 6 Hz, CH_3(10)], 1.48 [3H, s, CH_3(8)], 4.13 [1H, bt, J = 7 Hz,H(2)], 4.48 (2H, s, OCH_2Ph), 7.26 (5H, s, aromatic protons); MS: m/e 288 (M^+, $C_{18}H_{24}O_3$), 257 [$M^+-31(OCH_3)$], 228 (M^+-60), 200, 197 [M^+-91 (CH_2Ph)], 178, 165, 137, 109, 97, 95, 93, 91 (B^+, $^+CH_2Ph$). The benzyl ether (55 mg) was reduced with lithium alminium hydride [excess in ether (3 ml) at −15° C for 2 hr]. Silica gel [1 g, *n*-hexane-ethyl acetate (4:1)] chromatography of the crude material afforded pure XIV (48.3 mg, 97%), $[\alpha]_D$ −50° C (c = 1.00); IR (film); 3380, 3050, 1500, 735, 695 cm^{-1}; PMR: 0.92 [3H, d, J = 6 Hz, CH_3 (10)], 1.31 [3H, s, CH_3(8)], 3.42 (2H, s, CHaHO), 4.44 [2H, s, CHH_bO and H(2)], 7.26 (5H, s, aromatic protons); MS: m/e 260 (M^+, $C_{17}H_{24}O_2$), 245 (M^+-15), 242 (M^+-18), 229 (M^+-31), 191, 169 (M^+-91), 151, 123, 121, 109, 107, 97, 95, 93, 91 (B^+).

Oxidation of XIV. Pyridinium chlorochromate (Corey and Suggs, 1975) (60 mg) was suspended in anhydrous dichloromethane (0.35 ml), and then XIV (48 mg) in dichloromethane (0.25 ml) was added in one portion to the

stirred suspension at room temperature. After 2 hr, dry ether (2 ml) was added, and the resulting supernatant was decanted. The remaining gummy material was washed with ether. The combined ether solutions were concentrated to give a residue which was chromatographed over silica gel [1.5 g, *n*-hexane-ethyl acetate (9:1)]. Compound XV (38 mg, 80%), $[\alpha]_D$ +23.7° (c = 0.98); IR (film): 3060, 2780, 2680, 1715, 735, 695 cm^{-1}; PMR: 0.95 [3H, d, J = 6 Hz, CH_3(10)], 1.33 [3H, s, CH_3(8)], 4.25 [1H, bt, J = 6.5 Hz, H(2)], 4.48 (2H, s, OCH_2Ph), 7.27 (5H, s, aromatic protons), 9.50 (1H, s, CHO); MS: m/e 258 (M^+, $C_{17}H_{22}O_3$), 243 ($M^+ - 15$), 229 [$M^+ - 29$(CHO)], 230 [$M^+ - 30$-(HCHO)], 167 (B^+, $M^+ - 91$), 149, 137, 123, 121, 109, 107 98, 95, 93, 91.

Alcohol Va. Aldehyde XV (35 mg) in dry benzene (0.2 ml) was added to methylene triphenylphosphorane [0.243 mmol, prepared from 0.243 mmol of dimsyl sodium (2 M solution) and methyl triphenylphosphorium bromide (86 mg, 0.243 mmol) in 0.4 ml of dimethyl sulfoxide (DMSO)] in a little DMSO at room temperature and stirred at the temperature for 5 hr. The reaction mixture was poured into a mixture of ether (20 ml) and ice (5 g). The aqueous layer was extracted with ether which was combined with the original ether layer. The concentrate of the ether solution was chromatographed over silica gel (10 g) [benzene-*n*-hexane (2:1)] for affording pure olefin (24.6 mg, 71%), $[\alpha]_D$ +9.5° (c = 0.97); IR (film): 3060, 1810, 1630, 990, 910, 695 cm^{-1}; PMR: 0.90 [3H, d, J = 6 Hz, CH_3(10)], 1.34 [3H, s, CH_3(8)], 4.00 [1H, bt, J = 6.5 Hz, H(2)], 4.49 (2H, s, OCH_2Ph), 4.89 (1H, dd, J = 2, 17 Hz, ), 5.04 (1H, dd, J = 2, 11 Hz, ), 5.98 (1H, dd, J = 11, 17 Hz, ), 7.33 (5H, s, aromatic protons); MS: m/e 256 (M^+, $C_{18}H_{24}O$), 241 ($M^+ - 15$), 165 ($M^+ - 91$), 148, 139, 135, 133, 121, 109, 107, 95, 93, 91 (B^+); t_R: 23.4 at 160° C. The olefin (24 mg) was hydrogenated to give crude Va [10% Pd-C (20 mg) in ethyl acetate (1.5 ml), hydrogen gas at room temperature, 15 hr]. Silica gel (500 mg) chromatography [*n*-pentane-ethyl acetate (5:1)] of crude Va gave pure Va (15.2 mg, 95%), $[\alpha]_D$ +6.5° (c = 1.02): IR (film): 3300 cm^{-1}; PMR (90 MHz): 0.75 (3H, triplet, J = 7.4 Hz, $CH_2C\underline{H}_3$), 0.91 [3H, d, J = 6.6 Hz, CH_3(10)], 1.21 [3H, s, CH_3(8)], 1.24 (2H, m, $C\underline{H}_2CH_3$), 4.11 [1H, ddd, J = 7.9, 6.9, 1.7 Hz, H(2)]; MS: m/e 168 (M^+, $C_{11}H_{20}O$), 153 ($M^+ - 15$), 150 ($M^+ - 18$), 139 [$M^+ - 29$ (CH_2CH_3)], 135 ($M^+ - 33$), 121, 112, 99 (B^+), 97; t_R: 17.6.

Acetates (IIb, IVb, and Vb) and Propionate (IVc). Alcohols (IIIa, IVa, and Va) were converted into their acetates in the usual manner using acetic anhydride and pyridine. Since activity was observed in acetate IVb, the

corresponding propionate (IVc) was prepared with IVa, propionic anhydride, and pyridine. All of the esters were purified by HPLC eluting with *n*-hexane-ethyl acetate (4:1).

1R-(1α,2α,4α,5α,6α)]-4,6-Dimethylbicyclo[3·1·1]heptan-2-yl acetate (IIIb). $[\alpha]_D$ +4.6° (c = 0.50); IR (film): 1730, 1250^{-1}; PMR: 0.89 [3H, d, J = 6 Hz, CH_3(10)], 1.19 [3H, d, J = 6 Hz, CH_3(8)], 1.93 (3H, s, $COCH_3$), 5.00 [1H, bt, J = 7 Hz, H(2)]; MS: m/e 182 (M^+, $C_{11}H_{18}O_2$), 167 ($M^+ -15$), 140 ($M^+ -42$), 122 ($M^+ -60$), 107, 93 (B^+), 81, 79, 71, 43; t_R: 9.4.

1R-(1α, 2α, 4α, 5α, 6β)]-4, 6-Dimethylbicyclo[3 · 1 · 1]heptan-2-yl acetate (IVb). $[\alpha]_D$ + 33.0° (c = 1.00); IR (film): 1730, 1245 cm^{-1}; PMR: 0.82 [3H, d, J = 6 Hz, CH_3(9)], 0.88 [3H, d, J = 6, CH_3(10)], 1.95 (3H, s, $COCH_3$), 5.05 [1H, bt, J = 7 Hz, H(2)]; MS: m/e 182 (M^+, $C_{11}H_{18}O_2$), 167 ($M^+ -15$), 140 ($M^+ -42$), 122 ($M^+ -60$), 107, 93 (B^+), 81, 79, 71, 43; t_R: 10.2.

Propionate IVc. $[\alpha]_D$ +34.8° (c = 1.00); IR (film): 1730, 1190 cm^{-1}; PMR: 0.82 [3H, d, J = 6 Hz, CH_3(9)], 0.88 [3H, d, J = 6 Hz, CH_3(10)], 1.06 (3H, t, J = 8 Hz, $CH_2C\underline{H}_3$), 2.21 (2H, quartet, J = 8 Hz, $C\underline{H}_2CH_3$), 5.03 [1H, bt, J = 7 Hz, H(2)]; MS: 196 (M^+, $C_{12}H_{20}O_2$), 181 ($M^+ -15$), 167 ($M^+ -29$), 140, 112 [$M^+ -74(C_2H_5CO_2H)$], 107, 93 (B^+), 81, 80, 79, 57; t_R: 14.8.

1R-(1α, 2α, 4α, 5α)]-4, 6α-Dimethyl-6β-ethylbicyclo[3 · 1 · 1]heptan-2-yl acetate (Vb). $[\alpha]_D$ +4.3° (c = 1.15); IR (film): 1730, 1250 cm^{-1}; PMR: 0.87 (3H, t, J = 6 Hz, $CH_2C\underline{H}_3$), 0.90 [3H, d, J = 6 Hz, CH_3(10)], 1.20 [3H, s, CH_3(8)], 1.98 (3H, s, $COCH_3$), 5.10 [1H, bt, J = 7 Hz, H(2)]; MS: m/e 181 ($M^+ -29$), 168 ($M^+ -42$), 150 ($M^+ -60$), 139, 135, 121 (B^+), 99, 95, 93; t_R: 19.2.

Conformational Analysis

The analysis for IVa and Va was carried out by measuring coupling constants in the PMR spectra at 90 MHz with the chemical shift reagent, Eu(dpm)$_3$ (Nishino and Takayanagi, 1979b; Takayanagi and Nishino, 1982). A solution of 0.06 mmol of IVa and a little TMS were dissolved in 0.5 ml of $CDCl_3$ in a 0.5-mm measuring tube, and then 0.01-mmol portions of the shift reagent were added successively, until distinct separation of the proton signals was observed. In IVa, the addition of 0.5 mmol (0.8 molar equivalent) of the reagent was sufficient for the separation (Figure 3). In Va, the addition of 0.06 mmol (1.0 eq.) of the reagent was required for the adequate separation of the signals. Consequently, $J_{1,2}$, $J_{3\alpha,2}$, and $J_{3\beta,2}$ could be measured from the separated H-1, H-3α, and H-3β protons, respectively (Table 1).

Because of the insufficient quantity of IIIa, 0.01 mmol of the compound dissolved in 0.5 ml of $CDCl_3$ was subjected to a high-resolution spectrum (270 MHz) without the shift reagent. In the spectrum, coupling constants betwen H-2 and the neighboring protons distinctly appeared, especially when the proton signal was expanded (see Table 1).

The above three coupling constants observed in IIIa, IVa, and Va were

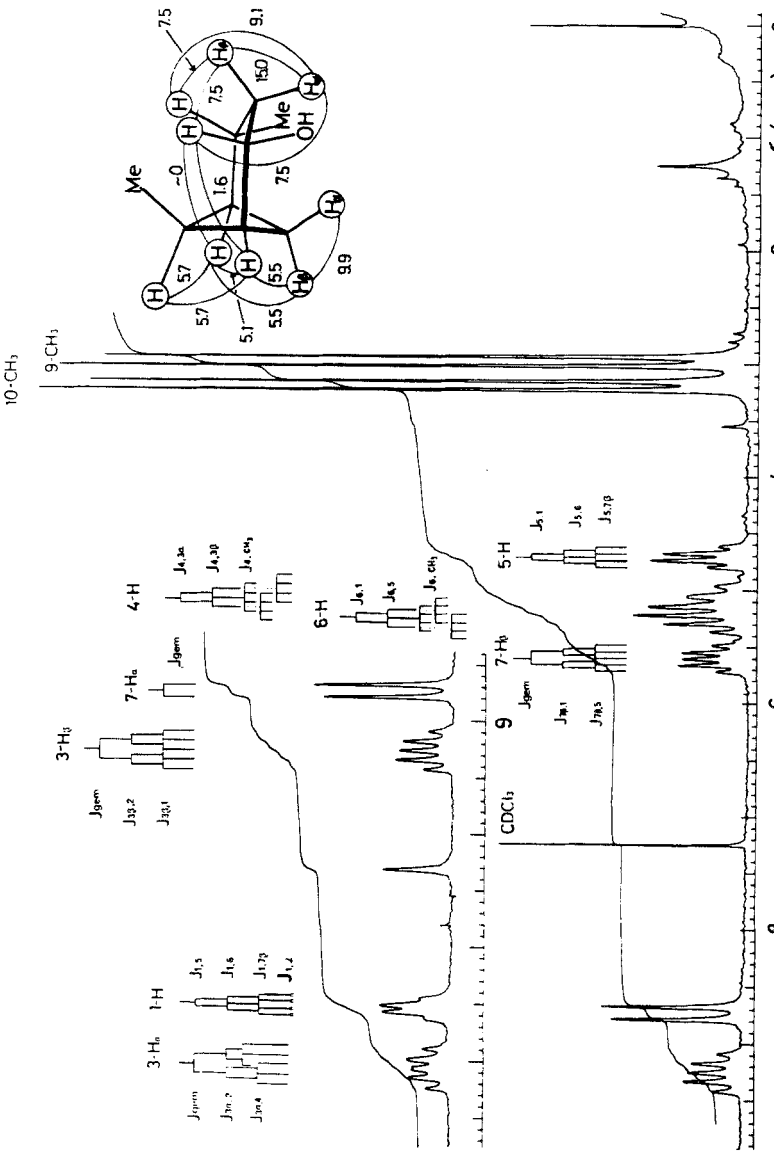
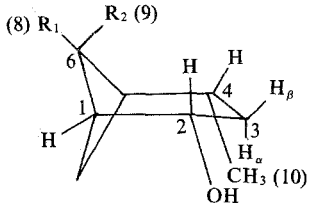


FIG. 3. The PMR of IVa complexed with Eu(dmp)$_3$ [Eu(dpm)$_3$/IVa = 0.8/1, in $CDCl_3$ at 90 MHz].

TABLE 1. CHEMICAL SHIFTS (ppm) OF METHYL GROUPS AND COUPLING CONSTANTS (Hz) BETWEEN PROTONS OBSERVED IN (+)-VERBANOL (IIa) AND THE PRESENT ALCOHOLS



	8-CH_3	9-CH_3	10-CH_3	$J_{1,2}$	$J_{3\alpha,2}$	$J_{3\beta,2}$
IIa: R_1=R_2=CH_3[a]	1.25 (s)	0.80 (s)	0.92 (d, J=6.6)	1.2	6.8	7.7
IIIa: R_1=CH_3, R_2=H[b]	1.21 (d, J=6.9)		0.91 (d, J=6.6)	1.6	7.0	7.2
IVa: R_1=H, R_2=CH_3[c]		0.80 (d, J=6.8)	0.91 (d, J=6.6)	1.6	7.5	7.5
Va: R_1=CH_3, R_2=CH_2CH_3[c]	1.21 (s)		0.91 (d, J=6.6)	1.7	6.9	7.9

[a]The spectrum was taken at 100 MHz, and reported in Nishino and Takayanagi (1979a).
[b]The spectrum was taken at 270 MHz.
[c]The spectra was taken at 90 MHz.

compared with those in IIa (Nishino and Takayanagi, 1979a) as shown in Table 1. Conformational inspection was carried out using the Dreiding model.

Behavioral Assay

The assay method, including sample preparation, followed Nishino et al. (1980) and Nishino and Takayanagi (1981a).

Doses of 1 or 0.5 mg of a synthesized alcohol or ester were tested first. The dose and the numbers of repetitions of the assay were dependent upon the quantity synthesized. When typical sexual display of males was observed at the above dosages, 0.1, 0.05, and 0.02 mg of the compound were assayed successively. The assay was performed in a special controlled-environment room (26° C, 40% relative humidity, and 7-hr day/17-hr night photoperiod) using a testing cage (24 × 30 × 9-cm) equipped with filter paper shelters. In the cage, 25 adult males had been housed for at least 1 month. The assay was initiated at 9:00 PM on Monday and Thursday under dimly lighted condition. The number of males displaying the sexual display was counted within 3 min.

EAG Recording

The recording method was described in a paper by Nishino et al. (1981b) in detail. An excised male or female antenna was fixed on a glass slide with small pieces of adhesive tape. The distal portion of the antenna was inserted into a recording electrode which was made up of a chloridized silver wire in a glass capillary filled with Ringer's solution. The proximal end of the antenna was connected with a stainless electrode functioning as an indifferent electrode. The EAG response was amplified with a microelectrode amplifier (DPZ-11, Dia-medical Ltd.) via probes. The EAG signal was visible on a storage-type oscilloscope (Tektronix 5115) after amplification with a biophysical amplifier (DPA-61M, Dia-medical Ltd.).

A specified quantity of a compound on a filter paper was placed in a 10-ml syringe, and the vapor of the compound was mixed in a continuous airstream (30 ml/sec) by depressing the syringe plunger. The airstream with the vapor was applied to the fixed antenna. Tests were repeated routinely at 20- to 30-sec intervals. One antenna was used for two tests of a series of the quantities (0.01, 0.02, 0.05, and 0.1 mg) of the compounds. Five antennae were consumed for the series, so that 10 tests were performed for a quantity. For the control, the same procedure was repeated without the odor. Camphor was employed as a reference of general odor.

Calculation of M/F Ratio and M/F Ratio Index (M/F I)

The calculation was performed according to Nishino et al. (1980) and Nishino and Takayanagi (1981b). In order to obtain more reliable M/F ratios, the average EAG amplitudes from 10 repetitions for the specified quantities (0.01, 0.02, 0.05, and 0.1 mg) of a compound (see Table 3) were plotted against the logarithmic scale of the quantities for male and female, and then lines were drawn for both sexes. Figure 4 shows the case of camphor as an example. The amplitudes corresponding to the 0.05-mg point (arrows in Figure 4) on the lines were used to calculate the M/F ratio. In order to give M/F I, the M/F ratio value of a compound was divided by that of camphor which is an easily available chemical for producing large EAG responses from both male and female antennae. The same procedure was carried out for all of the tested compounds.

RESULTS

Conformation. In (+)-verbanol (IIa), the C-8, C-9, and C-10 methyl protons resonated at 1.25, 0.80, and 0.92 ppm, respectively (Nishino and Takayanagi, 1979a). Although the C-6 of the alcohols IIIa, IVa, and Va was

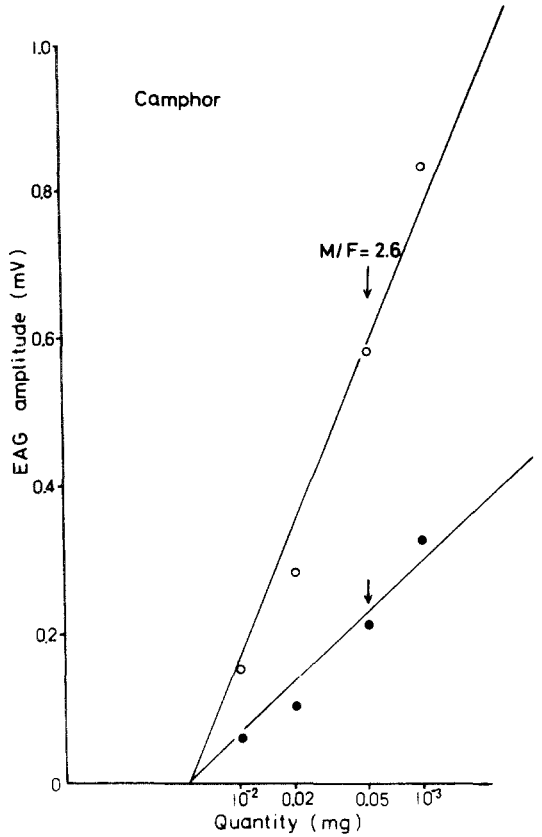


FIG. 4. Lines obtained from average EAG amplitudes of 10 tests at 0.01, 0.02, 0.05, and 0.10 mg of camphor for males (○) and female (●) of the American cockroach. For calculation of the M/F ratio, amplitudes at 0.05 mg (shown with arrows) on the male and female lines were used. Ordinate, EAG amplitude (mV). Abscissa, quantity (mg) of camphor on a logarithmic scale. The same procedure was carried out for the other compounds.

substituted by different groups than in the case of IIa, the monomethyl group at the C-6 of the alcohols displayed very similar chemical shifts to the corresponding methyl group of IIa. In IIIa and Va, the C-8 methyl protons were observed at 1.21 ppm showing reasonable splitting patterns, whereas in IVa there was a doublet signal due to the C-9 methyl protons at 0.80 ppm (Table 1).

The essential coupling constants for determining molecular conformation, which are $J_{1,2}$, $J_{3\alpha,2}$, and $J_{3\beta 2}$, were determined to be 1.2, 6.8, and 7.7 Hz in IIa, respectively (Nishino and Takayanagi, 1979b). On the basis of the constants,

IIa was assigned a bridged chair conformation in which the C-2–C-3–C-4 plane was bent down from the C-1–C-2–C-4–C-5 plane by ca. 15° as seen in Table 1. In the present work, the constants were measurable in the spectra with the shift reagent (IVa and Va) and in the high resolution spectrum (IIIa) without the reagent. The coupling constant values were quite similar to those of IIa (Table 1). Thus, the conformations of the present alcohols were considered to be bridged chair forms resembling that of IIa.

Behavioral Assay Results. The assay data are listed in Table 2. The synthesized alcohols demonstrated no pheromonal activity at the 1- or 0.5-mg doses. Among the acetates, IIIb (C-8 monomethyl) and Vb (ethyl at C-6) were also inactive at 0.5 mg, while IVb (C-9 monomethyl) showed weak activity at the same dosage level. On the other hand, the propionate (IVc) caused a potent activity at 0.02 mg.

Sexual display of the cockroach induced by 0.02 mg of IVc was quite similar to that of the previously found mimics with the 0.02-mg threshold level (Ib, Ic, IIb, IIc, etc.) (Nishino and Takayanagi, 1981a; Takayanagi and Nishino, 1982; Nishino et al., 1982), namely, short induction period (30–40 sec) for the response, long after effect (3 min) after elimination of the sample, and attractancy to the sample.

EAG Results. The compiled EAG data are listed in Table 3 together with M/F ratio and M/F ratio index (M/F I) values. Male antennae produced larger EAG responses than female antennae at any quantity with all of the esters. All the esters elicited similar EAG amplitudes (0.10–0.21 mV) at the

TABLE 2. SEX PHEROMONAL ACTIVITY AND M/F RATIO INDEX OF ESTERS

Compound	Quantity (mg)	Number of repetitions	Activity[a]	M/F I
Ib	0.02	20	9 ± 3[b]	2.1[c]
Ic	0.02	20	15 ± 4[b]	3.1[c]
IIb	0.02	20	15 ± 6[b]	3.1[c]
IIc	0.005	10	16 ± 2[d]	4.6[d]
IIIb	0.5	3	[e]	1.0
IVb	0.5	5	8 ± 2	1.6
IVc	0.02	10	13 ± 2	2.7
Vb	0.5	3	[e]	0.9

[a]Average number of cockroaches showing typical sexual display within 3 min in a group containing 25 males ± SD.
[b]Data from Nishino and Takayanagi (1981a).
[c]Data from Nishino and Takayanagi (1981b).
[d]Data from Nishino et al. (1982).
[e]No activity.

TABLE 3. EAG AMPLITUDES OF ADULT MALE AND FEMALE AMERICAN COCKROACH AT VARIOUS QUANTITIES OF COMPOUNDS, M/F RATIOS, AND M/F RATIO INDICES

Compound	Quantity (mg)	EAG amplitude[a]		M/F[b]	M/F I[c]
		Male	Female		
IIIb	0.01	0.10 ± 0.03	0.04 ± 0.03	2.7	1.0
	0.02	0.14 ± 0.05	0.05 ± 0.05		
	0.05	0.26 ± 0.06	0.09 ± 0.05		
	0.10	0.37 ± 0.08	0.12 ± 0.03		
IVb[d]	0.01	0.17 ± 0.04	0.03 ± 0.02	4.2	1.6
	0.02	0.28 ± 0.05	0.08 ± 0.04		
	0.05	0.48 ± 0.06	0.14 ± 0.06		
	0.10	0.94 ± 0.18	0.20 ± 0.05		
IVc[d]	0.01	0.21 ± 0.04	0.03 ± 0.02	6.9	2.7
	0.02	0.32 ± 0.04	0.04 ± 0.03		
	0.05	0.56 ± 0.09	0.08 ± 0.04		
	0.10	1.08 ± 0.14	0.15 ± 0.05		
Vb	0.01	0.10 ± 0.02	0.04 ± 0.03	2.4	0.9
	0.02	0.16 ± 0.04	0.03 ± 0.03		
	0.05	0.22 ± 0.06	0.11 ± 0.04		
	0.10	0.33 ± 0.05	0.15 ± 0.05		
Camphor	0.01	0.15 ± 0.04	0.06 ± 0.03	2.6	1
	0.02	0.28 ± 0.05	0.10 ± 0.04		
	0.05	0.58 ± 0.15	0.21 ± 0.04		
	0.10	0.83 ± 0.18	0.32 ± 0.07		

[a]Average response ± SD of 10 tests (in mV).
[b]The ratio was calculated using male and female EAG amplitudes at 50 μg on the induced lines (see Figure 4).
[c]The index was obtained by dividing M/F ratio with the ratio of camphor.
[d]Sexually active compounds (see Table 2).

0.1-mg dosage. However, at the 0.1-mg dosage, a large difference of the amplitude was observed between active (ca. 1.0 mV in IVa and IVc) and inactive esters (ca. 0.35 mV in IIIb and Vb). For the female EAG response, although the amplitudes increased according to the increase of dose quantity, all the amplitudes were recorded in the 0.03- to 0.02-mV range, implying a similar degree of general odorous property of the esters. Camphor, a typical general odor, strongly stimulated the male antennae as did IVb. This compound also elicited good responses from the female antenna.

The M/F I was intended to compensate for the variation of the M/F ratio values in different experiments by comparison with the ratio value of camphor as the standard (Nishino and Kimura, 1981). For example, M/F of IIc was reported to be 6.4 in the previous experiment (Nishino et al., 1982) in which

the ratio of camphor was 1.4, while the ratio of IVc was 6.9 in the present work and that of camphor was 2.6. Therefore, M/F I values were 4.6 (6.4/1.4) and 2.7 (6.9/2.6) for IIc and IVc, respectively. These values reflected well the pheromonal activity of the compounds.

As shown in Table 2, the order of the M/F I was as follows: IIc > Ic = IIb > IVc > Ib > IVb > IIIb = camphor ≃ Vb. This order represented a good correlation with the behavioral assay results (Table 2).

DISCUSSION

In our structure-activity investigations on the sex pheromone mimic, (+)-*trans*-verbenyl acetate (Ib), structurally important factors were elucidated as follows: (1) the presence of an α-oriented ester group at the C-2 (the carbonyl oxygen atom may act as an electron donor to an active site of the receptor) (Nishino and Takayanagi, 1981a), (2) the predominance of the bridged-chair conformation of the molecule to the Y-shape conformation (Takayanagi and Nishino, 1982), (3) the necessity of an alkyl (methyl or ethyl) group at the C-4 (in the bridged-chair conformation this alkyl group should be with α configuration) (Takayanagi and Nishino, 1982), and (4) the importance of the size of the alkyl group of the ester moiety [the predominance of ethyl group (propionate) to methyl group (acetate)] (Nishino et al., 1982).

In the present study, it is clear from the behavioral activity of IVb (active) and IIIb (inactive) that the C-9 methyl group is essential for sex pheromonal activity. The methyl group may be a structural requirement in spatial orientation of the molecule for fitting into the receptor, as seen in the case of the C-4 alkyl group (Takayanagi and Nishino, 1982). The C-9 methyl itself seemed to be the optimal size, since Vb with an ethyl instead of the methyl group was inactive. This may be due to be excessive bulkiness of the ethyl for the receptor space. On the other hand, the C-8 methyl group was assumed to be an unnecessary factor.

From the large difference of phermonal activity between the acetate IVb and the propionate IVc, it was expected that in IVc the precise fit of the propionate moiety to the corresponding active site of the receptor would provide perfect fits of the other important structural factors into the corresponding active sites.

It has been established that the monoterpenoid sex pheromone mimics possess both the sex pheromonal and general odor properties because of their pheromonal activity and significant EAG responses from both male and female antennae (Washio et al., 1976; Nishino and Takayanagi, 1981b). Therefore, male EAG responses stimulated by the mimics contain the responses from both general odor and sex pheromone receptors. In the M/F ratio, the general odorous property of a compound might be offset, so that net

pheromonal property of the compound appears in the ratio (Nishino et al., 1977b; 1980; Nishino and Takayanagi, 1981b). It is a pitfall to use only male EAG responses in expressing sex pheromonal activity of compounds. For example, although camphor has no pheromonal activity, this compound elicites strong EAG responses from male antennae. Moreover, a large amount of a general odor yields a larger response from male antennae than a small amount of sex pheromone. Hence, when the M/F ratio was used, the quantitative difference between compounds could be also offset.

Since the M/F ratio value varies in each experiment, the M/F I should be compared among the results from different EAG experiments (Nishino and Kimura, 1981). In this work, the order of M/F I showed the order of activity in the behavioral assay (Table 2). Thus, the ratio and the derived index were proved to be useful indicators for estimating sex pheromonal activity.

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IMPROVED PREPARATIONS OF ALKYNE NITRILES, ACETATES, AND ALCOHOLS
Application to the Synthesis of the Sex Pheromone Components of the Douglas Fir Tussock Moth and Peach Fruit Moth

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Abstract—One-pot conversions of terminal alkynes to cyanoethylated and cyanopropylated alkynes are described. Reactions of these nitriles with Grignard reagents produced alkynones, the reductions of which gave alkenones. The described routes provide 3-step syntheses of several insect sex or stimulatory pheromones, namely, those of the peach fruit moth (*Carposina niponensis* Walsingham), Douglas fir tussock moth (*Orgyia pseudotsugata* [McDunnough]), and housefly (*Musca domestica* L.). Additionally, potentially useful one-pot processes for the preparation of hydroxyalkylated and acetoxyalkylated alkynes are reported.

Key Words—Synthesis, nitriles, acetates, alcohols, esters, pheromone, attractant, sex pheromone, Douglas fir tussock moth, *Orgyia pseudotsugata*, peach fruit moth, *Carposina niponensis*.

INTRODUCTION

The components of the sex pheromone of the peach fruit moth (*Carposina niponensis* Walsingham) were established as a pair of γ,δ-unsaturated ketones, namely (*Z*)-7-nonadecen-11-one, I (Table 1), and (*Z*)-7-eicosen-11-one, II (Tamaki et al., 1977). Several syntheses of this pair of compounds have been reported (Tamaki et al., 1977; Tamada et al., 1978; Liu et al., 1981; Naoshima et al., 1981; Vig et al., 1981). Related semiochemicals are (*Z*)-6-heneicosen-11-one, III, the sex pheromone of the Douglas fir tussock moth [*Orgyia pseudotsugata* (McDunnough)] (Smith et al., 1975a), and (*Z*)-9-

TABLE 1. UNSATURATED KETONES IDENTIFIED AS INSECT PHEROMONES

$$CH_3(CH_2)_xCH=CH(CH_2)_yC(=O)(CH_2)_zCH_3$$

	x	y	z	
I (Z)	5	2	7	Peach fruit moth
II (Z)	5	2	8	Peach fruit moth
III (Z)	4	3	9	Douglas fir tussock moth
IV (Z)	7	3	8	Housefly

tricosen-14-one, IV, a stimulant obtained from female housefly (*Musca domestica* L.) (Uebel et al., 1978). Both of these compounds are δ,ε-unsaturated ketones and have likewise received the attention of synthetic chemists: III (Smith et al., 1975b; Kocienski and Cernigliaro, 1976; Mori et al., 1977; Henrick, 1977; Fetizon and Lazare, 1978; Kondo and Murahashi, 1979; Vig et al., 1980; Cahiez et al., 1980) and IV (Uebel et al., 1978).

The alkylation of 1-heptyne with 1-chloro-3-bromopropane has been reported (Henrick, 1977), although without experimental details. The chloropropylated derivative was isolated, converted to a magnesio derivative, and condensed with undecanal to produce an acetylenic alcohol. Reduction of the alkyne unit (to *Z*-alkene) and oxidation of carbinol to ketone completed one of the most effective syntheses of the Douglas fir tussock moth pheromone reported to date. In somewhat similar fashion, the components of the peach fruit moth pheromone were prepared by sequential hydroxyethylation of 1-octyne with discrete subsequent steps to the hydroxyl group with cyanide (Tamada et al., 1978). Condensations of the nitrile with appropriate Grignard reagents led to acetylenic ketones that were then reduced to the required (*Z*)-alkenones.

Potentially the most useful synthetic route that would be applicable to all of these compounds is that of acetylene alkylation to produce terminally functionalized alkyl acetylenes capable of in situ transformation into nitriles as intermediates (Figure 1). Grignard reactions of the nitriles to yield ketones followed by acetylene reduction, as mentioned above, would complete the syntheses. We report here the details of these contracted sequences (three steps) and their application to all four structures, as well as some additional information regarding displacements in haloalkylated alkynes. Included are chromatographic data that illustrate the superiority of liquid crystal stationary phases for gas–liquid chromatography of these relatively nonpolar compounds (Heath et al., 1979).

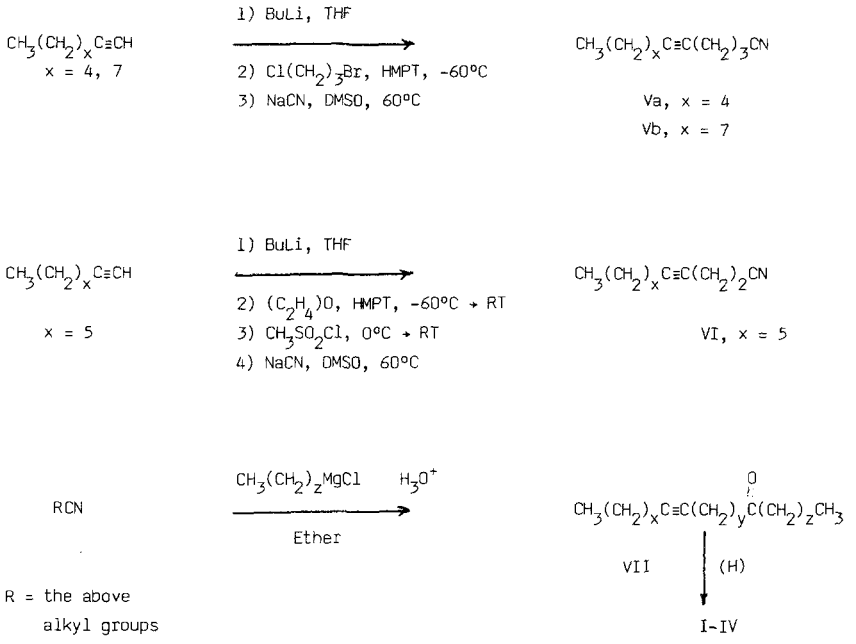

FIG. 1. Syntheses of the unsaturated ketones.

METHODS AND MATERIALS

Gas-liquid chromatography (GLC) was performed with a Varian 2400 instrument (flame ionization detection, He carrier) using capillary columns (0.25 mm ID) as indicated in Table 2. Infrared (IR) data were obtained with a Nicolet 7199-FT-IR system using CCl_4 solutions and ^1H nuclear magnetic resonance (NMR) data were obtained with a Nicolet 300-mHz FTNMR spectrometer (1% solutions in $CDCl_3$). Mass spectral data were obtained with a Finnegan model 3200 chemical-ionization mass spectrometer (isobutane) that was equipped with a chromatographic inlet (Varian model 1400) served by an OV-101 column, (0.25 mm × 31 m). Cesium salts were prepared from cesium carbonate and the corresponding acid. Water was removed azeotropically with benzene, and the salt was then dried in vacuo over P_2O_5. Potassium superoxide was ground before use but otherwise employed directly as purchased. Sodium iodide and cyanide were dried in vacuo over P_2O_5 before use.

5-Undecynoyl Nitrile, Va. Commercial 1-heptyne (3.3 ml, 25 mmol) was converted to the lithium acetylide with 2.2 M *n*-butyllithium (BuLi) in THF (15 ml) under nitrogen in the usual manner. The solution was cooled to

TABLE 2. GLC SEPARATIONS OF COMPOUNDS I–IV

	T (°C)	k'_Z	k'_E	α(R)	k'_{ynone}
A: CpCC (19 M)					
Compound I	190	7.41	7.76	1.047 (1.25)	9.00
Compound II	190	11.33	12.00	1.059 (1.3)	13.83
Compound III	200	9.71	10.53	1.085 (3.3)	10.88
Compound IV[a]	210				
B: OV-101 (31 M)					
Compound I	210	2.83	2.87	1.014 (<1.0)	3.17
Compound II	210	4.67	4.80	1.028 (<1.0)	5.17
Compound III	220	4.23	4.29	1.015 (<1.0)	4.65
Compound IV	220	8.77	8.77	1.000	9.37
C: SP-1000 (35 M)					
Compound I	170	4.26	4.26	1.000	6.82
Compound II	180	4.25	sh	1.0	7.33
Compound III	180	4.82	sh	1.0	7.30
Compound IV	200	5.33	5.50	1.031 (1.3)	8.50
D: SP-2340 (30 M)					
Compound III	180	2.25	2.25	1.000	3.25

[a]Above 210° C, the CpCC (liquid phase) is nearly isotropic and loses its ability to discriminate between isomeric substrates. The low volatility of compound IV precluded successful chromatography using the film thickness in this particular column.

$\leqslant -60°$ C and HMPT (10 ml) was injected. Commercial 1-bromo-3-chloropropane (2.7 ml, 25 mmol) was injected dropwise, and the resulting solution stirred at $\leqslant -60°$ C for 1 hr during which time copious amounts of (presumably) NaBr precipitated. The mixture was allowed to attain room temperature (homogeneous) and could be worked up in the usual fashion to give 1-chloro-4-decyne: 3.49 g (81%), bp 46–52° C (0.02 mm); NMR δ 0.88 (t, 3H, C$\underline{H}_3$CH$_2$) 1.80 (p, 2H, CH$_2$C$\underline{H}_2$CH$_2$), 2.15 (m, 2H, CH$_2$C≡C), 2.34 (m, 2H, C$\underline{H}_2$C≡C), 3.58 (t, 2H, C$\underline{H}_2$Cl) ppm; CIMS (*m/e*) 173, 175 (P+1).

Alternatively the reaction mixture containing the chloropropylated acetylene was supplemented with NaCN (2.5 g, 50 mmol), NaI (0.75 g, 5 mmol), and DMSO (12 ml) and then heated to 60–65° C overnight. After the usual work-up, the crude product was distilled to give Va = 2.50 g (63% from 1-heptyne), bp 75–77° C (0.05 mm); IR 2249 cm^{-1} (CN); NMR δ 0.90 (t, 3H, C$\underline{H}_3$CH$_2$), 1.82 (p, 2H, CH$_2$C$\underline{H}_2$CH$_2$); 2.13 (t, 2H, CH$_2$C≡C), 2.32 (t, 2H, NCCH$_2$CH$_2$C$\underline{H}_2$C≡C-elucidated by spin decoupling), 2.48 (t, 2H, CH$_2$C$\underline{H}_2$CH) ppm; CIMS (*m/e*) 164 (P+1).

5-Tetradecynoyl Nitrile, Vb. Similarly, 1-decyne was alkylated and then treated with NaCN–NaI and additional DMSO to give Vb (62%): bp 94–96° C (0.06 mm); IR 2250 cm^{-1}; NMR essentially as for Va; CIMS (*m/e*) 206 (P+1).

4-Undecynoyl Nitrile, VI. A solution of lithium octynylide was prepared (see above) from 1-octyne (12.0 ml, 81 mmol) in THF (60 ml). The solution was cooled to −78° C, and HMPT (15 ml) and ethylene oxide (4.5 ml from a precooled syringe, 90 mmol) were injected. The reaction mixture was stirred without a cooling bath through a subsequent low exotherm for 3 hr. The resulting lithiooxyethylated alkyne was converted to its mesylate by first cooling it to 0–5° C and then injecting into it methanesulfonyl chloride (10.2 ml, 132 mmol). Warming the mixture to 60° C for 3 hr converted the mesylate to 1-chloro-3-decyne which could be obtained by the usual work-up: 8.9 g (90%), bp 40–44° C (0.09 mm); NMR δ 0.88 (t, 3H, $C\underline{H}_3CH_2$), 2.15 (m, 2H, $CH_2C{\equiv}C$), 2.60 (m, 2H, $ClCH_2C\underline{H}_2C{\equiv}$), 3.58 (t, 2H, CH_2Cl) ppm; CIMS (*m/e*) 173, 175 (P+1).

Alternatively, the reaction mixture containing the chloroethylated alkyne was supplemented with DMSO (75 ml) and concentrated under reduced pressure removing ca. 80 ml distillate. Then THF (20 ml) and DMSO (25 ml) were injected, and NaCN (12.0 g, 0.245 mmol) and NaI (15.0 g, 0.10 mmol) were added. The resulting mixture was stirred at 60° C for 1.5 hr. The usual work-up gave VI: 6.1 g (48%), bp 60–62° C (0.001 mm); IR 2251 cm^{-1}; NMR δ 0.89 (t, 3H, $C\underline{H}_3CH_2$), 2.5 (t, 2H, $CH_2C\underline{H}_2C{\equiv}C$), 2.52 (s, 4H, $C{\equiv}CC\underline{H}_2C\underline{H}_2CN$) ppm; CIMS (*m/e*) 164 (P+1).

Preparation of Alkynones, VII. The appropriate 1-bromoalkane was converted to a Grignard reagent in THF in the usual fashion. The nitrile (24.5 mmol/40 mmol alkylbromide) was added dropwise to the organomagnesium (slightly exothermic). The resulting mixture was heated under reflux for 0.5 hr and then worked up by (1) slowly adding 4 N H_2SO_4 (40 mmol) while cooling the mixture, and (2) washing a hexane extract with 2 N HCl. Distillation provided a small forerun, and then 57–60% of the alkynone: for example, 7-nonadecyn-11-one (VII, $x = 5, y = 2, z = 7$) was obtained: 3.9 g (57%), bp 130–138° C (0.004 mm), rep. bp 135–138° C (0.45 mm) (Tamada et al., 1978), IR 1718 cm^{-1}; NMR δ 0.88 (bt, 6H, CH_3's), 2.11 (m, 2H, $CH_2C{\equiv}C$), 2.41 (bt, 4H, $RCH_2C{=}O$ and $C{\equiv}CC\underline{H}_2CH_2C{=}O$), 2.59 (bt, 2H, $C{\equiv}CCH_2C\underline{H}_2C{=}O$) ppm, CIMS (*m/e*) 279 (P+1). Spectral and chromatographic data for the other alkynones were in accord with assigned structures: VII ($x = 5, y = 2, z = 8$), mp 31–32° C; VII ($x = 4, y = 3, z = 9$), mp 25–26° C, rep. mp 26–27° C (Kocienski and Cernigliaro, 1976); VII ($x = 7, y = 3, z = 8$), mp 31–32° C, rep. mp 37–38° C (Uebel et al., 1978).

Preparation of (Z)-Alkenones, I–IV. Each alkynone was hydrogenated over Pd/$CaCO_3$ (Mozingo, 1955) in absolute ethanol. The reaction's progress was monitored by GLC (Table 2). The product was isolated in the usual manner. For example, (*Z*)-7-nonadecen-11-one, I, was obtained nearly quantitatively: bp 125–131° C (0.04 mm), rep. bp 125–126° C (0.65) (Tamada et al., 1978), IR 1716 cm^{-1}, NMR δ 0.88 (bt, 6H, $2CH_3$'s), 2.0 (m, 2H,

$RCH_2C{\equiv}C$), 2.3 (m, 2H, $O{=}CCH_2C\underline{H}_2C{=}C$), 2.4 (m, 4H, $CH_2[C{=}O]CH_2$), 5.34 (m, 2H, $HC{=}CH$); CIMS (*m/e*) 281 (P+1). Spectral and chromatographic data were in accord with the assigned structures: II, bp (short path) bath temp. 130° C (0.005 mm), rep. bp 135–140° C (0.45 mm) (Tamada et al., 1978); III, bp 150–154° C (0.002 mm), rep. bp (short path) 118° C (0.4) (Smith et al., 1975b); IV, not distilled. Products contained 4–7% *E* isomer.

Preparation of (*E*)-6-Heneicosen-11-one. The (*Z*)-alkenone (3.26 mmol) was treated with 2 M $NaNO_2$ (0.23 ml) and 6 M HNO_3 (150 μl) under nitrogen and at 70° C for 0.5 hr. The product (77% *E* by GLC) was obtained by the usual work-up and was recrystallized from acetone; mp 36–38° C, rep. mp 36–38° C (Smith et al., 1975b). This method has been found successful for γ,δ-unsaturated ketones, although not applied specifically to I or II.

4-Decyn-1-ol Acetate. A reaction mixture containing chloropropylated 1-heptyne as described above was supplemented with NaOAc (4 equiv.) and CsOAc (0.1 mol/100 ml), then warmed 60–65° C overnight. The product was worked up in the usual manner, treated neat briefly with pyridine (0.2 equiv.) and acetyl chloride (0.2 equiv.) to acetylate by-product alcohol, and distilled: 63% yield, bp 62–66° C (0.02 mm); IR 1743 cm^{-1}; NMR δ 0.90 (bt, 3H, $C\underline{H}_3CH_2$), 1.81 (p, 2H, $AcOCH_2C\underline{H}_2CH_2C{\equiv}C$), 2.05 (s, 3H, Ac), 2.13 (m, 2H, $RCH_2C{\equiv}C$), 2.25 (m, 2H, $AcOCH_2C\underline{H}_2CH_2C{\equiv}C$), 4.16 (t, 2H, $AcOCH_2$) ppm; CIMS (*m/e*) 211 (P+1).

4-Decyn-1-ol. A reaction mixture as described above was supplemented with sodium trifluoroacetate (2 equiv.) and the cesium salt (0.2 equiv.). The ratio of HMPT to lithium cation was adjusted to 4:1, and the resulting mixture was warmed to 60–65° C for 24 hr. The product was worked up in the usual manner with the crude mixture of alcohol and its trifluoroacetate ester in hexane shaken with 1.25 N NaOH for about 5 min (GLC monitoring). The alcohol was then distilled: 3.7 g (81%), bp 58–60° C (0.02 mm), IR 3613 cm^{-1}; NMR δ 0.90 (bt, 3H, $C\underline{H}_3CH_2$), 1.74 (p, 2H, $HOCH_2{-}_2CH_2C{\equiv}C$), 2.14 (m, 2H, $RCH_2C{\equiv}C$), 2.28 (m, 2H, $HOCH_2CH_2C\underline{H}_2C{\equiv}C$), 3.76 (t, 2H, $HOC\underline{H}_2$) ppm; CIMS (*m/e*) 169 (P+1).

DISCUSSION

Provided that the requisite unsymmetrical dihalides are available, selective replacement of one halogen by acetylides is useful. The solvent combination of tetrahydrofuran (THF) and hexamethylphosphoric triamide (HMPT) allows acetylides and alkyl halides to react exothermically (Normant, 1967). Even at ice bath temperatures, however, the reaction of 1-lithioheptyne with 1-bromo-3-chloropropane produced substantial amounts of 6,11-heptadecadiyne. This product arises from the reaction of chloroalkylated alkyne with a second molecule of alkyne and can only be attenuated by a more

selective replacement of the more reactive halogen. The solution of acetylide in THF was cooled to $\leqslant -60°$ C, and HMPT and dihalide were added directly. Displacement of bromide occurred essentially exclusively within the hour, affording the chloropropylated intermediate.

Displacement of primary chloride by cyanide is conventionally achieved under iodide catalysis in warm dimethyl sulfoxide (DMSO). The chief function of dipolar aprotic solvents in many types of organic reactions is to complex metal cations, rendering the organic nucleophile more reactive. Therefore, one would desire to alkylate the acetylene in DMSO, setting the stage for subsequent displacement by other organic/inorganic nucleophiles. Unfortunately, the acidity of DMSO is sufficient to produce competition between the dimsyl and acetylide anions (Smith and Kuehne, 1973). On the other hand, displacement of chloride by cyanide (iodide present) did not occur in the reaction mixtures obtained directly in alkylation reactions (60–65° C, 24 hr), despite successful, albeit slow, displacement of chloride in reactions employing purified chlorides. Perhaps the presence of substantial amounts of hexane from the butyllithium solutions acts to suppress introduction of cyanide ion into solution. The problem is readily overcome by adding DMSO to the mixture. In this connection it should be mentioned that the soluble (THF–HMPT) cyanide complex, LiCuClCN did not serve to affect displacement; i.e., probably there is no cyanide ion in equilibrium with the cuprate complex.

Preparation of a chloroethylated derivative from 1-decyne was not successfully accomplished with ethylene chlorobromide either by direct or inverse addition. Absence of the chain-extended 1-decen-3-yne from the reaction products was taken to indicate simply that the dihalide itself had suffered elimination. Reaction of the acetylide was therefore conducted with ethylene oxide intercepting the lithium alkoxide product with methanesulfonyl chloride. The mesylate could be isolated at that point or allowed to form the chloride by warming the reaction mixture briefly. Replacement reactions involving homopropargyl systems are subject to competition from elimination, which seemed to be slightly enhanced in the THF–HMPT solutions. Nevertheless, the addition of DMSO permitted the cyanide displacement to occur with a reasonable yield (48%) of the cyanoethylated compound. Reactions of purified chlorides in DMSO with NaCN produced less elimination and gave yields of 76–78% of replacement product. Rates of reaction and yields are markedly affected by solvent ratios. DMSO enhances solubilities of sodium salts, but reduces the solubility of an aliphatic substrate.

The nitriles, formed essentially as one-pot preparations, were then transformed to ketones with Grignard reagents in the usual manner. Although an excellent procedure for catalytic hydrogenation of alkynes to virtually pure (*Z*)-alkenes is avialable (Henrick, 1977), small amounts (*E*)-alkene were desired here for reference. Reductions to alkene were therefore conducted

with Lindlar catalyst (Mozingo, 1955), omitting the usual quinoline poisoning. Analyses of these alkenones for geometrical purity has been accomplished previously by epoxidation to produce diastereomeric epoxyketones that are separable by GLC (Smith et al., 1975a). The tabulated GLC data (Table 2) indicate that cholesterol parachlorocinnamate (CpCC), a liquid crystal phase, has the requisite discrimination between geometrical isomers and thereby offers a convenient method for assessing the exact constitution of the natural products directly and with great accuracy.

A procedure to produce an alkynol or its acetate from a terminal alkyne in a single step (pot) would be useful. The 2-carbon homologation using ethylene oxide, of course, accomplishes this directly. In the case of the chloropropylated derivative in situ displacement of the chloride with acetate could be accomplished readily with CsOAc (Table 3) and, as cesium salts are expensive, was probably best done using NaOAc with a small amount of the cesium salt as a catalyst. Cesium salts have been employed in dimethylformamide (DMF) for bimolecular displacement reactions (Wang et al., 1977); it appears that there is potential for their use as catalysts as well. Moreover, their utility is apparently not limited to use in DMF.

Reactions were conducted to affect direct conversion of a choropropylated alkyne to an alkynol also. Potassium superoxide (San Filippo et al., 1975) did produce the alcohol, albeit in low yield (Table 3). Additional DMSO and 18-crown-6 were required to complete the displacement of chloride. A more successul alternative involved the use of sodium trifluoroacetate with the cesium salt as a catalyst. Although the trifluoroacetate anion is by no means routinely employed as a nucleophile, displacement reactions

TABLE 3. DISPLACEMENTS OF CHLORIDE[a]

$$CH_3(CH_2)_4C{\equiv}C(CH_2)_3Cl \xrightarrow{X} CH_3(CH_2)_4C{\equiv}C(CH_2)_3Y$$

X	Y
CsOAc, 60°C, 6 hr	OAc (100% conversion)
KOAc, 18-Cr-6[b] 60°C, 8 hr	OAc (88% conversion)
NaOAc, 60°C, 8 hr, 20 hr	OAc (45, 93% conversion)
LiOAc, 60°C, 8 hr	OAc (45% conversion)
NaOAc, CsOAc[b], 60°C, 16 hr	OAc (98% conversion)
KO_2, 18-Cr-6[b], 60°C, 20 hr[c]	OH (97% conversion)
NaOTF[d], CsOTF[b], 60°C, 24 hr	OH (95% conversion)

[a] Displacement was affected in the reaction mixtures generated by alkylating 1-heptyne with 1-bromo-3-chloropropane.
[b] Employed as catalyst.
[c] Additional DMSO used.
[d] Additional HMPT used.

are possible, providing a useful conversion of halide to alcohol without the competing elimination seen with more basic nucleophiles. The trifluoroacetate ester was conveniently saponified by shaking it in hexane solution with aqueous base.

The *E* isomers of the pheromones have been synthesized previously in conjunction with the identification studies. This was generally accomplished by reduction of an acetylenic derivative with Na–liquid NH_3 and subsequent establishment of the ketone carbonyl group. Treatment of the *Z* isomers; i.e., the *Z*-enones, with nitrous acid (Litchfield et al., 1965) produced mixtures of enones (77 *E*:23 *Z*), from which the *E* isomers were conveniently obtained (58–61% overall yield) by recrystallization from acetone.

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SENSITIVITY OF ANTENNAE OF MALE AND FEMALE *Ips paraconfusus* (COLEOPTERA: SCOLYTIDAE) TO THEIR NATURAL AGGREGATION PHEROMONE AND ITS ENANTIOMERIC COMPONENTS

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Abstract—Antennae of male and female *Ips paraconfusus* were equally and highly sensitive to their male-produced, multicomponent aggregation pheromone. Female and male antennae were highly sensitive to the pheromonal component, (*S*)-(−)-ipsenol, but essentially insensitive to its antipode, (R)-(+)-ipsenol. Further, female and male antennae were more sensitive to the pheromonal component, (*S*)-*cis*-verbenol, than to its antipode, (*R*)-*cis*-verbenol. Dramatic sexual dimorphism in chiral sensitivity to the ipsdienol enantiomers was found, with female antennae being more sensitive to the conspecific pheromonal enantiomer, (*S*)-(+)-ipsdienol, and male antennae being more sensitive to the antipode, (*R*)-(−)-ipsdienol. Since (*R*)-(−)-ipsdienol is the principal pheromone of California *Ips pini* and interrupts *I. paraconfusus* aggregation, male antennae appear to be more sensitive to an interspecific allomone than a conspecific pheromone. Of the conspecific pheromonal enantiomers, both male and female antennae were most sensitive to (*S*)-(+)-ipsdienol, intermediately sensitive to (*S*)-(−)-ipsenol, and least sensitive to (*S*)-*cis*-verbenol. However, when enantiomeric sensitivities were compared to the estimated concentrations of these components in the natural pheromone, (*S*)-(−)-ipsenol tended to equal or approach the potency of (*S*)-(+)-ipsdienol as an antennal stimulant, while antennal responsiveness to (*S*)-*cis*-verbenol was dramatically less than for the other two pheromonal components. The behavioral implications of such physiological sensitivities are discussed in regard to perception of multicomponent synergistic pheromones and the relative efficacy of each component as an orientation cue.

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INTRODUCTION

It is now well established that multicomponent chiral pheromones coordinate mass aggregation and colonization of host-tree resources by bark beetles (Birch, 1978; Borden, 1974, 1977; Silverstein, 1979; Vité and Francke, 1976; Wood, 1982). In many species behavioral specificity of response to putative chiral pheromones has been determined (see Brand et al., 1979; Silverstein, 1979; Silverstein and Young, 1976), and in a few cases the enantiomeric composition of scolytid pheromones has been resolved (Birch et al., 1980; Borden et al., 1980; Brand et al., 1979; Fish et al., 1979; Lanier et al., 1980; Plummer et al., 1976; Renwick et al., 1976; Silverstein et al., 1966; Stewart, 1975; Stewart et al., 1977; Wood et al., 1976).

Although chiral specificity in both production and behavioral responsiveness to pheromones by bark beetles is now well documented, electrophysiological studies to determine the sensitivities of antennal receptors for the optical isomers of pheromones are much more limited. Most electrophysiological studies have used only racemic compounds (see Payne, 1979), while the few studies using enantiomers of pheromones have been limited in scope [e.g., only one component of a multicomponent chiral pheromone (Angst and Lanier, 1979; Dickens, 1978; Mustaparta et al., 1979, 1980) and/or tested on only one sex (Angst and Lanier, 1979; Mustaparta, 1979; Mustaparta et al., 1979)], or the optical purity of the enantiomers tested has been either unspecified (Mustaparta, 1979; Mustaparta et al., 1979) or relatively low (93%, Angst and Lanier, 1979; 92%, Mustaparta et al., 1980).

In this report electrophysiological techniques were used on antennae of male and female *Ips paraconfusus* Lanier to study some aspects of the olfactory reception of (1) the natural aggregation pheromone, (2) the highly pure enantiomeric components of its pheromone, and (3) the antipodes of the pheromone enantiomers.

The male produced aggregation pheromone of *I. paraconfusus* is a synergistic mixture of three chiral terpene alcohols: (*S*)-(−)-ipsenol, (*S*)-(+)-ipsdienol, and (1*S*,4*S*,5*S*)-*cis*-verbenol (Silverstein et al., 1966). The enantiomeric composition of the pheromonal components has been estimated to be: ≈97% (*S*)-(−)-/3% (*R*)-(+)-ipsenol; ≈94–95% (*S*)-(+)-/5–6% (*R*)-(−)-ipsdienol; and 100% (*S*)-*cis*-verbenol (L.E. Browne, personal communication; Fish et al., 1979; Plummer et al., 1976; Silverstein et al., 1966; Stewart, 1975).

The study was undertaken (1) to determine the sensitivity of antennal receptors for the chiral components of a multicomponent pheromone and (2)

to determine the sensory acuity of this olfactory system for the reception of one chiral component relative to the others. Such an analysis would help to understand the range of pheromonal and other chemically mediated orientations by a species whose species-specific aggregation requires synergism among its three pheromonal components in an environment where it coexists with several other species using one or more components and/or antipodes of its chiral pheromone.

METHODS AND MATERIALS

Source of Beetles

Naturally infested logs of ponderosa pine were collected in the McCloud Flats region of the Shasta-Trinity National Forest (Siskiyou County, California). Adult *I. paraconfusus* were collected daily as they emerged from these logs in the laboratory, separated as to sex, and stored at 4° C on strips of ponderosa pine phloem.

Isolation of Natural Aggregation Pheromones

The "natural" aggregation pheromone was isolated by condensing the volatiles produced both by the host tree and by actively boring male beetles, using a cryogenic liquefaction technique, or "cold trap" (Browne et al., 1974). The volatiles released by 95 *I. paraconfusus* males boring in a ponderosa pine log for 42 daylight trapping hours were condensed in a liquid nitrogen trap. The resulting aqueous solutions were saturated with sodium chloride, extracted with ether, and the combined ether extract was dried over anhydrous sodium sulfate and concentrated at 760 mm Hg to a total concentration of 342.0 beetle-min/μl of solution. One beetle-minute (bm) represents the amount of host volatiles and attractant produced by one beetle boring for one minute.

Sources and Purity of Enantiomers

Samples of (*S*)-(−)- and (*R*)-(+)-ipsenol (Figure 1) were provided by K. Mori (University of Tokyo) at 99% optical purity. The enantiomers of ipsdienol were resolved by B.J. Bergot (Zoecon Co., Palo Alto, California) to optical purity levels of 94.1% for the (*S*)-(+)-enantiomer and 97.8% for the (*R*)-(−)-enantiomer (see Birch et al., 1980). The enantiomers of *cis*-verbenol were purchased from Borregaard Industries Limited (Sarpsborg, Norway) with an optical purity cited as greater than 90%. The chemical purity of all compounds was 99% or greater, as determined by GLC analysis.

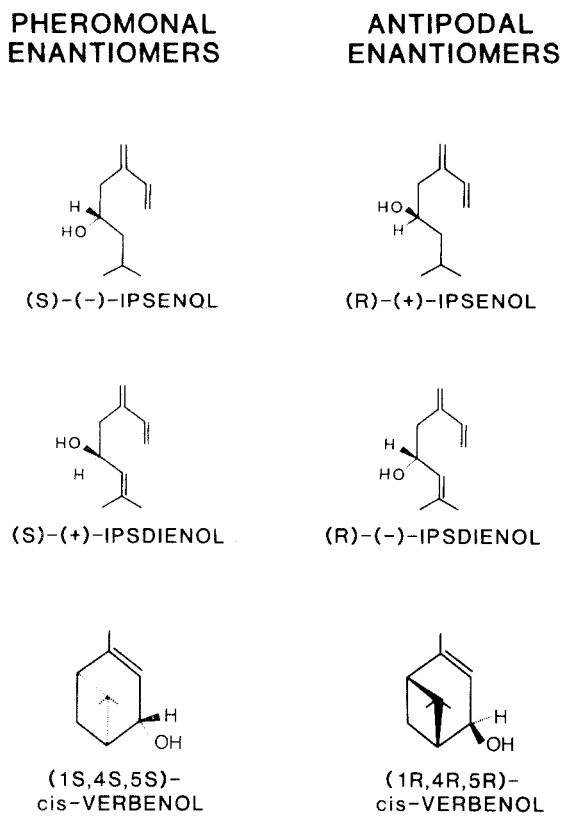

FIG. 1. Stereoisomeric configurations of the components of the aggregation pheromone of *Ips paraconfusus* and the antipodal enantiomers.

Electrophysiological Recording Technique

In the present study electroantennogram (EAG) recordings (Boeckh et al., 1965; Light and Birch, 1979a; Schneider, 1957) were obtained from intact antennae, using glass capillary Ag-AgCl electrodes filled with insect saline (NaCl, $CaCl_2$, KCl, and $NaHCO_3$ at 7.50, 0.21, 0.35, and 0.20 gm/liter, respectively) (Kaissling, as cited by Roelofs, 1977). Live beetles were immobilized in a hole in a Plexiglas block by a yoke that compressed the beetle's head against its pronotum (modified from Payne, 1970; similar to Angst and Lanier, 1979). Each antennal club was gently secured with its anterior (sensory) side up on double-sided sticky tape.

For each recording, the recording electrode was inserted within approximately the same apical region of the terminal antennal club segment. The ground electrode was positioned in the abdominal hemocoel. Such prepara-

tions gave reproducible EAGs for 1–8 hr. Electrodes were connected via a Grass model P16 differential high-impedance probe (10^{11} Ω) to a Grass model P16 DC microelectrode amplifier operated in the single-ended mode. Signals were fed to the upper beam of a Tektronix 5113 dual beam storage oscilloscope. EAG deflections were measured directly from the stored screen image or photographed with a Tektronix C5-A camera on Polaroid-type 667 film.

Stimulation Technique

Synthetic Compounds and Cold-Trap Condensate. The undiluted enantiomeric compounds were dissolved in *n*-pentane (Baker Photrex, 99.8% chemical purity) at dilutions from 1×10^{3} to 1×10^{-5} μg/μl. The concentrated *I. paraconfusus* cold-trap condensate was also dissolved in *n*-pentane at dilutions from 34.2 to 3.42×10^{-5} bm/μl.

From these solutions, dosage series were produced for each compound or condensate by pipetting 1-μl aliquots onto separate 1 × 2-cm pieces of fluted filter paper (Whatman No. 1). After application of each compound, the pentane was allowed to evaporate for 1 min, and the filter paper then was positioned within a Pasteur pipet cartridge. One hour prior to each experiment, new stimulus cartridges were made up, stored at 0° C, and then placed in an independently vented holding container during the course of each experiment.

Odor Delivery. The odor delivery system used was a modification of the technique described by Payne and Dickens (1976). Breathing-quality compressed air (Liquid Carbonics, <5 ppm hydrocarbons) was filtered through activated charcoal, humidified by bubbling through distilled water, and its flow regulated at 1.0 liter/min (Gilmont No. 13 Flowmeter). A three-way solenoid-operated valve (Asco 8320-62 Red Hat, 6 V DC) allowed the antennal preparation to be continuously exposed to clean air through one valve outlet when the solenoid was inactivated. Upon activation of the valve, clean air was immediately delivered through a given stimulus "cartridge" attached to the other valve outlet. Both the clean air and stimulus "cartridges" were placed within a common mixing chamber terminating in a nozzle, an automatic pipet tip (Centaur Chemical) with an orifice of about 2 mm diam. (air velocity at orifice estimated to be 5 m/sec). This common nozzle was attached to a Wild M-5 stereomicroscope and optically focused at a distance of approximately 20 mm from the antennal preparation and at about 45° from its longitudinal axis. All stimulus "puffs" were of 1.0 sec duration and were delivered via the three-way solenoid valve when activated by an electrical pulse from a stimulator circuit, which incorporated a solid-state, precision (±1% repeatability) adjustable time-delay relay (Potter Brumfield CDD21-300003). The on–off signal from the solenoid was fed into the lower beam of the oscilloscope.

It was assumed that the pheromonal evaporation rate, puff concentration, and number of pheromone molecules that impinged upon the antennal sensilla were proportional to the amount of the pheromone placed on the filter paper (Boeckh et al., 1965; Kaissling, 1971; Payne et al., 1970). The duration of stimulus puffs (1.0 sec), air flow rate (1.0 liter/min), physical factors (filter paper and cartridge size and position, etc.), and protocol for replicates were the same in all experiments in order to meet the requirements for EAG reproducibility (see Adler, 1971; Kaissling, 1971; Schneider, 1962).

Experimental Design and Evaluation of Results

A strict system of stimulus spacing was followed in order to reduce sensory adaptation in the receptors and to avoid absorptive overloading of the antenna (Payne et al., 1970; Payne, 1970, 1975). All stimulus presentations were followed by 1–3 min of clean air. Usually a pentane "control" stimulation of 1 sec duration was interspersed every 2–6 min of recording. All experiments began with the antenna being exposed to a 1-sec stimulation of 3.42 bm of male *I. paraconfusus* cold-trap condensate (in pentane) (henceforth referred to as 3.42 bm standard) followed by a control. The time from electrode implantation to each stimulation was recorded. The 3.42 bm standards were interspersed throughout the experiments to monitor the systematic time-dependent decrease in antennal responsiveness, suggested to be primarily caused by progressive dehydration of antennal preparations (Roelofs and Comeau, 1971; Schneider, 1962). A 3.42 bm standard dose was chosen because preliminary experiments showed that it elicited a significant behavioral response in males and females in both laboratory and field bioassays, and the estimated concentration of host terpenes and pheromones in a 3.42 bm dose would not cause sensory adaptation or antennal overloading (Light, unpublished data).

To compare the sensitivity of male and female antennae to the enantiomeric compounds and the pheromone cold-trap condensate, EAG amplitudes were recorded from 5–20 males and 5–29 females in response to an ascending series of doses (0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 10 μg, etc.) of each enantiomer on separate filter papers, with a pentane control and a 3.42 bm standard interspersed between every third or fourth stimulation. Due to the scarcity of the highly pure enantiomers of ipsdienol and ipsenol, tests could only be conducted up to the 1 μg and 10 μg levels, respectively, for these compounds. The order of presentation of different compounds was systematically randomized for each test insect.

EAG responses were evaluated by measuring the maximum amplitude of negative deflection (−mV) elicited by a given stimulus and then subtracting the amplitude of the response to the preceding pentane control. All cited EAGs have been "corrected" in this way to discount the possible antennal

chemoreception of pentane and the thermoreception, mechanoreception, electrical artifacts, and mechanical disturbances in the air flow due to the switching of the solenoid valve. The millivolt responses to all enantiomers and concentrations were converted to percentage values of the response of 3.42 bm standard. This conversion of each response to a percentage of the standard response allowed for comparison of responses within an individual and between different individuals. It also "normalized," or minimized, the variability in absolute sensitivity between individual preparations, the variability due to order and presentation of compounds, and the time-dependent variability in antennal responsiveness (Light and Birch, 1979a; Payne, 1975; Roelofs and Comeau, 1971).

Dosage ($\log_{10} \mu g$) vs. EAG response (as % of standard response) curves were plotted ($\bar{X} \pm$ SEM) for each compound and condensate. Mean responses of the same group of individuals to different stimuli at set concentrations were compared statistically using paired t tests and the nonparametric Wilcoxon signed rank test for matched pairs (Snedecor and Cochran, 1967). Mean responses of different groups of individuals to different stimuli were compared statistically using a t test and the nonparametric Mann-Whitney test (Snedecor and Cochran, 1967). Significant differences in sensitivity were assigned at $P < 0.05$.

EAG recordings have been used to infer which volatile odorants are perceived by receptor neurons on insect antennae (e.g., Boeckh et al., 1965; Kaissling, 1971; Roelofs and Comeau, 1971). The EAG is thought to be the expression of generator potentials of many simultaneously stimulated receptor cells with potentially different specificities (Boeckh et al., 1965; Kaissling, 1971; Schneider, 1962).

Dosage–EAG response curves to different compounds were compared to determine the relative sensitivity, affinity and efficacy of the particular compound to elicit an antennal response (Δ mV potential). The following sensitivity parameters were used to evaluate the sigmoidal EAG response curves: the threshold concentration (K_{th}), the maximum response concentration (K_{max}), the concentration when the response was one-half the response value of K_{max} (K_b), and the slope of the curve in its dynamic response range.

The definition suggested by Payne et al. (1970) for a significant mean EAG response was followed, as one having an amplitude greater than three times the mean standard error of the control above the mean response to the control. To delimit thresholds, Kaissling's (1971) definition of threshold as the concentration value with the lowest significant percentage of response was applied to the criterion set above by Payne et al. (1970) for significance above control. The K_{max} value was assigned at the point along the concentration axis where the slope of the sigmoidal curve changed from exponential to a slope nearing zero or to a plateau where the response amplitude did not change as concentration was increased logarithmically. The K_b value (or the concentra-

tion where response was half the response at K_{max}) was computed from Lineweaver-Burk plots where 1/response is plotted vs. 1/concentration and the x axis intercept is equal to $-1/K_b$ (Mitchell and Gregory, 1979). The slope of the exponential dynamic response range of each curve, when the velocity or rate of change in response as the logarithmic concentration increases is the greatest, was calculated.

RESULTS

Antennal Responsiveness to Standard and Control

The amplitudes of EAGs of male and female *I. paraconfusus* were not significantly different for the 3.42 bm standard dose of the pheromonal cold-trap condensate. Mean response (corrected for the pentane control) to the 3.42 bm standard was −2.01 mV (±0.15; $\overline{X}$ of 264 replications from 28 individuals) for female antennae and −2.39 mV (±0.20; $\overline{X}$ of 247 replications from 20 individuals) for male antennae (Figure 2A). Mean response to pentane controls was −0.57 mV (±0.06; $\overline{X}$ of 570 replications from 28 individuals) for female antennae and −0.76 mV (±0.10; $\overline{X}$ of 538 replications from 20 individuals) for male antennae. Thresholds, delimiting a significant response above responses to pentane controls, were 8.25% of response to standard for females and 12.84% of response to standard for males. Both thresholds are cited as a mean percentage of the response to the corrected 3.42 bm mean standard response. Mean responsiveness of intact antennae to stimulation by the pheromonal standard decreased over the time of experiment at a rate of approx. 0.003 mV/min for males and 0.002 mV/min for females.

Antennal Sensitivity

Natural Aggregation Pheromone. Male and female antennae were equally sensitive to the male-produced natural aggregation pheromone over the entire range of concentrations (Figure 2A and 2B). Antennal responses of both sexes exhibited virtually identical K_b values, response maximum values, and dynamic-response phase slopes (Table 1).

Ipsenol. Female and male antennae were highly sensitive to pheromonal (*S*)-(−)-ipsenol but essentially insensitive to its antipode, (*R*)-(+)-ipsenol (Figures 3A and 3B). Small responses recorded to the (*R*)-(+)-enantiomer can be accounted for by the presence of 1% of the (*S*)-(−)-enantiomer within the 99% optically pure (*R*)-(+)-ipsenol sample. Thus, threshold and K_b values are approximately two orders of magnitude (100×) higher for stimulation by the (*R*)-(+)-enantiomer than by the (*S*)-(−)-enantiomer, while the slopes of their dynamic response phase are nearly identical (Table 1).

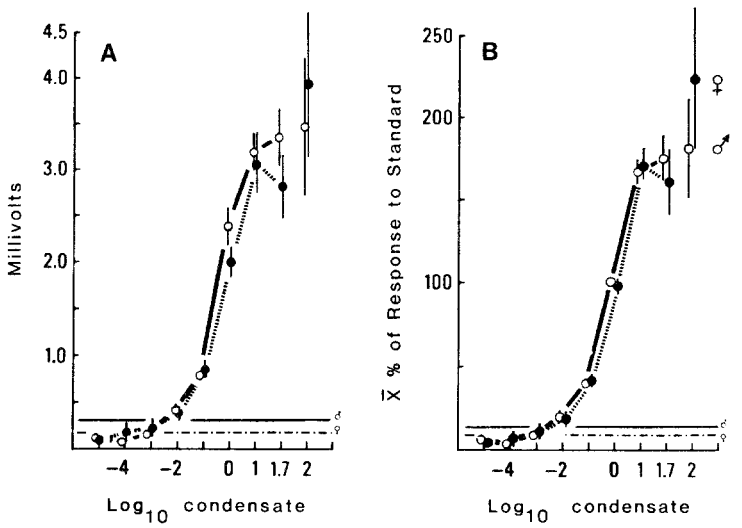

FIG. 2. Mean EAG responses of female (●) and male (○) *Ips paraconfusus* to a cold-trapped condensate of its natural conspecific aggregation pheromone and ponderosa pine volatiles. Log concentration steps are dilutions in pentane of a condensate of volatiles produced during 342 beetle-minutes of boring by male beetles (Log_{10} 1.7 = 50.0 bm dose). Responses are plotted as mean millivolt response (A) and as mean percentages of response (B) to a standard concentration of 3.42 beetle-minutes of the condensate (log_{10} 0). Points are means of recordings from 10–20 beetles; vertical lines are ±SEM. For clarity, points were positioned offset from the concentration markers. Horizontal lines show response thresholds for males (solid) and females (dash-dot), above which responses are significantly greater than to pentane solvent controls. Dose–response curves are not continuous to the highest concentration points because those points are means of independent samples from different individuals.

Ipsdienol. Female antennae were more sensitive to (*S*)-(+)-ipsdienol than to its (*R*)-(−)-antipode, as evidenced by significantly higher mean values (except at 1-μg dose) and by a lower threshold, higher responsiveness, and different response slopes (Figure 4A, Table 1). In contrast, male antennae were significantly more sensitive at lower (10^{-5} and 10^{-4} μg) and higher (1 μg) dosage levels to (*R*)-(−)-ipsdienol than to the dominant pheromonal component, the (*S*)-(+)-enantiomer (Figures 4B, Table 1).

cis-*Verbenol.* Dosage–response curves of female and male antennae had lower thresholds and K_b values and greater response maxima for stimulation by the pheromonal (1*S*, 4*S*, 5*S*)-*cis*-verbenol enantiomer than by the antipode (Table 1). Although there was a clear trend toward higher sensitivity to (*S*)-*cis*-verbenol by both females and males (Figures 5A and 5B), at only two stimulation levels (1 and 10 μg for females; 0.1 and 5000 μg for males) were the

TABLE 1. SENSITIVITY PARAMETERS[a] DERIVED FROM DOSE VS. MEAN EAG RESPONSE CURVES OF MALE AND FEMALE *Ips paraconfusus*

Compound	Sex	Threshold	K_b		Response maximum		Dynamic response phase
		Log_{10} conc. (μg)	Log_{10} conc. (μg)	Response to standard[b] (%)	Log_{10} conc. (μg)	Response to standard[b] (%)	Slope (% resp./ Log_{10} conc. (μg) step)
Male *I. paraconfusus*	M	−2.6	−0.2	90.0	1.0	167.1	63.6
Cold trap condensate	F	−3.8	−0.2	90.2	1.0	172.0	64.9
(*S*)-(−)-Ipsenol	M	−3.2	(0.4)[c]	(107.7)	No maximum reached[d]		30.9
	F	−2.3	(0.7)	(94.1)	No maximum reached		36.1
(*R*)-(+)-Ipsenol	M	−1.5	(2.7)	(100.0)	No maximum reached		26.4
	F	0.1	(2.7)	(100.0)	No maximum reached		37.9
Racemic Ipsenol	M	−2.2	1.1	107.1	3.7	202.4	37.3
	F	−2.8	0.8	94.1	2.0	179.7	41.2
(*S*)-(+)-Ipsdienol	M	−3.7	(0.0)	(109.8)	No maximum reached		28.5
	F	−5.0	(0.0)	(122.9)	No maximum reached		28.7
(*R*)-(−)-Ipsdienol	M	−5.0	(0.0)	(131.9)	No maximum reached		39.1
	M	−3.6	(−0.9)	(63.5)	0.0	118.6	34.7
Racemic ipsdienol	M	−5.0	0.8	144.0	3.7	275.0	51.3
	F	−5.0	0.4	123.3	3.0	238.2	52.3
(1*S*,4*S*,5*S*)-*cis*-Verbenol	M	−3.8	1.0	74.3	3.7	135.4	23.7
	F	−2.6	0.4	49.5	1.7	90.5	25.3
(1*R*,4*R*,5*R*)-*cis*-Verbenol	M	−0.8	1.5	60.8	3.7	108.6	20.9
	F	−1.6	1.2	38.0	1.7	67.0	31.6

[a]Sensitivity parameters as defined in Methods and Materials.
[b]Mean % response compared with response to the 3.42 bm cold-trap standard.
[c]K_b values in parentheses are based on interpolations from response maxima elicited by racemic compounds.
[d]The higher stimulus concentrations were not available for testing and thus no maximum response was reached.

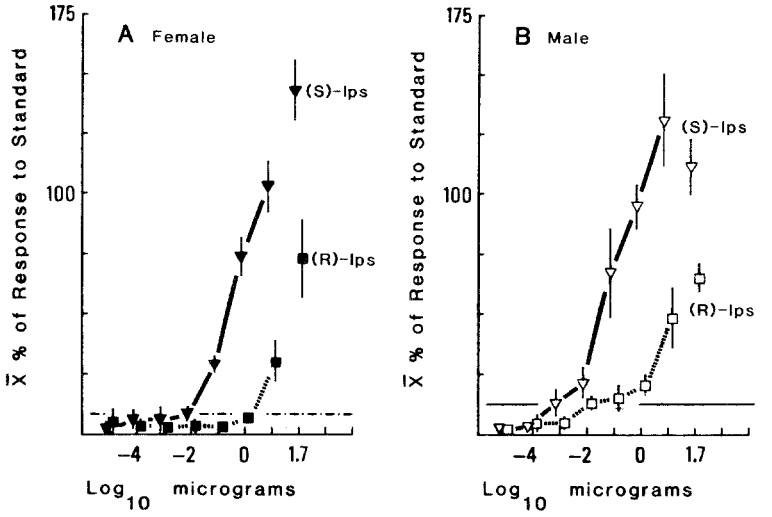

FIG. 3. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to (*S*)-(−)-ipsenol [(S)-Ips: ▼, ▽] and to (*R*)-(+)-ipsenol [(R)-Ips: ■, □]. Responses are plotted as mean percentages of the response to a standard of 3.42 beetle-minutes of the cold-trapped condensate. Points are means of recordings from 5–10 beetles; vertical lines are ±SEM. For clarity, points were positioned offset from the concentration markers. Horizontal lines show response thresholds for males (solid) and females (dash-dot), above which responses are significantly greater than to controls. Dose-response curves are not continuous to the highest concentration points because those points are means of independent samples from different individuals. (Log_{10} 1.7 = 50.0 μg dose).

differences significantly greater than those elicited by the (*R*)-enantiomer for each sex.

Relative Antennal Sensitivities

Female antennae had a significantly much higher sensitivity to (*S*)-(+)-ipsdienol than to (*S*)-(−)-ipsenol and, similarly, a significantly much higher sensitivity to (*S*)-(−)-ipsenol than to (*S*)-*cis*-verbenol at doses above threshold (Figure 6A). But male antennae tended to be only slightly, but not significantly, more sensitive to (*S*)-(+)-ipsdienol than to (*S*)-(−)-ipsenol, while male responses to both these pheromonal components were significantly much greater than those to (*S*)-*cis*-verbenol at doses above threshold (Figure 6B).

Concentration levels of the three pheromonal components of *I. paraconfusus* in a similar cold-trap condensate were estimated by GLC analysis to be

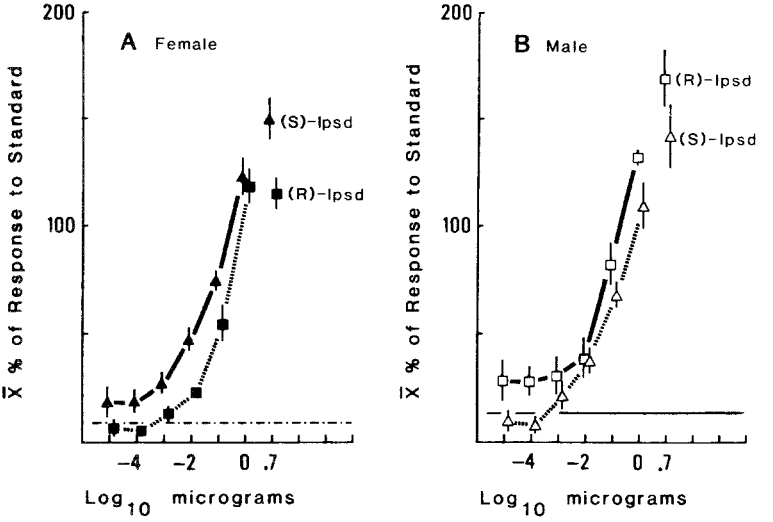

FIG. 4. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to (S)-(−)-ipsdienol [(S)-Ipsd: ▲, △] and (*R*)-(−)-ipsdienol [(R)-Ipsd: ■, □] (see Figure 3) ($\log_{10}$ 0.7 = 5.0 μg dose).

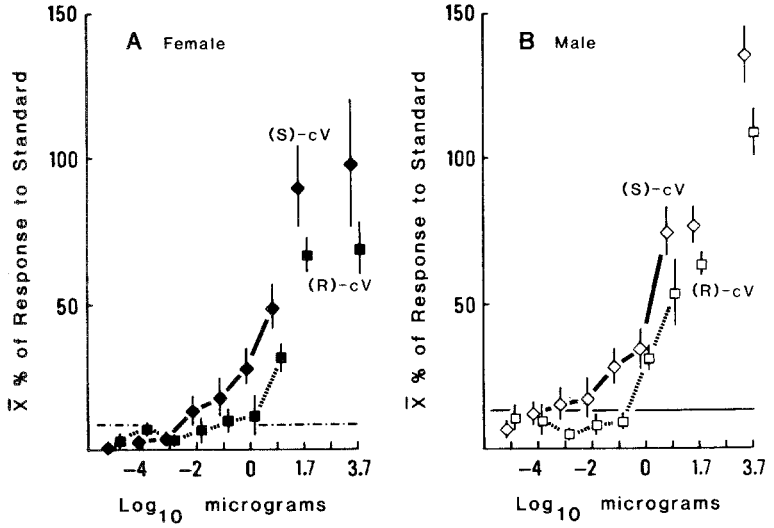

FIG. 5. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to (1*S*,4*S*,5*R*)-*cis*-verbenol [(S)-cV: ◆, ◇] and (1*R*,4*R*,5*R*)*cis*-verbenol [(R)-cV: ■, □] (see Figure 3) ($\log_{10}$ 1.7 = 50.0 μg dose and $\log_{10}$ 3.7 = 5000.0 μg dose).

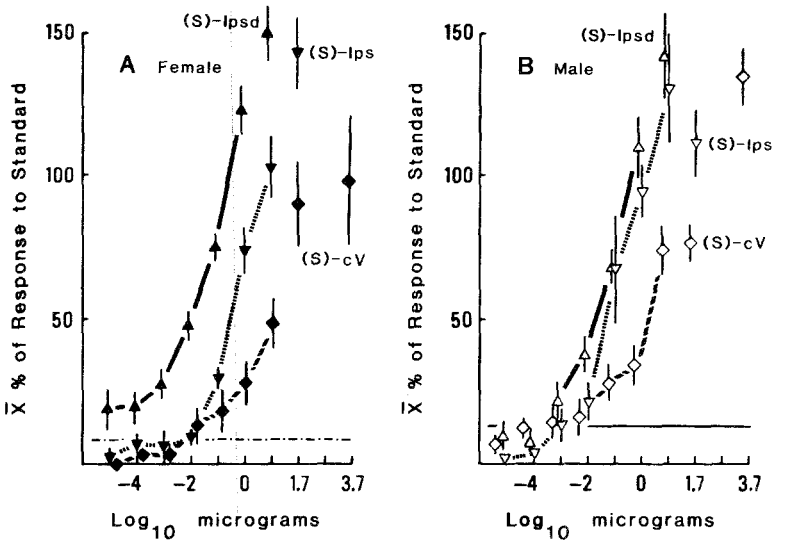

FIG. 6. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to the pheromonal components: (S)-(+)-ipsdienol [(S)-Ipsd: ▲, △]; (S)-(−)-ipsenol [(S)-Ips: ▼, ▽]; and (1*S*,4*S*,5*S*)-*cis*-verbenol, [(S)-cV: ◆, ◇] (see Figure 3).

approx. 0.014 μm/bm of (*S*)-(−)-ipsenol, 0.00086 μg/bm of (*S*)-(+)-ipsdienol, and 0.00021 μg/bm of (*S*)-*cis*-verbenol (L.E. Browne, personal communication; Light, unpublished data). When the EAG responses to these pheromonal components and the natural aggregation pheromone are replotted vs. their estimated natural rates of occurrence per beetle-minute of cold-trap condensate (over a concentration abscissa based on an estimated level of 1 bm = $\log_{10}$ 0), a more accurate estimate of the natural response to the pheromones was obtained (Figures 7A and 7B). At these "natural" levels of occurrence, (*S*)-(−)-ipsenol tended to be a slightly, but not significantly, stronger stimulant than (*S*)-(+)-ipsdienol of male antennae (Figure 7B); while at doses below 10 bm equivalents, (*S*)-(+)-ipsdienol was still a significantly more potent stimulant than (*S*)-(−)-ipsenol of female antennae (Figure 7A). Either of these pheromones accounts for approximately half the responsiveness to the complex cold-trap condensate. The antennal responsiveness to (*S*)-*cis*-verbenol was dramatically less than for the other two pheromonal components. Responses to (*S*)-*cis*-verbenol did not exceed threshold until equivalent concentrations of greater than 10.0 bm were reached for females and 1.0 bm for males (Figures 7A and 7B).

DISCUSSION

Natural Aggregation Pheromone. The antennae of male and female *I. paraconfusus* were found to be equally and highly sensitive to their conspecific

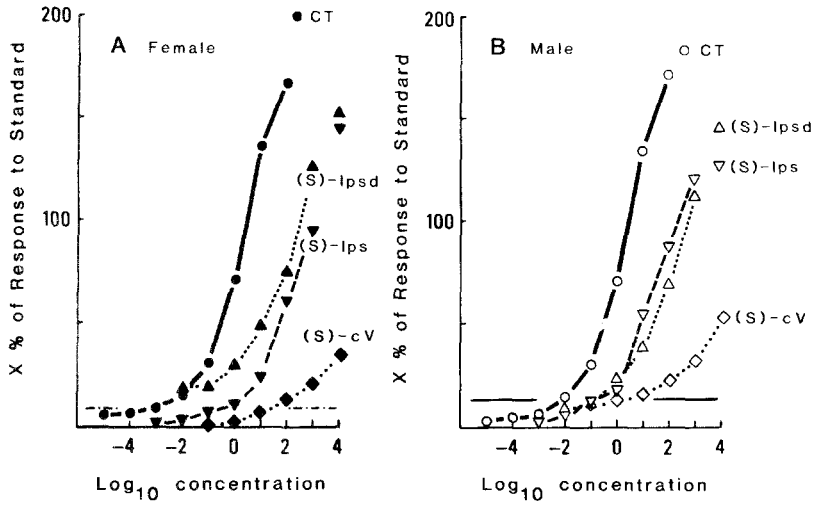

FIG. 7. Mean percent EAG responses of female (A) and male (B) *Ips paraconfusus* vs. both (1) a dosage series of its cold-trapped (CT: ●, ○) pheromonal and host condensate (bm/μl), and (2) independent concentration series of each of the pheromone components at their estimated natural levels (μg) present per beetle-minute (bm) of CT condensate. Thus for this figure the dose–response curves (figure 6) for each of the pheromonal components has been appropriately displaced along the x axis so that the present log concentration scale is simultaneously equivalent, for example, at $\log_{10}$ 0 to 1.0 bm of CT condensate, 0.014 μg of (*S*)-(−)-ipsenol (▼, ▽), 0.0009 μg of (*S*)-(+)-ipsdienol (▲, △), and 0.0002 μg (1*S*,4*S*,5*S*)-*cis*-verbenol (◆, ◇). Responses are plotted as mean percentages of the response to a standard of 3.42 beetle-minutes of the cold-trapped condensate. Points are means of recordings from 5–10 beetles. For clarity, no lines delineating the SEM were plotted (see Figures 2 and 6). Horizontal lines show response thresholds for males (solid) and females (dash-dot), above which responses are significantly greater than to controls. Dose–response curves are not continuous to the highest concentration points in most cases because those points are means of independent samples from different individuals.

natural aggregation pheromone. However, the natural aggregation pheromone used here is a complex condensation in which the terpene alcohol pheromones and other compounds produced by the male beetles (and/or their microorganisms, see Brand et al., 1979) are minor components, while the major constituents are various volatile terpenes from ponderosa pine (up to ten compounds that accounted for 79% by weight of the condensate as determined by GLC analysis; Light, unpublished data). Perception of two major ponderosa pine terpenes, myrcene and *alpha*-pinene, which are the precursors of the pheromones, is described elsewhere (Light, ibid.).

Ipsenol. Data presented both here and from differential adaptation

experiments (Light, in preparation) suggest that antennae of male and female *I. paraconfusus* have populations of receptor neurons highly sensitive to and possessing acceptors with specific affinities for the (*S*)-(−)-ipsenol enantiomer. On the other hand, the antennae of both sexes appear to be devoid of (or have very few) receptors responsive to the (*R*)-(+)-ipsenol enantiomer. No evidence is available to suggest that any *Ips* species responds behaviorally to or produces primarily (*R*)-(+)-ipsenol. Ipsenol is produced by many *Ips* species (Vité et al., 1972) and functions as a pheromonal attractant (Bakke, 1978; Birch et al., 1977b; Lanier and Burkholder, 1974; Silverstein et al., 1966; Vité et al., 1976a,c; Wood, 1970; Wood et al., 1967; 1968), allomonal interruptant (Birch and Wood, 1975; Birch and Light, 1977), or kairomonal attractant (Hedden et al., 1976; Wood et al., 1968). However, the enantiomeric composition of ipsenol has been determined only for *I. paraconfusus* which produces approx. 97% (*S*)-(−)-ipsenol (L.E. Browne, personal communication; Fish et al., 1979; Silverstein et al., 1966). The (*R*)-(+)-ipsenol enantiomer appears not to elicit receptor responses in any *Ips* species so far tested, i.e., both sexes of *I. paraconfusus* (Light and Birch, 1979b) and *I. typographus* (Hansen, 1983), or female *I. pini* (Mustaparta, unpublished data). Moreover, the apparent behavioral effects of ipsenol on all *Ips* species tested to date can be attributed to the (*S*)-(−)-ipsenol enantiomer alone (Birch et al., 1977a; Light and Birch, 1979b; Vité, 1978; Vité et al., 1976a). Thus, it appears that since (*R*)-(+)-ipsenol is not used by any congeneric competitor, there has been no selection pressure upon the gene pool to acquire and maintain a means of perceiving this compound by any *Ips* species.

It should be emphasized here that the mere identification within an isolated crude pheromone of the presence of a minor percentage of the antipodal enantiomer of a pheromonal component is not reason enough to assume reception, perception, or behavioral responsiveness by conspecifics to this minor antipodal product. In *Dendroctonus brevicomis* and *D. frontalis* 10% and 15%, respectively, of the frontalin produced is in the (1*R*, 5*S*)-(+)-configuration (Stewart et al., 1977), which in both species is nonattractive and noninterruptive (Payne, 1979; Payne et al., 1982; Silverstein, 1979; Wood et al., 1976) and in *D. frontalis* elicits significantly smaller EAG responses and fewer action potentials than the (1*S*, 5*R*)-(−)-enantiomer (Payne, 1979; Payne et al., 1982). This is further illustrated in the apparently total nonreception of the approx. 3% (*R*)-(+)-ipsenol present in the *I. paraconfusus* pheromone. Although the 3% of (*R*)-(+)-ipsenol is a very minor percentage of total ipsenol produced by *I. paraconfusus*, this "minor" percentage represents approx. 0.014 μg/bm, or a concentration 16 times greater than the estimated total ipsdienol present in the multicomponent pheromone.

Ipsdienol. In northern California two sympatric species, *I. paraconfusus* and *I. pini* Say, compete for the same host resource, ponderosa pine, and both utilize ipsdienol as an aggregation pheromone. Male *I. paraconfusus* produce

predominately (94–95%) the (*S*)-(+)-ipsdienol enantiomer (L.E. Browne, personal communication; Plummer et al., 1976; Silverstein et al., 1966; Stewart, 1975), while male California *I. pini* produce approx. 100% (*R*)-(−)-ipsdienol (Birch et al., 1980; Stewart, 1975). The response of each species to its natural pheromone is interrupted by either the natural pheromone of the other (Birch and Wood, 1975) or the enantiomer of ipsdienol predominantly present in the pheromone of the other species (Birch et al., 1980; Light and Birch, 1979b). A small percentage (between 2.2 and 5%) of the (*S*)-(+)-enantiomer can completely interrupt the normal pheromonal response to (*R*)-(−)-ipsdienol in California *I. pini* (Birch et al., 1980). Conversely, (*R*)-(−)-ipsdienol, when released at rates comparable to those produced by male *I. pini*, allomonally interrupts the attraction of both sexes of *I. paraconfusus* to its natural pheromone (Light and Birch, 1979b). Thus, for *I. paraconfusus*, (*S*)-(+)-ipsdienol functions as a component of its conspecific aggregation pheromone, while (*R*)-(−)-ipsdienol functions as an interspecific allomone, interrupting the aggregation of *I. paraconfusus* on resources colonized by its competitor, *I. pini*.

The significantly greater sensitivity of female *I. paraconfusus* antennae to (*S*)-(+)-ipsdienol than to (*R*)-(−)-ipsdienol as reported here (Figure 4a), is probably due to a greater number of acceptors for reception of the dominant pheromonal enantiomer. This conclusion is based on the suggestion of Payne (1975, 1979) that the EAG response maximum provides a relative measure of the total number of antennal acceptors for one given stimulus as compared to another. Further, this conclusion agrees with the results from single-cell recordings by Mustaparta et al. (1980), who found that of the 11 "ipsdienol specialized cells" recorded from female *I. paraconfusus*, nine were specifically sensitive to (*S*)-(+)-ipsdienol, but only two were specialized for reception of (*R*)-(−)-ipsdienol. They concluded that *I. paraconfusus* (as well as western *I. pini*) have "more cells sensitive to the enantiomer that acts as an aggregation pheromone than the opposite one that acts as an allomonal aggregation interruptant." However, their conclusion is based on only 11 recordings solely from female beetles. Conclusions about overall olfactory input to the brain based on such a small number of single-cell recordings from an unknown number of individuals and from sensilla of unknown morphological type are speculative. Suggestions of overall antennal sensitivity may, however, be made guardedly from EAG recordings because they are thought to summate a portion of the overall antennal response and therefore reflect a cross-section of the olfactory input to the brain (Boeckh et al., 1965; Kaissling, 1971). Similar conclusions about the sensitivity of populations of antennal receptors in female *I. pini* based on single-cell recordings (Mustaparta et al., 1977, 1979) were corroborated by EAG studies (Angst and Lanier, 1979).

In the present study using highly pure enantiomers, males were found to have a higher sensitivity for (*R*)-(−)-ipsdienol than pheromonal (*S*)-(+)-

ipsdienol (Figure 4B). Moreover, in comparison to results from a concurrent study, the sensitivity of male antennae to (*R*)-(−)-ipsdienol was far greater than the sensitivies of antennae of either sex to any of the other 16 individual semiochemicals tested (Light, 1983). The higher sensitivity (i.e., lower threshold, higher responsiveness, and steeper slope) to (*R*)-(−)-ipsdienol suggests possibly that the male antennae have a greater number of acceptors for, and/or acceptors with a higher affinity for, (*R*)-(−)-ipsdienol than for its (*S*)-(+) antipode. Acceptor affinity has been suggested as being proportional to the slope of the EAG dynamic response phase (Payne and Finn, 1977) and reversely proportional to the K_b and the threshold.

Since (*R*)-(−)-ipsdienol has been identified both as the principal aggregation pheromone of California *I. pini* (Birch et al., 1980; Stewart, 1975) and shown to interrupt *I. paraconfusus* aggregation (Light and Birch, 1979b); then the higher sensitivity of antennae of male *I. paraconfusus* to the (*R*)-(−)- than the (*S*)-(+)-enantiomer of ipsdienol appears to be a unique instance of an insect having a higher sensitivity for an interspecific allomonal interruptant than for its own pheromonal attractant. In addition, this differential sensitivity to the enantiomers of ipsdienol could possibly also represent a higher sensitivity by male antennae for a "minor pheromonal" rather than a major pheromonal component. The only evidence that (*R*)-(−)-ipsdienol might be a minor pheromonal component for *I. paraconfusus* is its presence in small amounts in the male-produced frass. But this presence appears to be as a biosynthetic by-product and not as an end-product because male *I. paraconfusus* convert myrcene to the predominant pheromonal component, (*S*)-(−)-ipsenol, through the intermediary precursor, (*R*)-(−)-ipsdienol (Fish et al., 1979), which is found only in residual amounts (approx. 4–5%) in the final pheromone-laden frass product (L.E. Browne, personal communication; Plummer et al., 1976; Stewart, 1975). Critical behavioral experiments have yet to be undertaken to determine whether the small percentage of (*R*)-(−)-ipsdienol, normally present within the pheromonal triplet of *I. paraconfusus*, enhances or facilitates conspecific aggregation. Thus at this time, (*R*)-(−)-ipsdienol cannot be classified as an aggregation pheromone of *I. paraconfusus*. Therefore, the minor presence of a compound that in other contexts elicits behavioral interruption, i.e., (*R*)-(−)-ipsdienol, within the multicomponent *I. paraconfusus* aggregation pheromone is suggested to be "compensated" for or "tolerated" by responding conspecifics due to the predominance of pheromonal attractant, the (*S*)-(+)-enantiomer of ipsdienol.

cis-*Verbenol*. Both male and female antennae appear to have a greater number of acceptors with a higher affinity for their pheromonal component (*S*)-*cis*-verbenol than for its antipode. The (*S*)- and (*R*)-*cis*-verbenol response curves parallel each other, with a rather constant shift of one order of magnitude less sensitivity for the (*R*)-*cis*-verbenol enantiomer, but the two response curves have different response maxima. This suggests that antennae

are truly sensitive to the less effective (*R*)-*cis*-verbenol enantiomer, and are not just responding to the approx. 10% impurity of the (*S*)-enantiomer present in the approx. 90% optically pure samples.

This higher sensitivity to the pheromonal (*S*)-enantiomer of *cis*-verbenol over its antipode agrees with the findings by Mustaparta (1979) based on the differential sensitivity of a limited number of *cis*-verbenol-specialized receptor neurons found on antennae of female *I. paraconfusus*. Electrophysiological evidence presented here from both sexes of *I. paraconfusus* also agrees with that from female *I. pini* (Mustaparta, 1979) and male and female *I. typographus* (Dickens, 1978, 1981; Hansen, 1983) in that antennae are responsive to both enantiomers, although they are more sensitive to (*S*)-*cis*-verbenol than its antipode.

I. paraconfusus is the only *Ips* species for which the pheromonal chirality of *cis*-verbenol has been determined (Silverstein et al., 1966). The aggregation of many *Ips* species is synergized by (*S*)-*cis*-verbenol or racemic *cis*-verbenol (Bakke, 1976, 1978; Bakke et al., 1977; Birch et al., 1977b; Byers and Wood, 1981; Silverstein, et al., 1966; Vité and Franke, 1976; Vité et al., 1976b,c; Wood et al., 1967, 1968), although no *Ips* species is known to produce or be differentially attracted to (*R*)-*cis*-verbenol (see Brand et al., 1979; Renwick et al., 1976; Silverstein, 1979; Vité, 1978).

Antipodal interruption by an enantiomer of *cis*-verbenol has been suggested only twice, and in each case, the interruption was either not significant (*I. acuminatus*, Bakke, 1978) or occurred only when the (*R*)-*cis*-verbenol enantiomer was delivered at rates 10 or 20 times more than that of the attractive (*S*)-*cis*-verbenol enantiomer (*I. calligraphus*, Vité et al., 1976b). However, Dickens (1981) found recently that (*R*)-*cis*-verbenol caused no inhibition of *I. typographus* aggregation, even when released at rates 10 and 20 times greater than those of the attractive (*S*)-*cis*-verbenol enantiomer.

Odor Discrimination and Information Coding. Based both on the evidence of high sensitivity and specificity (differential or cross-adaptation experiments, Light, in preparation), the evidence presented here supports the suggestion that the chiral components of the multicomponent aggregation pheromone of *I. paraconfusus* in particular, and *Ips* species in general, are perceived individually by means of "labeled lines" (Boeckh, 1977; Mountcastle, 1974) of received and transmitted neural information from receptors possessing specific acceptors for only one of the components (Dickens, 1979; Mustaparta, 1979; Payne, 1979). This proposed system of information coding is in agreement with single-cell recordings from individual receptor neurons of female *I. paraconfusus* that were classified into specialized populations of cells (labeled lines) based on their relative chemoreceptive affinity and efficacy to stimulation by the pheromonal components (Mustaparta, 1979). Thus, in *Ips*, the behavioral phenomenon of obligatory synergism of different components of the aggregation pheromone appears not to be due to peripheral

interaction of different compounds on the same receptors (Roelofs and Comeau, 1971) but on convergence of the axons of different labeled lines on interneurons in the central nervous system.

Relative Sensitivity to Pheromonal Components. Differential antennal sensitivity and specialization for the three components of the aggregation pheromone occurs in *I. paraconfusus*. Female antennae were 10–100 times more sensitive to (*S*)-(+)-ipsdienol than to (*S*)-(−)-ipsenol, and 1000 times more sensitive to (*S*)-(+)-ipsdienol than to (*S*)-*cis*-verbenol. The differences in male sensitivity to these compounds were not as great; males being 5–10 times more sensitive to (*S*)-(+)-ipsdienol than to (*S*)-(−)-ipsenol, and 100 times more sensitive to (*S*)-(+)-ipsdienol than to (*S*)-*cis*-verbenol.

A truer insight into how the multicomponent aggregation pheromone of *I. paraconfusus* might be perceived is obtained from an analysis of antennal sensitivity vs. the estimated natural concentration levels of these components in the pheromonal cold-trap condensate. (*S*)-(−)-Ipsenol is the predominant pheromone component present in both frass (Wood et al., 1967) and cold-trap condensate (L.E. Browne, personal communication), with (*S*)-(+)-ipsdienol approximately 10 times less and (*S*)-*cis*-verbenol 50 times less prevalent than (*S*)-(−)-ipsenol. At these estimated natural levels, when a beetle antenna is stimulated by the pheromone condensate, the relatively higher concentration of (*S*)-(−)-ipsenol present elicits a nearly equal response (i.e., a similar percentage of the magnitude of the DC potential deflection) as that of the less prevalent (*S*)-(+)-ipsdienol present (Figure 7A and 7B). Thus, although (*S*)-(+)-ipsdienol was found to be the more potent antennal stimulant, at the estimated natural levels of occurrence (*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol contribute to a nearly equal extent to the overall antennal sensation elicited by the natural pheromone bouquet.

(*S*)-*cis*-Verbenol, at its estimated natural level, contributes significantly less to the overall antennal responsiveness to cold-trap condensate than does either ipsdienol or ipsenol. This suggests that if (*S*)-*cis*-verbenol does act as a synergistic component of the aggregation pheromone of *I. paraconfusus*, as demonstrated by both field (Wood et al., 1967, 1968) and laboratory bioassays (Byers and Wood, 1981; Silverstein et al., 1966), then its environmental zone of perception or active space, within which it has a behavioral influence on species-specific aggregation, is of a short-range nature, possibly modulating the long-range attraction to the other two more predominant or "primary" components (see Seabrook, 1977, 1978).

Orientation and taxis are orchestrated in part by the animal's sensory capabilities and the nature, timing, and placement of stimuli. Odor molecules may instigate a behavioral orientation response, but only within a "sphere of influence" in time and space dictated by the insect's ability to perceive both the presence of the odor and changes in the concentration of the odor in the environment. The ability to perceive an odor molecule is a function of the

threshold at which the odor is discriminated as being present and/or different from other stimuli, and by the differential responsiveness to increasing concentrations of the odor. A low threshold for a particular odor molecule would increase the ability of the insect to perceive the compound in low concentrations at greater distances from its source (see review by Seabrook, 1977, 1978). Differential sensitivity to a compound over a wide range of concentrations allows the insect to perceive the gross and subtle changes in concentration of an odor as it is carried by the wind from its source and aids in the orientation of the insect to the odor source over distance (Seabrook, 1977, 1978).

Thus, because of low thresholds, steep slopes to response curves, and high maximal sensitivity, both the pheromonal components (*S*)-(+)-ipsdienol and (*S*)-(−)-ipsenol are capable of being perceived and oriented to from a distance by *I. paraconfusus*. However, the lower evaporation rate, higher thresholds, shallower response slopes, and low sensitivity maxima suggest that (*S*)-*cis*-verbenol might synergize and/or modulate short-distance orientation behavior.

Such physiologically based suggestions on the environmental range of influence of pheromonal components on flight initiation and inflight orientation of bark beetles need to be corroborated through field orientation and laboratory flight-tunnel studies using highly pure pheromonal enantiomers.

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SENSITIVITY OF ANTENNAE OF MALE AND FEMALE *Ips paraconfusus* (COLEOPTERA: SCOLYTIDAE) TO ITS PHEROMONE AND OTHER BEHAVIOR-MODIFYING CHEMICALS

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Abstract—The antennal sensitivities of both male and female *Ips paraconfusus* were found generally to be greatest for conspecific aggregation pheromones (ipsdienol, ipsenol); intermediate for an additional conspecific pheromone (*cis*-verbenol), an aggregation synergist (2-phenylethanol), and pheromones/allomones of sympatric species (trans-verbenol, verbenone, and frontalin); and lowest for both host terpenes (alpha-pinene and myrcene) and other bark beetle-produced odorants (*exo*-brevicomin and linalool). Of the enantiomeric compounds tested, antennae of both sexes did not differ in sensitivity between the *trans*-verbenol enantiomers at low dosage levels; but at higher dosages, the conspecific-produced enantiomer, (1*R*,4*S*,5*R*)-(+)-*trans*-verbenol, elicited larger mean EAG responses than its antipode, (1*S*,4*R*,5*S*)-(−)-trans-verbenol. At the mid-dosage range, female antennae tended to be slightly more responsive to (*S*)-(−)-verbenone than to (*R*)-(+)-verbenone, while male antennae were equally responsive to stimulations by either verbenone enantiomer. In field bioassays there was a large and significant reduction in trap catches of *I. paraconfusus* on traps where the (*S*)-(−)- or (*R*)-(+)-enantiomers of verbenone were evaporated beside logs containing boring conspecific males. Only when the (*S*)-(−)-enantiomer of verbenone was evaporated beside logs containing boring males did the sex ratio of *I. paraconfusus* trapped shift from female-dominated to male-dominated attraction. Thus both physiological and behavioral data suggest a differential chiral sensitivity of female beetles for the verbenone enantiomers. The relative sensitivities between different chiral compounds derived from one or the other of the

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common precursoral host terpenes, (*S*)-(−)- and (*R*)-(+)alpha-pinene or myrcene, are discussed.

Key Words—Coleoptera, Scolytidae, bark beetle, *Ips paraconfusus*, pheromone, enantiomer, electrophysiology, electroantennogram, interruption, allomone.

INTRODUCTION

Host-tree selection, mass attack, and establishment phases of the colonization behavior of bark beetles (Coleoptera: Scolytidae) are dependent upon the perception of various behavior-modifying chemicals (BMCs). Besides the key role of aggregation pheromones in colonization behavior, other BMCs have been demonstrated to be, or implicated as, olfactory cues that are required for or modify: host-tree selection, aggregative mass attack, intraspecific and interspecific resource space and food partitioning, and interspecific breeding isolation (Birch, 1978; Bordon, 1974, 1977, 1982; Lanier and Burkholder, 1974; Lanier and Wood, 1975; Vité and Francke, 1976; Wood, 1970, 1972, 1982). Although much is known about how these compounds modify behavior, relatively little is known about the relative degree of chemoreceptive sensitivity to chiral and achiral bark beetle BMCs (for review see Payne, 1979).

During tree colonization, pine bark beetles are in intimate contact with many monoterpene hydrocarbons that are present in the oleoresin of the host tree's phloem and xylem tissues. Tree monterpenes have been suggested as either long-range "primary attractants" (Pearson, 1931, see Moeck et al., 1981; Wood, 1972) or possibly "chemostimulants" and "feeding-stimulants" encountered in phloem tissue (Elkinton and Wood, 1980; Wood, 1982) that are utilized by "pioneer beetles" to select a susceptible host for aggregative attack. Furthermore, in many cases, tree terpenes are the biosynthetic precursors of bark beetle pheromones.

It has been demonstrated that volatile compounds, presumably associated with the boring activity in ponderosa pine of either male *Ips pini* (Say) (Birch and Wood, 1975) or female *Dendroctonus brevicomis* Le Conte (Byers and Wood, 1980), will interrupt the attraction of *Ips paraconfusus* Lanier to sources of its conspecific aggregation pheromone. The compounds, linalool, frontalin, *exo*-brevicomin, *trans*-verbenol, and verbenone, have been suggested as either the putative allomones responsible for interspecific interruption between these sympatric resource competitors or as BMCs that might enhance the interspecific perceptual recognition of the presence of a resource competitor.

To gain a better understanding of the olfactory perception and and the potential ecological importance of both intra- and interspecific BMCs, the

summated receptor potential responses elicited by these various chiral and achiral BMCs were recorded by means of the electroantennogram technique from antennae of both male and female *I. paraconfusus*. The BMCs tested in this study include: (1) host-tree monoterpenes (myrcene and (*R*)-(+)-*alpha*-pinene), (2) a synergist of the aggregation pheromone (2-phenylethanol), and (3) both pheromones (frontalin, *exo*-brevicomin, and enantiomers of *trans*-verbenol) and allomones (enantiomers of verbenone) produced by sympatric species that attack the same host tree, ponderosa pine.

METHODS AND MATERIALS

Source of Beetles. Naturally infested logs of ponderosa pine were collected in the McCloud Flats region of the Shasta-Trinity National Forest (Siskiyou County, California). Adult *I. paraconfusus* beetles were collected daily as they emerged from these logs in the laboratory, separated as to sex, and stored at 4° C on strips of ponderosa pine phloem in which they tunneled and fed. A day prior to their use, they were removed from the phloem and placed on moist paper towels. Those that possessed both antennae and legs, and were active when warmed to room temperature, were used in electrophysiological or field experiments.

Sources and Purities of Behavior-Modifying Chemicals. The following BMCs (Figure 1) were purchased from Borregaard Industries Limited (Sarpsborg, Norway) at given chemical and optical purities: racemic ipsdienol (93.8% purity), racemic ipsenol (95% purity), both the (1*S*, 4*S*, 5*S*)- and (1*R*,4*R*,5*R*)-*cis*-verbenol enantiomers (99% purity, optical purity 90% minimum), both the (1*S*,4*R*,5*S*)-(−)- and (1*R*,4*S*,5*R*)-(+)-*trans*-verbenol enantiomers (95% purity, optical purity 90% min.), and both the (1*S*,5*S*)-(−)- and (1*R*,5*R*)-(+)-verbenone enantiomers (99.5% purity, optical purity 90% min.). Racemic linalool (97.7% purity), 2-phenylethanol (99.8% purity), racemic *exo*-brevicomin (98.8% purity), and racemic frontalin (99.4% purity) were all purchased from Chemical Samples Co. In addition, myrcene (95% purity) was obtained from Aldrich Chemical Co. and (1*R*,5*R*)-(+)-*alpha*-pinene (95% purity, optical purity 95%) from K&K Laboratories. The chemical purity of all compounds, as cited by sources, was corroborated by GLC analysis.

Electrophysiological Recording Techniques. The neural responses of male and female *I. paraconfusus* to the various BMCs were quantified by measuring the amplitudes (in millivolts) of the elicited summated receptor potentials of electroantennograms (EAGs) from intact antennae (Schneider, 1957; Boeckh et al., 1965). Stimulation, recording, and analysis techniques used in this study were the same as those described earlier (Light and Birch, 1979a; Light, 1983). A pentane control and a standard concentration of

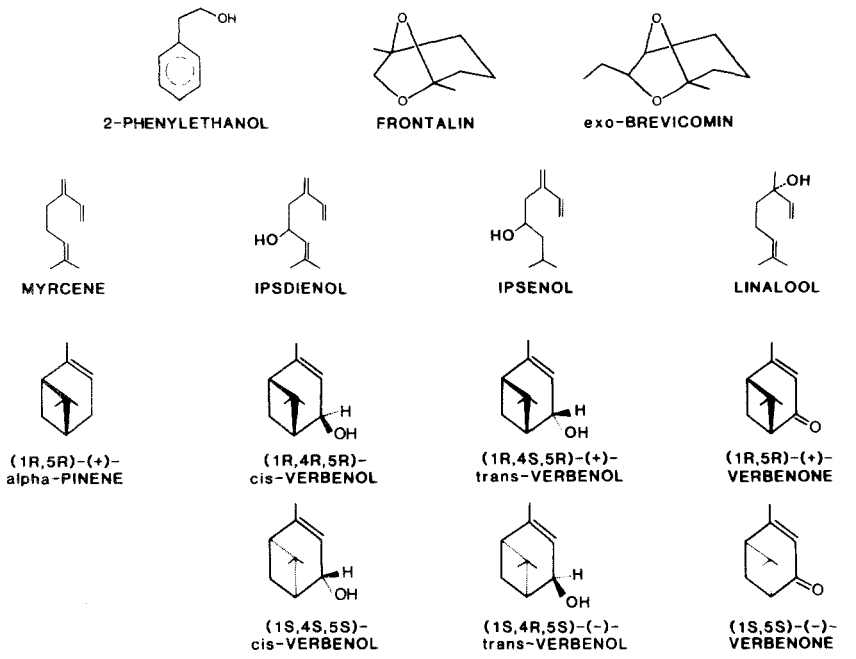

FIG. 1. Structural and enantiomeric configurations of the behavior-modifying chemicals of Scolytidae that were tested in this study.

natural aggregation pheromone of *I. paraconfusus* were interspersed between each stimulation by a serial dilution in pentane of a particular BMC. The natural aggregation pheromone of *I. paraconfusus* was obtained by condensing the volatile compounds produced by male beetles (the pheromone-producing sex) boring in ponderosa pine, removing the water, and making a concentrated solution in pentane (Browne et al., 1974). The "standard cold-trap concentration" represented the volatiles produced by a single beetle boring for a period of 3.42 min. All responses to the racemic and enantiomeric BMCs were expressed as a percentage of the response to the cold-trapped standard. Mean EAG response magnitude derived from replications upon 5–10 individual beetles were compared by the t test statistic and assigned significance at $P < 0.05$ level.

Field Experiments. The ability of the enantiomers of verbenone to interrupt or inhibit the aggregation response of *I. paraconfusus* to sources of its natural aggregation pheromone was tested at McCloud Flats, Siskiyou County, California, following established procedures (Birch and Wood, 1975; Birch and Light, 1977; Birch et al., 1977, 1980; Light and Birch, 1979b). Attractive *I. paraconfusus* bolts were produced by introducing 30 male beetles into predrilled holes in small ponderosa pine logs (30 × 15 cm). Control logs

contained no beetles. Attracted wild beetles were prevented from penetrating the logs by wrapping each log in fine metal screen. The treatment logs were placed inside cylindrical wire-mesh traps coated with Stikem Special® and elevated on pipe standards 1 m above ground level. Each enantiomer of verbenone was evaporated neat from a small open glass vial (5 mm ID × 35 mm) suspended upright inside an inverted 35-mm aluminum film canister with a perforated lid. A treatment consisted of two canisters containing the same enantiomer hung on opposite sides of a trap. The rates of evaporation for each enantiomer were approximately 2 mg/day from each vial, as measured by weight loss.

Treatments were arranged in a line about 30 m apart in forested areas where beetles were known to be flying, as determined by the catch at male-infested bolts. The verbenone enantiomeric treatments were compared with male-infested bolts as standards. Beetles were picked from traps twice each day, and the traps were then rotated to other randomized positions. In this design the number of beetles caught on each trap during the same time period constituted one replication of the test. To minimize the effects of positional variation on trap catches, treatments were relocated between replications so that no treatment appeared twice in the same position. After the field tests, the treatment logs were returned to the laboratory, frozen, and then dissected to determine the percentage of actively boring males and the average length of each gallery. Trap catches were analyzed statistically using Wilcoxon's sign rank test for matched pairs, while the sex ratios (♂ : ♀) of the treatment catches were analyzed by the Chi square test (Snedecor and Cochran, 1967). Significance level as assigned at $P < 0.05$.

RESULTS

Antennal Sensitivity to BMCs

2-Phenylethanol. Female and male antennae did not differ significantly in sensitivity to the purative aggregation synergist, 2-phenylethanol (Figure 2A; Table 1). Although 2-pheylethanol tended to elicit greater mean EAG responses in female than male antennae, the variability of the mean responses of females was quite high.

Linalool. Both male and female antennae had low sensitivity to racemic linalool and were not significantly different in their responses (Figure 2B; Table 1).

Frontalin and exo-*Brevicomin.* Over a wide concentration range, the *Dendroctonus* pheromones frontalin and *exo*-brevicomin evoked EAG responses of similar magnitude. But at higher concentration levels both sexes were significantly more sensitive to frontalin than *exo*-brevicomin (Figures 3A and 3B; Table 1).

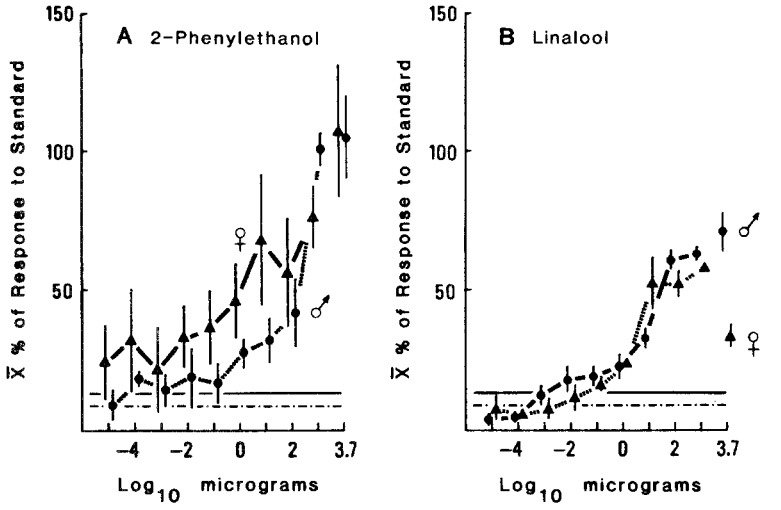

FIG. 2. Dose–EAG response curves of female (▲) and male (●) *Ips paraconfusus* to 2-phenylethanol (A) and to racemic linalool (B). Responses are plotted as mean percentages of the response to a standard of 3.42 beetle-minutes of the cold-trapped condensate. Points are means of recordings from 5–10 beetles; vertical lines are ±SEM. For clarity, points were positioned offset from the concentration markers. Horizontal lines show response thresholds for males (solid) and females (dash-dot), above which responses are significantly greater than to controls. The dose–response curves are not continuous to the highest concentration points because those points are means of independent samples from different individuals. (Log_{10} 3.7 = 5000.0 μg dose).

trans-Verbenol. At low concentration levels, the enantiomers of *trans*-verbenol elicited antennal responses that were not significantly different for either sex (Figures 4A and 4B; Table 1). Although female antennae were highly sensitive to both *trans*-verbenol enantiomers (as evident from their low 1×10^{-5} μg thresholds), females tended to be slightly, but not significantly, more sensitive to the (1*S*,4*R*,5*S*)-(−)-enantiomer than to its antipode at stimulus concentrations lower than 1×10^{-1} μg, while they were more sensitive to the (1*R*,4*S*,5*R*)-(+)-enantiomer at the highest concentration level (Figure 4A). Similarly, male antennae were more sensitive to (1*R*,4*S*,5*R*)-(+)-*trans*-verbenol than the (−)-enantiomer at concentrations equal to or greater than 10 μg (Figure 4B).

Verbenone. At the mid-concentration range (0.01–1 μg), female EAG responses tended to be slightly greater to (*S*)-(−)-verbenone than to (*R*)-(+)-verbenone, although the differences were not significant (Figure 5A), while the slope of the response curve to (*R*)-(+)-verbenone was twice that of the

TABLE 1. SENSITIVITY PARAMETERS[a] DERIVED FROM DOSE VS. MEAN EAG RESPONSE CURVES OF MALE AND FEMALE *Ips paraconfusus*

Compound	Sex	Threshold	K_b		Response maximum		Dynamic response phase
		Log_{10} conc. (μg)	Log_{10} conc. (μg)	Response to Standard[b] (%)	Log_{10} conc. (μg)	Response to Standard[b] (%)	Slope (% Resp./ Log_{10} Conc. (μg) Step)
2-Phenylethanol	M	−4.6	2.3	56.8	3.0	100.7	20.0
	F	−5.0	2.1	57.9	3.7	107.4	12.7
Racemic	M	−2.9	1.2	37.0	2.0	61.0	9.9
linalool	F	−2.8	0.2	30.4	1.0	52.4	13.0
Racemic	M	−3.9	1.8	67.9	3.0	122.9	26.3
frontalin	F	−3.5	1.3	49.7	3.7	91.0	14.6
Racemic	M	−1.6	0.1	33.3	2.0	53.6	12.2
exo-brevicomin	F	−3.8	−0.1	32.4	2.0	56.3	13.2
(1*R*, 4*S*, 5*R*)-(+)-	M	−1.0	0.7	71.0	2.0	129.1	41.2
trans -verbenol	F	−5.0	0.5	55.9	2.0	103.3	29.8
(1*S*, 4*R*, 5*S*)-(−)-	M	−1.5	1.0	59.2	2.0	105.4	29.8
trans-verbenol	F	−5.0	0.1	41.9	1.0	75.4	26.5
(1*R*, 5*R*)-(+)-	M	−2.4	1.3	72.7	3.7	132.5	45.8
verbenone	F	−2.2	0.5	41.4	2.0	74.3	22.7
(1*S*, 5*S*)-(−)-	M	−3.2	1.2	69.4	3.7	125.8	28.4
verbenone	F	−2.8	0.5	41.8	3.7	75.2	11.2
Myrcene	M	−1.9	1.1	54.4	2.0	95.8	35.8
	F	−5.0	1.1	52.0	2.0	95.5	33.8
(1*R*, 5*R*)-(+)-	M	−3.4	0.9	52.4	2.0	91.9	23.8
alpha-pinene	F	−3.9	0.7	50.9	2.0	93.4	24.7

[a] As defined in Methods and Materials (Light, 1983).
[b] Percent response as compared with response to the standard, 3.42 beetle-min cold trap condensate of male *I. paraconfusus* boring in ponderosa pine.

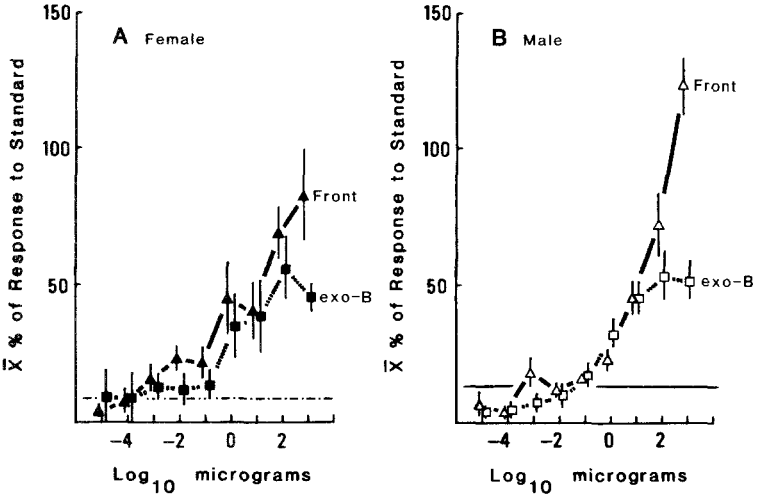

FIG. 3. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to racemic frontalin (Front: ▲, △), and racemic *exo*- brevicomin, (exo-B: ■, □) (see Figure 2).

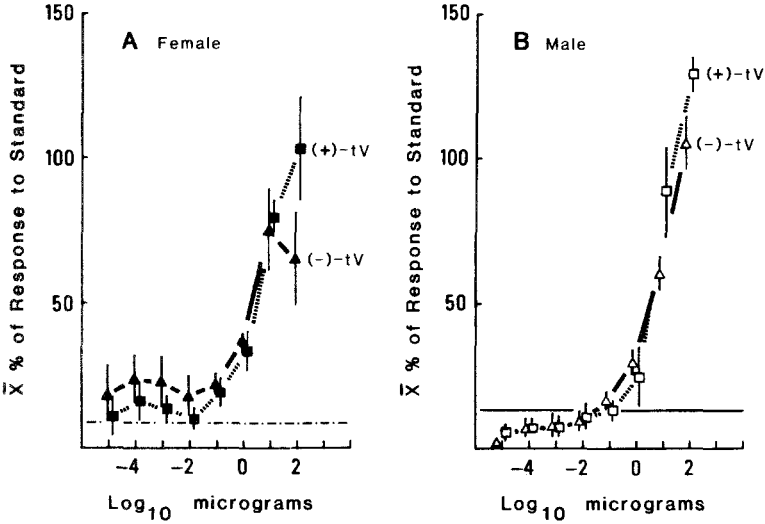

FIG. 4. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to (1*R*, 4*S*, 5*R*)-(+)-*trans*-verbenol, [(+)-tV: ■, □], and (1*S*, 4*R*, 5*S*)-(−)-*trans*-verbenol, [(−)-tV: ▲, △] (see Figure 2).

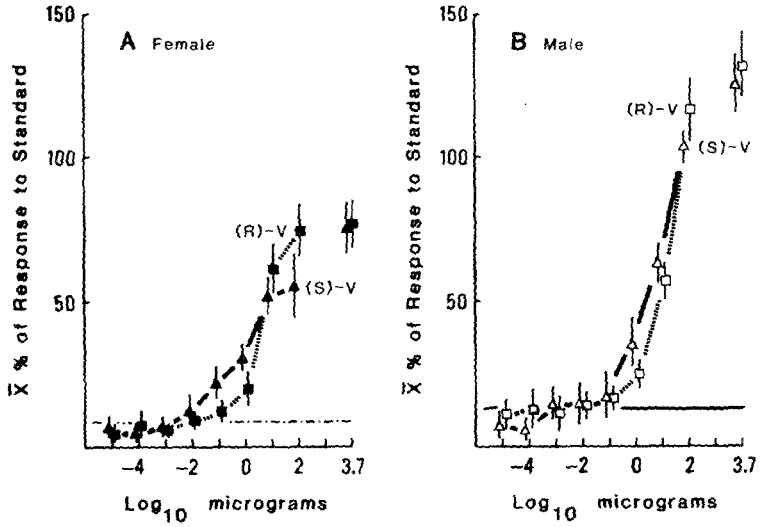

FIG. 5. Dose–EAG response curve of female (A) and male (B) *Ips paraconfusus* to (1*R*, 5*R*)-(−)-verbenone, [(R)-V: ■, □], and (1*S*, 5*S*)-(−)-verbenone, [(S)-V: ▲ , △] (see Figure 2).

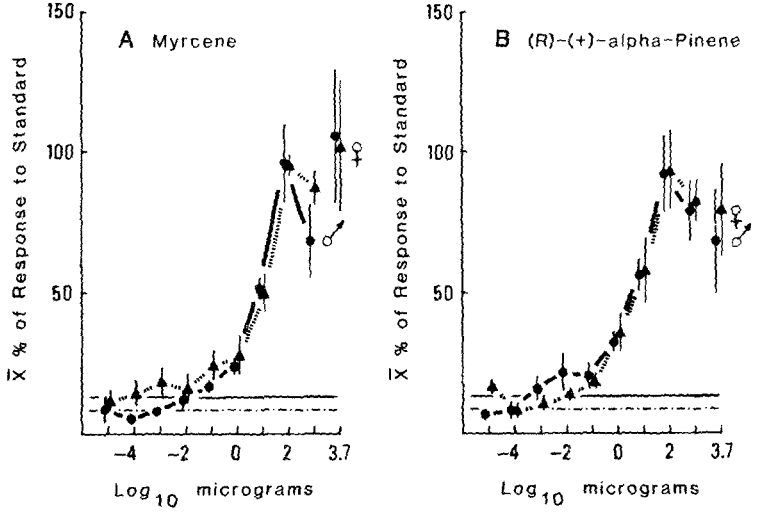

FIG. 6. Dose–EAG response curves of female (▲) and male (●) *Ips paraconfusus* to the BMC precursors myrcene (A) and (1*R*, 5*R*)-(+)-*alpha*-pinene (B) (see Figure 2).

(*S*)-(−)-enantiomer (Table 1). Male antennae were equally responsive to stimulation by either verbenone enantiomer (Figure 5B; Table 1).

Myrcene and alpha-Pinene. Female antennae were significantly more sensitive than male antennae to the monoterpene myrcene at low concentrations (1×10^{-4} and 1×10^{-3} μg), but at higher stimulus concentrations their responses did not differ in EAG amplitude (Figure 6A; Table 1). (*R*)-(+)-*alpha*-Pinene elicited equal response magnitudes in male and female antennae (Figure 6B; Table 1). Further, the antennal responses to myrcene and (*R*)-(+)-*alpha*-pinene were not significantly different for either sex (Table 1).

Relative Antennal Sensitivities

When BMCs derived from the same biosynthetic precursor are compared, there is evident a large variance in selective sensitivities than was generally found between the enantiomers of a compound. Of the racemic forms of the myrcene-derived compounds, the sensitives of both female and male antennae were highest for the pheromonal components, ipsdienol and ipsenol, intermediate for myrcene, and lowest for linalool (Figures 7A and 7B). Of the compounds presumably derived from (1*R*,5*R*)-(+)-*alpha*-pinene, female and male antennal sensitivity was generally greatest for the *I. paraconfusus*-produced compound (1*R*,4*S*,5*R*)-(+)-*trans*-verbenol, intermediate for (*R*)-(+)-*alpha*-pinene and (1*R*,5*R*)-(+)-verbenone, and least for (*R*)-*cis*-verbenol (Figures 8A and 8B). Similarly, of the three compounds

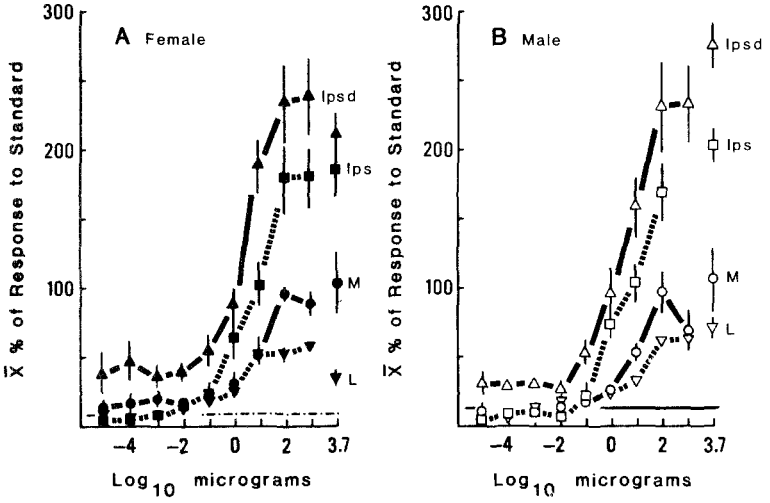

FIG. 7. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to the BMCs derived from myrcene (M: ●, ○); racemic ipsdienol (Ipsd: ▲, △); racemic ipsenol (Ips: ■, □); and racemic linalool (L: ▼, ▽) (see Figure 2).

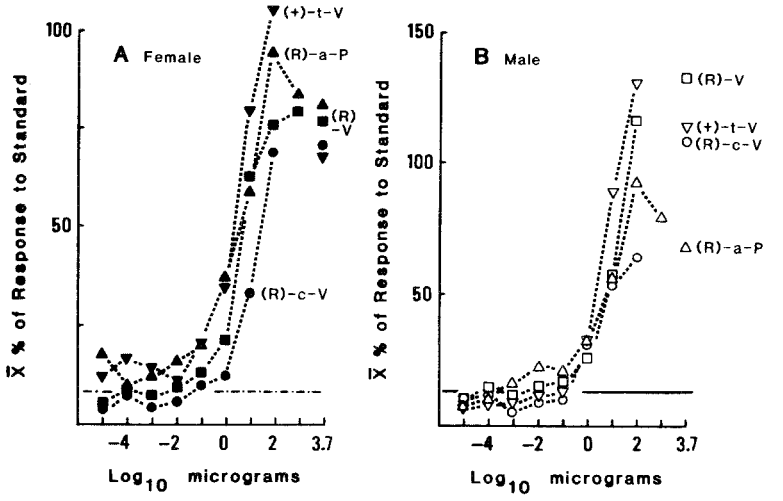

FIG. 8. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to the enentiomeric BMCs derived from (1*R*, 5*R*)-(+)-*alpha*-pinene [(R)-a-P: ▲, △]: (1*R*, 4*S*, 5*R*)-(+)-*trans*-verbenol [(+)-t-V: ▼, ▽]; (1*R*, 5*R*)-(+)-verbenone [(R)-V: ■, □]; and (1*R*, 4*R*, 5*R*)-*cis*-verbenol [(R)-c-V: ●, ○] (see Figure 2). For clarity, no lines delineating the SEM were plotted (see Figures 4, 5, and 6).

derived from (1*S*,5*S*)-(−)-*alpha*-pinene, the antennal sensitivity of females was greatest for (1*S*,4*R*,5*S*)-(−)-*trans*-verbenol, while intermediate for (*S*)-(−)-verbenone and (*S*)-*cis*-verbenol (Figure 9A). However, at the highest concentrations tested, (*S*)-*cis*-verbenol elicited significantly greater EAG responses. While male antennae were equally sensitive to (*S*)-*cis*-verbenol, (1*S*,4*R*,5*S*)-(−)-*trans*-verbenol, and (*S*)-(−)-verbenone, they were less sensitive to (*S*)-*cis*-verbenol at the highest stimulation level below saturation (Figure 9B).

Field Interruption Bioassay

In field bioassays there was a large and significant reduction in trap catches of *I. paraconfusus* on traps where the (*S*)-(−)- or (*R*)-(+)-enantiomers of verbenone were evaporated beside logs containing boring conspecific males (Table 2). The (*R*)-(+)-verbenone treatment did not significantly affect the sex ratio of beetles trapped. But when (*S*)-(−)-verbenone was evaporated beside logs containing boring males, the sex ratio of *I. paraconfusus* beetles trapped was significantly shifted away from the usual female-dominated attraction to a reversed ratio of male-dominated attraction (Table 2). Because of the low numbers of beetles caught on the verbenone treatment traps, the relevance of such significant sex ratio

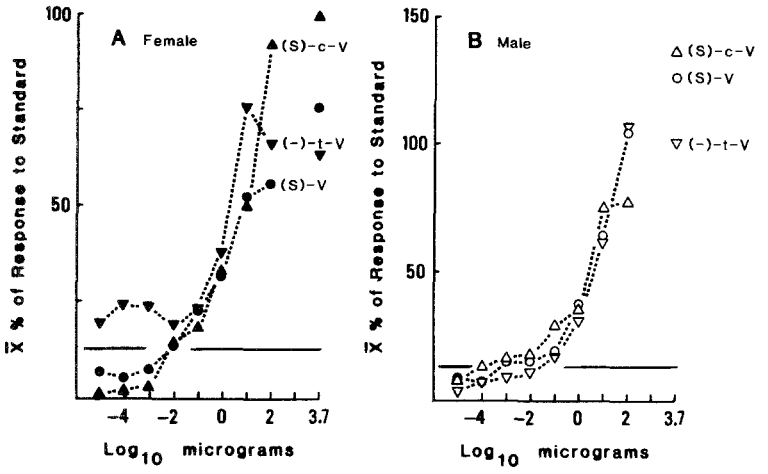

FIG. 9. Dose–EAG response curves of female (A) and male (B) *Ips paraconfusus* to the enantiomeric BMCs derived from (1*S*, 5*S*)-(−)-*alpha*-pinene: (1*S*, 4*S*, 5*S*)-*cis*-verbenol [(S)-c-V: ▲, △]; (1*S*, 4*R*, 5*S*)-(−)-*trans*-verbenol [(−)-t-V: ▼, ▽]; and (1*S*, 5*S*)-(−)-verbenone [(S)-V: ●, ○] (see Figure 2). For clarity, no lines delineating the SEM were plotted (see Figures 4, 5, and 6).

differences must be viewed cautiously. No other endemic species were caught with any of the treatments.

There were no differences in the percentage of actively boring beetles in each bolt (average 87%) nor in gallery length (3.5–5.0 cm) between bolts with beetles alone, and bolts containing beetles plus the enantiomeric treatments. Thus, it is more likely that the response interruption caused by either

TABLE 2. FIELD RESPONSE OF *I. paraconfusus* TO MALES BORING IN PONDEROSA PINE LOG ALONE AND COMBINED WITH (*S*)-(−)-VERBENONE OR (*R*)-(+)-VERBENONE (MCCLOUD FLATS, CALIFORNIA, JULY 11–16, 1978)

Treatment	Mean trapped	Range	Replications	Sex ratio (male–female)
30 male *I. paraconfusus*	46.4a[a]	(7–146)	10	1:2.39
30 male *I. paraconfusus* + (*S*)-(−)-verbenone	1.8b	(0– 4)	5	1:0.13[b]
30 male *I. paraconfusus* + (*R*)-(+)-verbenone	1.4b	(0– 3)	5	1:1.33
Control log	0.0c	—	10	—

[a] Means followed by different letters are significantly different from the other responses at $P < 0.05$ (Wilcoxon sign rank tests).

[b] Significantly different from all other sex ratios at $P < 0.05$ (chi-square test).

enantiomer of verbenone was a result of discrimination by the responding beetles and not to inhibition of pheromone production, as measured by the boring activity of males.

DISCUSSION

Antennal Sensitivity

Generally, the antennal sensitivity of *Ips paraconfusus* was found to be greatest for conspecific attractant pheromones, intermediate for both pheromones and interruptant allomones of sympatric species, and least for host terpenes, as was similarly found for male and female *D. frontalis* (Dickens and Payne, 1977, 1978; Payne, 1970, 1971, 1975), male and female *D. brevicomis* (Payne, 1975), male and female *I. typographus* (Hansen, 1983), female I. *pini* (Angst and Lanier, 1979; Mustaparta et al., 1977, 1979, 1980; Mustaparta, 1979), and female *I. paraconfusus* (Mustaparta, 1979; Mustaparta et al., 1980). In studies on female *Ips* antennae, Mustaparta (1979; Mustaparta et al., 1979, 1980) found predominantly "specialized" receptor neurons with acceptors highly sensitive and specific for a particular BMC. Thus in *Ips* species differential EAG responsiveness should reflect the relative numbers and/or summated reactivities (mV/acceptor–odor molecule activated complex) of different populations of "specialist" acceptors and receptors, and not relative affinity and reactivity of the different compounds with the same acceptor population, as was postulated for *D. frontalis* (Dickens, 1979; Dickens and Payne, 1977, 1978; Payne, 1979; Payne and Dickens, 1976).

2-Phenylethanol. Renwick et al. (1976b) isolated 2-phenylethanol from hindguts of male, but not female, *I. paraconfusus*. The addition of 2-phenylethanol to a natural source of the aggregation pheromone of *I. paraconfusus* enhanced the catch of the responding beetles by 47%, without significantly affecting the sex ratio of responding beetles (Renwick et al., 1976b). Because 2-phenylethanol is produced during the stage of greatest attraction to beetle frass and has an enhancement effect, Renwick et al. (1976b) suggested that "2-phenylethanol plays some role in aggregation of *I. paraconfusus*."

Although Renwick et al. (1976b) found that 2-phenylethanol did not change the sex ratio of beetles responding to aggregation pheromone, in the present electrophysiological study females tended to be more sensitive than males to 2-phenylethanol, although not significantly so. Both sexes had very low thresholds (between 1×10^{-4} and 1×10^{-5} μg, Table 1) for perception of 2-phenylethanol, comparable to those for the potent pheromone ipsdienol (Light and Birch, 1982), indicating that both sexes possess the capability to sense this compound from a distance. However, the slope of the dose–response curve for each sex was much more gradual than those for the aggregation pheromones ipsdienol and ipsenol (Figures 2A, 7A, and 7B),

suggesting that it is probably not an adequate olfactory cue for chemo- or anemotaxis where perception of subtle and abrupt changes in odor concentrations are thought to be required (Seabrook, 1977, 1978).

Linalool. Linalool has been isolated from volatiles produced by female *I. paraconfusus* and from frass produced by male *I. pini* (Young et al., 1973a), although the enantiomeric configuration of natural linalool has not been determined. Racemic linalool did not significantly enhance or interrupt the natural attractiveness of *I. paraconfusus* to male-infested logs (Birch and Wood, 1975; Birch and Light, 1977).

Of all compounds tested here, antennae were least sensitive to stimulation by racemic linalool, which corroborates the inactivity of this compound in the field (Birch and Wood, 1975; Birch and Light, 1977). In a similar EAG study, Angst and Lanier (1979) found that female *I. pini* antennae had a very low sensitivity for linalool. Further, in single-cell recordings on sensilla of unknown morphology from female *I. pini* and *I. paraconfusus* antennal clubs, Mustaparta (1979) found a "small number" of cells "moderately activated" but not seemingly "specialized" for linalool.

Frontalin and exo-*Brevicomin.* The bicyclic ketals, frontalin and *exo*-brevicomin, are produced by many *Dendroctonus* species but are not produced by any *Ips* species examined so far (Silverstein and Young, 1976; Wood, 1982). The aggregation pheromone of the sympatric and resource competitive species, *D. brevicomis*, has been identified as a synergistic, multicomponent mixture of *exo*-brevicomin (Silverstein et al., 1968), frontalin (Kinzer et al., 1969), and the tree terpene, myrcene (Bedard et al., 1969).

The greater maximum responsiveness to (±)-frontalin than (±)-*exo*-brevicomin reported here for both *I. paraconfusus* sexes maybe indicative of a greater number of acceptors present for reception of (±)-frontalin. This conclusion is based on the suggestion of Payne (1975, 1979) that the EAG response maximum provides a relative measure of the total number of antennal acceptors for one given stimulus as compared to another. The keen reception of frontalin, a compound produced only by *Dendroctonus* species, suggests an adaptation in *I. paraconfusus* for the perception of intergeneric resource competitors, although neither *exo*-brevicomin nor frontalin interrupts the aggregation of *I. paraconfusus*. Curiously, both male and female *D. brevicomis* were found to be more sensitive to (±)-*exo*-brevicomin than to (±)-frontalin (Payne, 1975).

Trans-Verbenol. trans-Verbenol has been identified as a natural product present in hindguts or frass of numerous *Ips* species, including *I. paraconfusus* (Silverstein and Young, 1976; Vité et al., 1972). In only one *Ips* species has *trans*-verbenol been suggested to modify the behavior of beetles, that being the European species, *I. typographus*, in which *trans*-verbenol has been inconclusively suggested as a pheromone that synergizes aggregation behavior (Bakke, 1976).

The production of *trans*-verbenol appears to be nearly ubiquitous in *Dendroctonus* species, having been identified as an aggregation pheromone in the sympatric and resource-competitive species, *D. ponderosae* (Pitman et al., 1968; Edson, 1978), as an aggregation synergist in *D. frontalis* and *D. pseudotsugae* (Pitman and Vité, 1970), and as an aggregation interruptant in *D. brevicomis* (Wood, 1972; Bedard et al., 1980). The chirality of natural *trans*-verbenol is virtually unknown, having been determined once for *D. frontalis* as a 60% (+) and 40% (−) mixture of enantiomers (Plummer et al., 1976) and suggested as approximately 100% (1*R*,4*S*,5*R*)-(+)-*trans*-verbenol for *I. paraconfusus* (Renwick et al. 1976a). In neither case has behavioral specificity for the enantiomers been determined.

Based on the greater maximum responsiveness, both male and female antennae appear to posses a larger number of acceptors and/or receptors specialized for the reception of the *I. paraconfusus* produced (1*R*,4*S*,5*R*)-(+)-enantiomer of *trans*-verbenol than its antipode. However, although female antennae have very low response thresholds for either *trans*-verbenol enantiomer, the higher responsiveness to the (1*S*,4*R*,5*S*)-(−)-enantiomer at lower concentrations could be suggestive of a higher acceptor affinity for the (−)-enantiomer than the (+)-enantiomer. There is presently no evidence that *trans*-verbenol either enhances or interrupts aggregation of any North American *Ips* species. Thus the adaptiveness of an *Ips* species being receptive to the enantiomers of *trans*-verbenol is still speculative. Since many *Ips* and *Dendroctonus* species produce *trans*-verbenol and female *I. paraconfusus* are keenly sensitive to the (−)-enantiomer that is not produced by conspecifics, then, as with other BMCs, the perception of *trans*-verbenol might increase the ability of *I. paraconfusus* to "recognize" the presence of transspecifics, including potential resource competitors, and/or "distinguish" which species is present.

Verbenone. Verbenone is a common product of many *Dendroctonus* species where it appears to function as an "epideictic" pheromone (an intraspecific regulator of attack density on a limited resource; Prokopy, 1981) interrupting conspecific aggregation (Bedard et al., 1980; Payne et al., 1978; Rudinsky, 1973; Wood, 1972, 1982). However, verbenone is known to be produced by only two *Ips* species, male *I. pini* (Stewart, 1975) and male *I. confusus* (Young et al., 1973b), where its effects on intraspecific behavior have not been determined. And in no scolytid species has the chiralty of the naturally produced verbenone been determined.

It has been well established that the pheromones of *I. paraconfusus* and *I. pini* mutually interupt each other species' conspecific aggregation (Birch and Wood, 1975; Birch and Light, 1977; Birch et al., 1977, 1980; Light and Birch, 1979b). Recently, Byers and Wood (1980, 1981) have demonstrated that the natural pheromones of resource-competitive *I. paraconfusus* and *D. brevicomis* are also mutually interruptive to each other's conspecific aggregation.

Verbenone, an epideictic pheromone of both male and female *D. brevicomis* (Bedard et al., 1980; Borden, 1974; Renwick and Vité, 1970; Wood, 1972, 1982), and not the pheromones frontalin and *exo*-brevicomin, appears to be the allomonal BMC that interrupts the aggregation of *I. paraconfusus*. This was determined by Byers and Wood (1980, 1981), who demonstrated that racemic verbenone interrupted the aggregation of both male and female *I. paraconfusus*, with a tendency to interrupt aggregation of females to a greater extent than males. Their findings, in which racemic verbenone interrupted aggregation, agree with the present findings in that (*R*)-(+)-verbenone interrupted aggregation of both sexes without changing the normal sex ratio of beetles attracted, while (*S*)-(−)-verbenone interrupted the response of females to a much greater extent than males (Table 2). Because the verbenone enantiomers are such potent interruptants of aggregation, interpretation of a sexual dimorphic behavior, based on the sex ratio of a sampling containing so few trapped beetles, must be tentative at this time. This possible differential behavioral sensitivity of females is correlated with female antennal receptors having a greater affinity for, and sensitivity to, the (*S*)-(−)- than the (*R*)-(+)-enantiomer of verbenone at concentrations just above threshold. At this level sensory information is most critical for orientation to or, in this case, avoidance of an odor source. The equal and keen sensitivity of male antennae to both verbenone enantiomers is also reflected in the equal extent of interruption of male beetle aggregation by the verbenone enantiomers (Table 2). These combined findings suggest that verbenone might act on *I. paraconfusus* as an interspecific (from *I. pini*) and intergeneric (from *D. brevicomis*) allomonalinterruptant, enhancing resource utilization for the allomone-releasing species, by decreasing or eliminating direct resource competition by the receptive species, *I. paraconfusus*.

Relative Sensitivities to BMCs

The monoterpenes *alpha*-pinene and myrcene are most often detoxified through an allylic hydroxylation mechanism in the hindgut of bark beetles to form numerous oxygenated monoterpene products (for review see Brand et al., 1979; Wood, 1982). Renwick et al. (1976a) found the stereospecific production of geometric and optical isomers of the verbenols in both adult male and female *I. paraconfusus* when the beetles were exposed to the enantiomers of *alpha*-pinene. Analysis of hindguts of both sexes treated with (*S*)-(−)-*alpha*-pinene showed the presence of predominantly (1*S*,4*S*,5*S*)-*cis*-verbenol, an aggregation pheromonal component, while (1*R*,4*S*,5*R*)-(+)-*trans*-verbenol was found to be the predominant product after (*R*)-(+)-*alpha*-pinene exposure. Myrcene has been found to by hydrolyzed to the pheromones ipsdienol and ipsenol in male but not female *I. paracofusus* (Byers et al., 1979; Fish et al., 1979; Hendry et al., 1980; Hughes, 1974).

The antennal sensitivity of both *I. paraconfusus* sexes to the myrcene

derived compounds was: ipsdienol >> ispenol >>> myrcene > linalool. This sensitivity ranking generally agrees with the findings of Mustaparta (1979), based on single-cell recordings, in which she estimated the proportions of receptor neurons specialized for these myrcene-derived compounds present on female antennae of *I. paraconfusus*. Similarly, the same sensitivity ranking to myrcene-derived compounds was found for female *I. pini* through both EAG (Angst and Lanier, 1979) and single-cell recordings (Mustaparta et al., 1977, 1979; Mustaparta, 1979). In female *I. paraconfusus*, Mustaparta (1979) found large numbers of receptors highly specialized for one or the other pheromone, ipsdienol or ipsenol. Contrary to the present EAG findings, where there appears to be a greater number of antennal acceptors for ipsdienol than ipsenol, Mustaparta estimated that there are equal numbers of receptors present for ipsdienol and ipsenol.

Antennal sensitivity of both males and females to the (*R*)-(+)-*alpha*-pinene-derived compounds was: (1*R*, 4*S*, 5*R*)-(+)-*trans*-verbenol ≥ (*R*)-(+)-verbenone = (*R*)-(+)-*alpha*-pinene ≥ (*R*)-*cis*-verbenol. In contrast, the differential sensitivity of both males and females to the (*S*)-(−)-*alpha*-pinene-derived compounds emphasizes a higher relative sensitivity to (*S*)-*cis*-verbenol. The antennal sensitivity of females was: (1*S*, 4*R*, 5*S*)-(−)-*trans*-verbenol ≥ (*S*)-*cis*-verbenol ≥ (*S*)-(−)-verbenone, while the sensitivity of male antennae was approximately equal for the three (*S*)-(−)-*alpha*-pinene derivatives. In studies using racemic mixtures, Mustaparta et al. (1979) classified the *cis*-verbenol, *trans*-verbenol, and verbenone responding receptor neurons of female *I. paraconfusus* under one grouping of specialized receptors because of their general responsiveness to all of these *alpha*-pinene-derived compounds. However, Mustaparta et al. (1979) and Mustaparta (1979) also observed that other different cells varied in their sensitivity to verbenone and the geometric isomers of verbenol. Similar to the present study, Angst and Lanier (1979) determined that both New York and Idaho ecotypes of female *I. pini* were slightly more sensitive to racemic *trans*-verbenol than racemic verbenone.

A generalized ranking of the antennal sensitivity of both sexes of *I. paraconfusus* for the tested BMCs was: ipsdienol >> ipsenol >>> *cis*-verbenol ≈ *trans*-verbenol ≈ verbenone ≈ 2-phenylethanol ≈ frontalin ≈ *alpha*-pinene = myrcene > *exo*-brevicomin ≥ linalool, which generally corroborates the findings on female *I. pini* (Angst and Lanier, 1979; Mustparta et al., 1979). Of the 95 receptors recorded from female *I. pini* antennae, Mustaparta et al. (1979) found different proportions of specialized cells: 47% ipsdienol, 16% ipsenol, 6% myrcene, 4% *alpha*-pinene, 3% linalool and camphor, and 24% combined for *trans*-verbenol, *cis*-verbenol, and verbenone.

Although sensitivity of *I. paraconfusus* to transspecific allomones and pheromones was much lower than for the conspecific pheromones (ipsdienol and ipsenol), the threshold and magnitude characteristics of reception of these

transspecific BMCs are well within the range thought to be necessary for olfactory perception and orientation from an ecologically relevant distance (Seabrook, 1977, 1978). This emphasizes the importance of the perception of allomones, as has been suggested by the established potency of allomones in interrupting interspecific cross-aggregation, even when the concentration of the allomone is less than 5% that of the pheromone (Birch et al., 1980; Borden et al., 1976, 1980). Interestingly, within the group of intra- and interspecific BMCs that produced intermediate EAG responses in *I. paraconfusus* antennae is (*S*)-*cis*-verbenol, a synergistic component of its aggregation pheromone. Thus if EAG response parameters are a true reflection of the overall olfactory input to the brain and they are correlated with elicited behaviors, then the observed near-equality in EAG sensitivity between a conspecific synergist (2-phenylethanol), pheromones [*trans*-verbenol, frontalin, *exo*-brevicomin, and (*R*)-*cis*-verbenol], and allomones (verbenone enantiomers) of other sympatric *Ips* and *Dendroctonus* species, and host tree monoterpenes (myrcene and *alpha*-pinene) suggests, apparently, that it is adaptive for *I. paraconfusus*, and presumably bark beetles in general, to rely on a balanced and diversified perception of both intraspecific pheromones and trans-specific and environmental olfactory cues.

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IDENTIFICATION OF NEW COMPONENTS FROM ANAL GLANDS OF *Tapinoma simrothi* PHEONICIUM

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Abstract—In addition to 2-methyl-4-heptanone, 6-methyl-5-hepten-2-one, and iridodials, the anal gland exudate of the dolichoderine ant, *Tapinoma simrothi*, contains 4-heptanone and 4-hydroxy-4-methyl-2-pentanone. The secretion functions as an alarm pheromone.

Key Words—*Tapinoma simrothi*, Hymenoptera, Formicidae, Dolichoderinae, 4-heptanone, 2-methyl-4-heptanone, 6-methyl-5-hepten-2-one, 4-hydroxy-4-methyl-2-pentanone, iridodials, ant, ketones, aldehydes, alcohol, gland anal, alarm, pheromone.

INTRODUCTION

Tapinoma simrothi pheonicium (Emery, 1925) is the most common of the four dolichoderine ant species that are present throughout Israel. It is found in the temperate zones as well as in the oases of the desert, and it is a major house pest. These ants are notorious for the large mounds, sometimes as high as half a meter, which they build during the winter. Each mound is carved with galleries forming a well-aerated solarium where the workers rear the brood. If a mound is accidentally destroyed, the ants enter in alarm frenzy, some rushing to the exposed areas with open mandibles, biting fiercely at any moving object; meanwhile other workers carry the brood to the safety of deeper undisturbed galleries. At this time a characteristic sweet smell usually permeates the air around the mound. The smell has been traced to the chemicals emanating from a pair of anal glands located at the tip of the abdomen. This typical *Tapinoma* smell had been noted as early as 1921 by Stumper, who reported that *T. erraticum* "smelled like a methylheptenone" although he did not conduct any chemical analysis. Later, the presence of

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6-methyl-5-hepten-2-one in the anal glands of another *Tapinoma* species, *T. nigerrimum*, was confirmed (Trave and Pavan, 1956; Pavan and Trave, 1958). There it is accompanied by 2-methyl-4-heptanone and the cyclopentanoids, iridodial and iridomyrmecin. In this paper we report the identification of two other acyclic ketones that are produced by the anal glands of *T. simrothi*.

METHODS AND MATERIALS

Insects. The ants were collected near Tel-Aviv, Israel, and transferred to artificial nests in the laboratory. Each nest was placed on a special foraging table (Hefetz and Lloyd, 1982) where the ants were allowed to feed on honey and maggots. Anal gland extracts were prepared by dissecting the glands of chilled ants under cold distilled water and then extracting the mixture with methylene chloride.

Chemical Analyses. Analyses were conducted on an LKB-2091 combined gas chromatograph–mass spectrometer using a 1.8-m 10% SP-1000 column programed from 55 to 220°C at 10°/min and also on a 1.8-m 1% OV-17 column programed from 55 to 300°C at 10°/min. The components of the extract were identified by comparison of their GC retention times and mass spectra to those of authentic samples as well as by cochromatography with the suspected compounds. The chemicals 4-heptanone, 6-methyl-5-hepten-2-one, and 4-hydroxy-4-methyl-2-pentanone were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin, and 2-methyl-4-heptanone from ICN Pharmaceuticals, Inc., New York.

Behavioral Assays. Observations of the alarm defense system of *T. simrothi* were made both in the field and in the laboratory. The laboratory assays were conducted on the foraging table. Alarm reaction was first elicited by tapping lightly on the table, and the behavior of the ants was followed. Fifteen minutes later, when the ants had calmed down, their reaction to anal gland extract or to various chemicals was studied. The compounds in methylene chloride were applied to small cardboard pieces (1 × 0.5 cm) distributed around the table. Besides the compounds identified in the secretion (6-methyl-5-hepten-2-one, 2-methyl-4-heptanone, 4-heptanone and 4-hydroxy-4-methyl-2-pentanone), two control substances, methylene chloride and citral, were also tested.

RESULTS

Chemistry

Figure 1 represents the GC tracing of the anal gland extract on a 10% SP-1000 column.

Peak 1. The component eluting first had a molecular ion at m/z 114 (22%), a base peak at m/z 43, a major fragment ion at m/z 71 (85) and

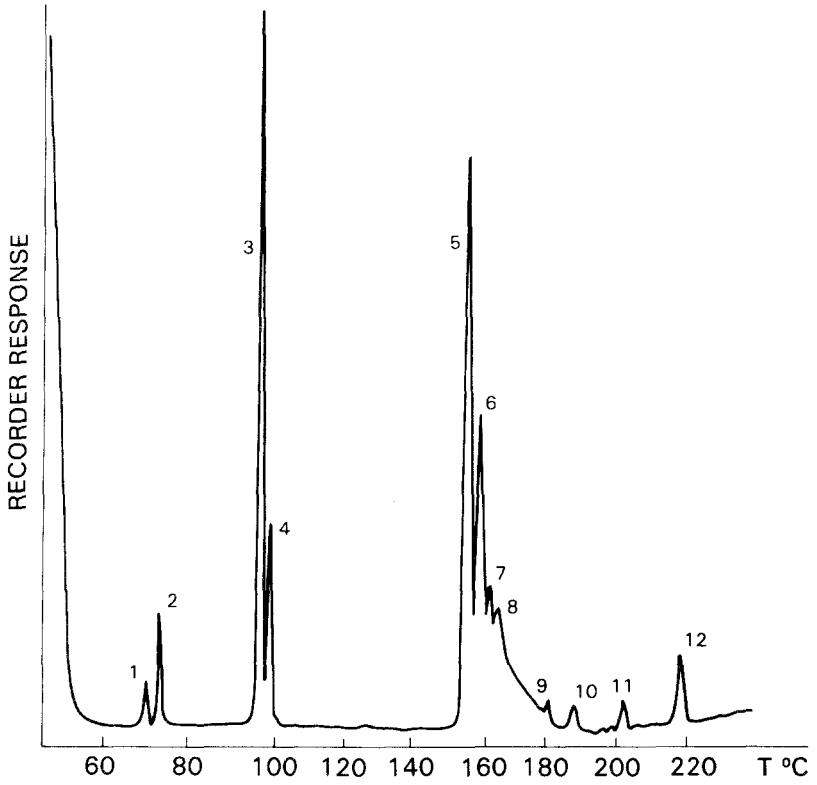

FIG. 1. Gas chromatogram of the anal gland extract of *Tapinoma simrothi*, on a 1.8-m 10% SP-1000 column programed from 55 to 220° C at 10° / min. Peak 1: 4-heptanone; 2: 2-methyl-4-heptanone; 3: 6-methyl-5-hepten-2-one; 4: 4-hydroxy-4-methyl-2-pentanone; 5, 6, 7, 8: iridodials; 9: iridomyrmecin; 10, 11, 12: alkanes.

additional ions at m/z 41 (24), 55 (4), 57 (2), 58 (7), 86 (2), and 99 (2). The mass spectrum and GC retention time were identical to those of authentic 4-heptanone.

Peak 2. The spectrum exhibited a molecular ion at m/z 128 (25%) and significant fragments at m/z 41 (40), 43 (80), 57 (100), 58 (31), 71 (75), 85 (75), and 113 (8), and was identical to the spectrum of authentic 2-methyl-4-heptanone; comparison of the GC retention times and cochromatography confirmed this assignment.

Peak 3. The spectrum of the major component of the extract was identical to that of 6-methyl-5-hepten-2-one with characteristic ions at m/z 41 (52), 43 (100), 55 (34), 58 (17), 69 (33), 71 (12), 83 (10), 93 (10), 108 (33), 111 (16), and 126 (10). An authentic sample of this ketone had the same GC retention time as the unknown.

Peak 4. The spectrum of this constituent exhibited fragment ions at m/z

41 (7), 43 (100), 58 (18), 59 (36), 71 (1), 86 (2), 98 (3), and 101 (111). The absence of a molecular ion and presence of fragments at 101 (M − 15) and 98 (M − 18) suggested an alcohol of molecular weight 116. The spectrum corresponded to that published in the EPA/NIH Mass Spectral Data Base (Heller and Milne, 1978) for 4-hydroxy-4-methyl-2-pentanone (diacetone alcohol). This was checked with an authentic sample of this hydroxyketone which also possessed the same retention time as peak 4. It should be mentioned that the spectrum given for this compound in the Registry of Mass Spectral Data (Stenhagen et al., 1974a) is erroneous. It consists of a superimposition of the spectra of 4-hydroxy-4-methyl-2-pentanone and of its dehydration product 4-methyl-3-penten-2-one. On a 1.8-m 1% (instead of 10%) SP-1000 column this compound coeluted with 6-methyl-5-hepten-2-one, and on a 1.8-m 1% OV-17 column it eluted immediately after 4-heptanone as a shoulder on the main peak.

Peaks 5, 6, 7, and 8. These four compounds showed the same mass spectrum (M^+ 168) which was identical to that published for iridodial (Cavill et al., 1976).

Peak 9. This trace component appeared to be iridomyrmecin by comparison with the spectrum published in the Registry of Mass Spectral Data (Stenhagen et al., 1974b).

Peaks 10, 11, and 12. These components exhibited mass spectra typical of branched alkanes (C_{22} to C_{26}) and probably originated from the cuticle.

Quantitation of Ketones in T. simrothi

The relative proportions of the constituents in the secretion determined from GC peak areas for ketones (1, 2, 3, 4), total iridodials (5, 6, 7, 8), and iridomyrmecin (9), were 1:1.8:18:4.5:33:0.5.

The amount of ketones per ant was estimated by comparing GC peaks from several anal gland extracts (20–30 ants) to calibrated GC peaks from dilutions of 6-methyl-5-hepten-2-one. This indicated that each ant contained between 50 and 100 ng of total ketonic material.

Behavior

In the field it was noted that any disturbance of the ant colony was accompanied by the release of a sweet terpenoid odor resembling that of 6-methyl-5-hepten-2-one. Destruction of a mound brought about alarm frenzy in the colony, the ants coming out of the nest in an aggressive stance and biting all moving objects in their vicinity. If the ants were disturbed while foraging in the field, their well-formed trail was dispersed at once and they ran around wildly looking for possible enemies. This alarm response was of short duration; it abated within 5 min and faded completely after 10 min.

In the laboratory the response of the ants to physical disturbance, such as

tapping on the foraging table, was quick movement toward the disturbed area with open mandibles and raised abdomens. A similar reaction was observed when the ants were exposed to anal gland exudate applied to a piece of cardboard placed on the table. In an attempt to decide which of the compounds found in the glands was responsible for this behavior, the ant colony was exposed to each of the pure components in turn and also to various combinations of the individual components. Unfortunately it was difficult to interpret the results unambiguously. Once the ants were excited, they immediately raise their abdomens, probably to emit the alarm pheromones from their anal glands; this action in turn excites other ants in the colony. Although the reaction of the ants to all four ketones tested was extremely rapid, preliminary observations suggest that 6-methyl-5-hepten-2-one is the most potent of the ketones and 4-hydroxy-4-methyl-2-pentanone the least. Response to the citral control was much milder; this compound provoked interest but no alarm or abdomen raising. Judging from its rapid fade-out, it did not induce alarm pheromone release.

The ants behaved quite differently when the gland extract was applied at the nest entrance. After a period of excitement, the ants retreated into the nest and aggregated into a ball; they did not come out of the nest until most of the material had evaporated some 15 min later. The synthetic mixture of ketones brought about the same reaction.

DISCUSSION

The chemistry of the anal gland secretion of *T. simrothi* presents many similarities to that of other dolichoderine ants and especially to other *Tapinoma* species. Various isomers of iridodial have been reported as extractives of a number of dolichoderine ants (Blum and Hermann, 1978a, and references therein) as well as of some beetles (Abou-Donia et al., 1971; Bellas et al., 1974; Vidari et al., 1973). In the ants, it is usually accompanied by 6-methyl-5-hepten-2-one, as in the case of *Tapinoma nigerrimum* (Trave and Pavan, 1956) and *T. sessile* (McGurk et al., 1968). This ketone has also been detected in the mandibular gland secretions of many formicine ants such as *Polyrhachis simplex* (Hefetz and Lloyd, 1982), *Calomyrmex* (Brown and Moore, 1979), *Formica* species (Duffield et al., 1977), and others (see references in Blum and Hermann, 1978b).

The two 4-alkanones are not as widely distributed as 6-methyl-5-hepten-2-one. To date, 2-methyl-4-heptanone has been detected in only one other *Tapinoma* species, *T. nigerrimum* (Trave and Pavan, 1956), and it may be genus-specific; 4-heptanone has been found recently in the mandibular secretions of two ants, the myrmicine *Zacryptocerus varians* (Olubajo et al., 1980) and the formicine *Polyrhachis simplex* (Hefetz and Lloyd, 1982).

To our knowledge, 4-hydroxy-4-methyl-2-pentanone has not been previously identified as a component of any arthropod secretion. It has been reported in the volatiles of several plants such as gardenia flowers (Tsuneya et al., 1980), the berries of arctic bramble (Kallio, 1976), the oil of a *Pogostemon* sp. (Bedekar et al., 1979), and others. As one of 99 ketones tested (Blum et al., 1971), it elicited very little alarm response in the ant *Pogonomyrmex badius* compared to the natural pheromone 4-methyl-2-heptanone, and in a field study of the response of tsetse flies (Glossinidae) to various chemicals (Vale, 1980), it was found to have mildly attractive properties for the flies.

A condensation product of acetone, 4-hydroxy-4-methyl-2-pentanone, is often present as a trace impurity in dried acetone. However, the anal glands or their extract were never at any time in contact with acetone, so the finding of this ketone should be not regarded as an artifact. It did provoke alarm behavior in *T. simrothi*, although not as much as 6-methyl-5-hepten-2-one, and conceivably it is a genuine alarm pheromone for this ant.

The anal glands of dolichoderine ants are well known manufacturers of chemical alarm signals; they form a "parsimonious glandular system with both a communicative and defensive function" (Blum, 1969). In *T. simrothi* the effect of its alarm pheromones was twofold. When gland extract was applied to the foraging area, it recruited workers to the emitting source and provoked aggressive behavior but, when applied to the nest entrance, it induced the ants to retreat deep into the nest in panic alarm.

Although they are the major constituents of the anal gland secretion, the iridodial isomers do not seem to play a significant role as alarm releasers. Indeed the synthetic mixture of the four ketones appeared to induce the same aggressive alarm response as the total glandular extract. As others have suggested (Blum and Hermann, 1978a, and references therein), the iridodials which can polymerize rapidly may serve as carrier or fixative for the more volatile ketones in the secretion.

The response of the ants to the total ketone mixture is not significantly different from their response to 6-methyl-5-hepten-2-one alone which is not surprising since this ketone dominates the secretion. It is not possible at this time to assign a specific role to each of the ketones. They all seemed to be potent alarm releasers and to provoke immediate emission of alarm pheromones from the anal glands, thus making it difficult to separate the action of the individual ketone tested from that of the total blend.

In contrast to the behavior of colonies of *T. sessile* which were observed to abandon the nest and emigrate to new sites after exposure to methylheptenone (Wilson and Pavan, 1959), the alarm response of *T. simrothi* seemed to fade out quickly and leave no after-effect; the ants resumed their normal behavior soon after exposure to the pheromones.

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STRUCTURE–ACTIVITY CORRELATIONS AMONG ANALOGS OF 4-METHYL-3-HEPTANOL, A PHEROMONE COMPONENT OF THE EUROPEAN ELM BARK BEETLE (*Scolytus multistriatus*)

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Abstract—A number of analogs of the title compound (**1**), with several different functional groups in place of the 3-OH and with a variety of substituents, were tested for biological acitivity by a laboratory walking-beetle assay. The electroantennogram (EAG) response was determined for many of these, as well. Field tests with baited sticky traps were carried out on compounds with activity in the walking-beetle assay and/or that gave a high EAG response. Structure–activity correlations with parameters reflecting hydrophobic, steric, electronic, and van der Waals interactions with olfactory receptors were examined primarily on the basis of the behavioral tests. Electronic substituent effects on the 3-position functional group and steric effects were found to correlate best. It is suggested that the strength of a hydrogen bond to the 3-oxygen or 3-nitrogen (as proton acceptor) is important in chemoreception by receptors that are involved in the behavioral response.

Key Words—Structure–activity relationships, pheromone, *Scolytus multistriatus*, *Coleoptera*, *Scolytidae*, Dutch elm disease, electroantennogram, chemoreception, 4-methyl-3-heptanol, analogs, attractant, aggregation, beetle, bark beetle, alcohols, ketones, esters, epoxides, carboxylic acids, amines, isothiocyanates, halides, azides.

INTRODUCTION

An examination of the chemical requirements for pheromone recognition during initial contact between pheromone molecule and macromolecular

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receptors can give insight into the mechanism of olfaction. For insect pheromones, this knowledge can also provide a foundation for the design of antagonists or more potent attractants.

It is generally accepted that molecular size and shape are important for insect pheromone chemoreception. Activity is found to depend critically on such things as the length of the hydrocarbon chain, the positions of methyl groups, double bonds, and other functional groups, and the configuration of the molecule such as the arrangement about a double bond or epoxide moiety (e.g., Priesner, 1977; Jacobson, 1970; Adler, 1972). Not unexpectedly, receptor sites are asymmetric as evidenced by the often stark differences in activity of enantiomers (Silverstein, 1979). There is also evidence that pheromone molecules are incorporated in a specific conformation (Bestmann, 1980; Kostelc, 1979; Chapman, 1978).

Apart from stereochemical requirements, other chemical factors that could potentially be involved have received little attention. Olfactory receptors are likely to be proteinaceous (Beets, 1973; Ryan, 1978; Fesenko, 1979; Riddiford, 1970). Electronic charge–charge attraction, hydrogen bonding, hydrophobic bonding, and van der Waals forces are potentially important in binding to such receptor macromolecules. Some of these interactions have been proposed for the reception of odorants and tastants in humans (Tancredi, 1979; Beets, 1973, 1978; Ohloff, 1980) and are of recognized general importance in chemoreception (Hansch, 1973, 1979).

In insect chemoreception, several reports hint at the importance of factors other than stereochemical. For example, substitution of $-CH_2-O-CH_2-$ for $-CH_2-CH_2-CH_2-$ within a long aliphatic chain causes suppression of pheromone activity (Carrol, 1980). Substitution of $-O-\overset{O}{\overset{\|}{C}}H$ for $-CH_2-\overset{O}{\overset{\|}{C}}H$ causes strong suppression in one case but not in another (Mitchell, 1978). Activity has been correlated with the charge density on the pyrrole nitrogen atom of the trail pheromone of the ant *Atta texana* (Caputo, 1979). 4-Methyl-3-heptanone is 10^5 times more effective than the corresponding alcohol in triggering the alarm response of *A. texana* (Moser, 1968). Sensory perception of analogs of the feeding stimulant methyl eugenol by the Oriental fruit fly is inhanced by substituents that donate electron density into the aromatic ring, and activity also correlates with a hydrophobic bonding parameter (Metcalf, 1981).

In this study we examine some physicochemical factors for the chemoreception of 4-methyl-3-heptanol (**1**, Table 1), which together with (−)-α-multristriatin and the host-produced synergist (−)-α-cubebene, comprise the aggregation pheromone of the European elm bark beetle. *Scolytus multistriatus* (Marsham), a major vector of Dutch elm disease in Europe and North

America (Lanier, 1976). In most analogs tested we have maintained the carbon skeleton of **1** in order to minimize size and shape factors.

METHODS AND MATERIALS

Walking-Beetle Assay

The walking-beetle olfactometer described by Moeck (1970) was used except that the runway on which the beetles were placed was open instead of glass-roofed. Pheromone components and analogs in *n*-hexane (HPLC grade) were spotted on strips of filter paper and placed in a 250-ml glass bottle connected in line with a stream of untreated laboratory air at 120 ml/min. Beetles were induced by light to walk through a narrow stream of this air (0.5–1 cm wide, determined with smoke), which was blown perpendicular to their paths from an outlet 3–6 cm away. Beetles that were diverted from their walk to the light source and turned or walked directly toward the source of the chemical-laden air stream, or that made a 360° turn within or near the stream were considered positive responders (Lanier, 1977). Beetles displaying ambiguous behavior were retested. Beetles were tested 5–10 at a time in a group of 50, during a total time of 10–13 min. Each group was allowed to rest in clean air for at least 1 hr before retesting. The natural pheromone mixture was conveyed through tubing separate from that of analogs. The "natural pheromone" is defined as the attractive mixture of synthetic 4-methyl-3-heptanol (mixture of diastereomers, Aldrich Chem. Co.), synthetic multistriatin (35% α, Albany Int.) and natural (−)-α-cubebene (98 or 70%, distilled from cubeb oil, Albany Int.). All components of the apparatus in contact with vapor-laden air were changed for each new sample and thoroughly cleaned before reuse.

Beetles were obtained from the U.S. Forest Service, Northeastern Forest Experiment Station, Delaware, Ohio, and were 4–7 days old at the time of use. They were conditioned by exposure to room fluorescent light for 8 or more hours. Only males were used, since females were found to be less responsive in this assay. The response to the natural pheromone varied from batch to batch of beetles and sometimes from day to day (Lanier, 1978); trials were conducted with analogs only when the response to the natural pheromone was $\geq$45% and at least four times greater than the control which consisted of the other two components of the pheromone: α-cubebene plus α-multistriatin. Some batches of beetles gave a high response to the control. In these cases, a satisfactory difference between control and the natural pheromone could be obtained by eliminating the synergist, α-cubebene, from the control and the test mixtures.

Electroantennogram (EAG) Response

The beetles used in the EAG tests emerged in the laboratory from field-collected (Syracuse, New York) brood wood and were kept refrigerated until tested. Testing occurred within 5 days of emergence. A technique modified from Payne (1970) was used to secure beetles for recording. "Omega dot" glass capillaries pulled to a tip diameter of approximately 10 μm and filled with a weak saline solution (Roelofs, 1976) were used in conjunction with chlorinated silver wires to form salt bridge electrodes. Standard methods were used to amplify and display the response potentials (Payne, 1970).

Administration of stimuli was accomplished by injecting a "puff" of chemical-laden air into a pure air stream moving at a rate of 1000 ml/min that continually bathed the antenna. The "puff" was delivered by a second air stream (1000 ml/min) controlled by a solenoid valve that was allowed to open for approximately 250 msec. To minimize contaminants, all air channels were constructed of glass tubing with Teflon® joints. The air streams were also filtered through molecular seives (PM-100, Hewlett Packard) and dehydrated with Bluegel. A vacuum immediately behind the preparation removed odorized air. Between stimulations, the beetles were allowed to recover for 2–5 min, depending on the intensity of the response, until the response potential returned to baseline level.

The standard concentration of stimulant in air was obtained by the following procedure. A known volume of neat compound was pipetted to the bottom of a 250-ml ground-glass joint Erlenmeyer flask containing 150 glass beads (1 mm diameter) to aid in dispersion of the chemical. The flask was then quickly capped with a special all-glass cap and shaken until the compounds were observed to "evaporate" completely (see below). The special cap contained inlet and outlet glass tubing positioned so that, when connected in line with the air stream, the "puff" of fresh air would enter at the bottom of the flask and displace the same volume of chemical-laden air out at the top, to eventually reach the antenna.

Field Trials

Field trials were conducted in St. Louis Park, Minnesota, and Syracuse, New York, from June through September, 1978, 1980, and 1981. Traps consisted of white sheets of heavy paper (66 × 45.5 cm^2) coated with Stickem Special®. These were stapled to utility poles at a height of about 3 m (Lanier, 1976) in a 5 row × 5 trap array. Each row included one trap baited with the natural pheromone and one trap baited with the control, α-multistriatin plus α-cubebene. Baits were contained in capped 2-cc polyethylene vials. Diffusion through the polyethylene provides a slow release of chemical. Analogs were contained in separate vials. Absorption of analogs into the polyethylene was

verified by inspection. In addition, analogs were field-tested in open vials for a 7-day period with no significant change in the results.

Partition Experiments

Partition coefficients of analogs relative to 4-methyl-3-heptanol (**1**) were determined in octanol–water. Typically, 10 μl each of analog and **1** and 0.25 ml each of distilled *n*-octanol and distilled water were placed in a 0.7-ml sealed glass vial and shaken to equilibrium. Both layers were then assayed by GC (2.44 m × 2 mm 5% FFAP, 1.63 m × 2 mm 10% SE-30, or 1.63 m × 2 mm OV-17, on Chromosorb W). Peak heights or peak areas relative to **1** were used to calculate the relative partition coefficient. In cases where separation of peaks was difficult, 4-methyl-4-hepten-3-ol (**7** in Table 1), whose partition coefficient relative to **1** was known, was used as the reference. Experiments were run in duplicate.

Chemicals

The following analogs were purchased commercially: (Numbers as in Table 1) **7**, **30**, **33**, **34**, and **35** (Albany Int.); **31** (Saber Lab.); **8** (Farchan). All other compounds were synthesized as described below. Elemental analyses (Galbraith Lab., Knoxville, Tennessee) for C, H, N, S, and halogen, as applicable, were within ±0.4% for all newly synthesized compounds except **18**. Proton NMR spectra (Brüker 270 MHz) were taken in $CDCl_3$ except where noted. IR spectra were taken neat (Perkin-Elmer 283). Mass spectra were recorded on an AEI MS-30 at the Chemistry Department of the University of Minnesota, and are reported as taken under electron impact (EI) or chemical ionization (CI) conditions. All compounds were verified to be free of 4-methyl-3-heptanol by GC.

2-Fluoro-4-methyl-3-heptan-one (**12**) *and -ol* (**2**). The ketone **12** was made from the synthesis in situ of the sodium salt of 2-hydroxymethylene-4-methyl-3-heptanone prepared from **11** by the procedure according to Weisenborn et al. (1954) for hydroxymethylenecholestenone except that the reaction was run under nitrogen for 20 hr. After destroying excess NaH with methanol, the product was fluorinated (Edwards 1959) by bubbling gaseous $FClO_3$ at a moderate rate until the reaction mixture gave a neutral color to wet pH paper. The $FClO_3$ was swept away with nitrogen, and the reaction mixture was poured into water and extracted with hexane in the usual manner. The extract was dried ($MgSO_4$) and concentrated by fractional distillation through a glass helices-packed column to remove solvent, and then distilled (bp 69–71°, 27 mm), 56% yield. NMR showed about equal amounts of two diastereoisomers: δ0.91 (t, 3, C7-H_3), 1.09 (two d, 3, C4-CH_3), 1.30 (m, 2, C6-H_2), 1.49 (two d of d, 3, J_{FH} = 24 Hz, C1-H_3), 1.69 (m, 2, C5-H_2), 3.03 (m,

1, C4-H), 4.95 and 4.96 (two q and two q, 1, J_{FH} = 49 Hz, C2-H). IR: 1727 (C=O), 982 and 1085 cm^{-1} (s, CF). MS (EI, 70 eV): *m/e* 146 (M^+), 104 ($M-CH_2=CHCH_3$), 99 ($M-CH_3CHF$).

The alcohol **2** was made from **12** by reduction with 1.1 equivalents $NaBH_4$ in ethanol followed by work-up with ether and distillation (bp 67–69°, 11 mm). Yield 91%. NMR showed approximately equal amounts of 4 diastereoisomers (CCl_4): δ 0.91 (m, 6, C4-CH_3 and C7-H_3), 1.29 (two d of d, J_{HF} = 24.3, C1-H_3), 2.4 and 2.3 (variable, two br. s, 1, OH), 3.08–3.45 (m's, 1, C3-H), 4.59 (m, 1, J = 47.1, 47.5 and 49 Hz, C2-H). IR: 3450 (s, br., OH), 980–1150 cm^{-1} (s, multimax, C-O, C-F). MS (CI, pos., NH_3): *m/e* 166 (M + 18); (EI, 70 eV) 129 (M−F), 101 ($M-CH_3CHF$).

2,2-Difluoro-4-methyl-3-heptan-one (**13**) *and -ol* (**3**). To a degassed, water-free solution of diisopropyl amine (0.157 mol) in freshly distilled tetrahydrofuran (150 ml) at 0° was added slowly a solution of *n*-butyllithium in hexane (0.12 mol). The solution was cooled to −78° and a degassed THF solution of 2-fluoro-4-methyl-3-heptanone (**12**) (0.11 mol) was added dropwise over 2 hr. This solution was transferred to an addition funnel and added dropwise over 3 hr to an oxygen-free solution of THF (25 ml) at 0° that was kept saturated with gaseous $FClO_3$. After stirring for an additional hour and then purging with N_2, the reaction mixture was poured into water and taken up with pentane. The organic layer was washed with 2 N HCl to remove diisopropyl amine and, after washing with water and drying ($MgSO_4$), was fractionated at atmospheric pressure through a glass helices-packed column up to 70°. The pot residue was placed on a column of silica gel and eluted with pentane to give the desired product after distillation of the pentane (16% yield). Unreacted starting material **12** was recovered from the column after increasing the eluent gradually to 10% ether in pentane (12% yield). NMR of **13**: δ0.90 (t, 3, C7-H_3), 1.13 (d, 3, C4-CH_3), 1.29 (m, 4, C5-H_2 and C6-H_2), 1.68 (t, 3, J_{FH} = 19.5 Hz, C1-H_3), and 3.10 (m, 1, C4-H). IR: 1745 (C=O), 1140 and 1205 cm^{-1} (s, CF_2). MS (CI, pos, NH_3): *m/e* 182 (M + 18); (EI, 70 eV) 99 ($M-CH_3CF_2$), 71 ($C_5H_{11}^+$).

The corresponding alcohol **3** was made from **13** by $NaBH_4$ reduction in absolute ethanol, worked up with ether in the usual manner, and distilled to a colorless liquid (bp 48°, 5–5.5 mm) 88%. NMR showed equal amounts of two diastereoisomers: δ 0.91 and 0.97 (t and two d, 6, C7-H_3 and C4-H_3), 1.32 (m, C5-H_2, C6-H_2), 1.62 and 1.63 (t and t, 3, J_{FH} = 19.1 Hz, C1-H_3), ~1.7 (m, C4-H), 2.11 and 2.01 (d and d, 1, J = 5.9 Hz, OH), 3.56, (m, 1, C3-H). IR: 3430 (s, br., OH), 1140 cm^{-1} (s, CF_2). MS (CI, pos., NH_3): *m/e* 184 (M+18); (EI, 70 eV) 101 ($M-CH_3CF_2$).

1,1,1,2,2,-Pentafluoro-4-methyl-3-heptan-one (**14**) *and -ol* (**4**). The Grignard reagent of 2-bromopentane (0.4 mol) was prepared in ether under N_2 in the usual manner and filtered. To this was added dropwise over 1.5 hr $CF_3CF_2CO_2H$ (0.135 mol) in ether (50 ml) and the mixture refluxed for 2 hr

and then poured over HCl (75 ml) in ice (600 ml). The product was extracted with ether in the usual manner. The ether was removed at atmospheric pressure in a fractionating column and the pot residue fractionated under vacuum to give the desired product (bp 54°, 82 mm) plus other uncharacterized higher boiling products. Yield 30%. NMR: δ0.92 (t, 3, C7-H_3), 1.19 (d, 3, C4-CH_3), 1.34 and 1.74 (m and m, 4, —CH_2CH_2—), 3.09 (m, 1, C4-H). IR: 1755 (C=O), 1210 (s, broad) and 1330 cm^{-1} (CF_3CF_2—). MS (CI, pos., NH_3): *m/e* 236 (M+18); (EI, 70 eV) 99 ($M-C_2F_5$), 147 ($M-C_5H_{11}$).

The alcohol **4** was prepared from **14** by $NaBH_4$ reduction in absolute ethanol and work-up with ether. The ether was stripped by rotoevaporation and the product distilled (bp 66°, 33 mm). Yield 82%. NMR showed equal amounts of two diastereoisomers: δ0.92 and 1.04 (t and two d, 5, C7-H_3 and C4-CH_3), 1.38 (m, 4, C6-H_2 and C5-H_2), 2.01 (br, m, 1, C4-H), 2.6 (variable) (two d, 1, J = 8.7 Hz, OH), 3.94 (m, 1, C3-H). IR: 3430 (s, br, OH), 1340 (w) and 1200 cm^{-1} (s, br, CF_3CF_2—). MS (EI, 70 eV): *m/e* 201 (M^+-F), 101 ($M^+-C_2F_5$).

2-Chloro- (**16**) *and 4-Chloro-4-methyl-3-heptanone* (**17**) *and 2-Chloro-4-methyl-3-heptanol* (**5**). The ketones were synthesized by chlorination of 4-methyl-3-heptanone (**11**) with $CuCl_2 \cdot H_2O$ and LiCl in DMF at 90° for 3 hr according to the procedure of Werthemann and Johnson (1970). The product, containing about 90% pure **16** and **17**, was further purified by chromatography on silica gel (20:1 hexane–ether) to yield a fraction containing **16** and one containing a 2:1 mixture of **16** and **17** (bp 64–66°, 3–3.5 mm).

16: NMR: δ0.90 (t, 3, C7-H_3), 1.11 (d, 3, C4-CH_3), 1.29 and ca. 1.65 (m and m, C6-H_2 and C5-H_2), 1.59 (d, 3, C1-H_3), 3.00 (m, 1, C4-H), 4.46 (two q, 1, C2-H). MS (EI, 70 eV): *m/e* 162, 164 (M^+), 99 (M—CH_3CHCl).

17: NMR δ0.93 (C7-H_3), 1.10 (two d, C1-H_3), 1.62 (s, C4-CH_3), ca. 1.9 (m, C5-CH_2), 2.78 and 2.91 (m, C2-H_2, J_{gem} = 18.7 Hz). IR of mixture of **16** and **17**: 1728 and 1718 cm^{-1} (C=O).

Alcohol **5** was prepared by $NaBH_4$ reduction of **16** in absolute ethanol, worked up with ether in the usual manner and distilled bulb-to-bulb. NMR: δ0.91 (m, 6, C4-CH_3 and C7-H_3), 1.56 (d, C1-H_3), 3.20 3.34, and 3.49 (three m, 1, C3-H), 4.24 (m, 1, C2-H). MS (EI, 70 eV): *m/e* 101 (M—CH_3CHCl), 93 and 95 ($M-C_5H_{11}$).

3,4-Dimethyl-3-heptanol (**6**). To a solution of 4-methyl-3-heptanone (**11**) in degassed ether was added CH_3MgBr in ether dropwise with mechanical stirring. After stirring an additional 0.5 hr, saturated NH_4Cl was added, slowly at first, until the clear ether solution separated from a gummy white solid. The white solid was extracted with ether and the combined organic solutions were washed and dried and then stripped of solvent by rotary evaporation. Fractional distillation gave a colorless liquid (bp 68–69°, 11 mm). Yield 80%. NMR showed approximately equal amounts of two diastereoisomers: δ0.90 (m, 9, C1-H_3, C4-CH_3 and C7-H_3), 1.06 and 1.07 (two

s, 3, C3-CH_3), 1.19 (s, OH), 1.49 and 1.19 (m, all CH_2 and CH). IR: 3460 (OH), 1150 cm^{-1} (C—O). MS (CI, pos., NH_3): *m/e* 162 (M+18); (EI, 70 eV) 129 (M−CH_3), 115 (M−C_2H_5), 73 (M−C_5H_{11}).

4-Hydroxy-4-methyl-3-heptanone (**22**) *and 4-Methyl-3,4-heptanediol* (**9**). A mixture of 4-methyl-3-heptene (**34**, 59%*E*, 8 mmol), $NaClO_3$ (9 mmol), and OsO_4 (4 mg) in ethanol–water (3:1) was brought to reflux and more ethanol was added if necessary to bring the mixture to homogeneity. After olefin had been consumed (24 hr), the cooled reaction mixture was diluted with an equal volume of saturated NaCl and extracted with ether. The ether layer was washed with saturated NaCl, dried ($MgSO_4$), and evaporated to a colorless oil containing from 1:1 to 1:2 mixture of ketone (**22**) and diol (**9**). The ketone was isolated by preparative GLC (2.44 m × 3 mm 5% FFAP on Chromosorb A). The diol was obtained by $NaBH_4$ reduction of the crude mixture in absolute ethanol, work-up in the usual manner with ether, and distillation of the viscous oil (bp 66–68°, 0.1–0.2 mm).

22 NMR: δ0.89 (t, 3, C7-H_3), 1.11 (t, 3, C1-H_3), 1.35 (s, 3, C4-CH_3), ca. 1.4 and 1.67 (m and m, 4, —CH_2—CH_2—), 2.53 (two q, 2, C2-H_2). MS (CI, pos., NH_3): *m/e* 162 (M+18).

9 NMR showed a 1.7:1 ratio of diastereoisomers: δ0.93 (m, 3, C7-H_3), 1.03 (t, 3, C1-H_3), 1.09 and 1.16 (two s, 3, C4-CH_3, ratio 1.7:1), 1.39 (m, 6, all —CH_2—), 1.80-2.06 (variable) (two d and two s, 2, OH), 3.31 (m, 1, C3-H). IR: 3410 (s, br, OH), 1000-1160 cm^{-1} (br., multimax, C—O). MS (CI, pos., NH_3): *m/e* 164 (M+18); (EI, 70 eV) 87 (M—$C_5H_{10}OH$).

2-Methylpentan-1-ol (**10**). A solution of 2-methylpentanoic acid (8.8 mmol) in ether was added slowly to a suspension of lithium aluminum hydride (87 mmol) in ether under nitrogen and then refluxed overnight. Excess reagent was destroyed by the cautious addition of water and then 10% H_2SO_4 was added to obtain a clear, two-phase mixture. Work-up with ether in the usual manner, followed by distillation, yielded 72% of pure alcohol (bp 66°, 19 mm). NMR: δ0.90 and 0.91 (t and d, 6, C5-H_3 and C2-CH_3), 1.69 (br., s, OH), 3.45 (br. m, 2, CH_2OH). MS (CI, pos., NH_3): *m/e* 120 (M+18); (EI, 70 eV) 84 (M−H_2O).

4-Methyl-3-heptanone (**11**). 4-Methyl-3-heptanol (**1**) was oxidized with chromic acid in acetone according to the procedure of Meinwald et al. (1973). The product, after removal of solvent, was vacuum distilled (bp 74–75°, 38 mm) in 80% yield. NMR: δ0.90 (t, 3, C7-H_3), 1.04 and 1.06 (t and d, 6, C1-H_3 and C4-CH_3), 1.29 and 1.62 (m and m, 4, —CH_2CH_2—), 2.46 (two q, $J_{gem} \simeq 17$ Hz, C2-H_2), 2.53 (m, C4-H). IR: 1720 cm^{-1} (C=O). MS (EI, 70 eV): *m/e* 128 (M^+), 99 (M−C_2H_5), 86 (M−CH_2=$CHCH_3$), 57 (M−C_5H_{11}).

4-Bromo-4-methyl-3-heptanone (**15**). 4-Methyl-3-heptanone (**11**), bromine, and cyclohexene oxide in CCl_4 were irradiated with a 75-W tungsten lamp for 1 hr at room temperature (Calo, 1977). The solvent was evaporated, and the oil was placed on a column of silica gel and eluted with hexane–ether

(10:1). The desired fraction was stripped of solvent and distilled under vacuum (bp 62°, 4.5 mm). NMR (neat): δ0.96 and 1.12 (t and t, 6, C1-H_3 and C7-H_3), 1.38 (m, 2, C6-H_2), 1.81 (s, 3, C4-CH_3), 2.05 (m, 2, J_{gem} = 14 Hz, C5-H_2), 2.8 (q of q, 2, J_{gem} = 17.5 Hz, C2-H_2). MS (EI, 70 eV): *m/e* 206 and 208 (M^+), 164 and 166 (M—CH_2=$CHCH_3$), 57 ($C_2H_5CO^+$).

3,4-Heptanedione (**18**). The procedure was that of Sharpless et al. (1971). The product was fractionally distilled giving a yellow liquid (bp 54–55°, 18 mm). Yield, 24% of product about 93% pure. NMR (acetone-d_6): δ0.91 (t, 3, C7-H_3), 1.01 (t, 3, C1-H_3), 1.57 (m, 2, C6-H_2), 2.71 and 2.76 (t and t, 4, C5-H_2 and C2-H_2). IR: 1720 cm^{-1} (C=O). MS (EI, 20 eV): *m/e* 128 (M^+), 71 ($C_3H_7CO^+$), 57 ($C_2H_5CO^+$).

(E)-*4-Methyl-4-hepten-3-one* (**19**). CrO_3 (0.56 mol) was added in portions to ice cold pyridine (450 ml) with mechanical stirring. After the temperature subsided again to 3°, the solution was allowed to warm to room temperature at which time 4-methyl-4-hepten-3-ol (**7**, >95% *E*) was added (0.2 mol) and stirring continued until GC indicated that starting material had been consumed (4 hr). The reaction mixture was then partitioned between ether and water. The ether layer was washed with 1 N HCl until the aqueous phase became acidic, washed with water and then 5% K_2CO_3, dried ($CaCl_2$), and stripped of solvent. The resulting liquid (82% yield of 92+% purity) was fractionally distilled to a slightly yellow liquid (bp 68–69°, 16 mm). Only one vinyl proton resonance was seen by NMR and its chemical shift was indicative of the *E* isomer (Jackman, 1969). NMR: δ 1.09 and 1.08 (t and t, 6, C1-H_3 and C7-H_3), 1.77 (s, 3, C4-CH_3), 2.25 (m, 2, C6-H_2), 2.69 (q, 2, C2-H_2), 6.62 (t, 1, vinyl). IR: 1678 and 1712 (C=O), 1624 and 1640 cm^{-1} (C=C). MS (EI, 70 eV): *m/e* 126 (M^+), 97 (M—C_2H_5), 69 (97−CO).

(E)-*2-Fluoro-4-methyl-4-hepten-3-one* (**20**). A solution of 2,4-dibromo-4-methyl-3-heptanone (**38**) (7 mmol), KF (21 mmol), and 18-crown-6 (0.07 mmol) in CH_3CN (5 ml) was refluxed for 21 hr until GC indicated that starting material and the transient intermediate **21** had been converted to product. The reaction mixture was partitioned between ether and water and worked up in the usual manner. The liquid remaining after evaporation of the solvent (about 75% **20**) was placed on a column of silica gel and eluted with 20:1 hexane–ether to give, after removal of solvent and bulb-to-bulb distillation, pure **20** (bp 74°, 21 mm). Configuration about C=C bond is based on analogy to **19**. NMR: δ 1.09 (t, 3, C7-H_3), 1.53 (q, 3, J_{FH} = 24.3 Hz, C1-H_3), 1.81 (s, 3, C4-CH_3), 2.29 (m, 2, C6-H_2), 5.55 (two q, 1, J_{FH} = 48.5 Hz, C2-H), 6.67 (t, 1, vinyl), IR: 1685 (C=O), 1635 (C=O), 1050 cm^{-1} (ms, broad, C-F). MS (EI, 70 eV): *m/e* 144 (M^+), 97 (M—C_2H_4F), 69 (97−CO).

(E)-*2-Bromo-4-methyl-4-hepten-3-one* (**21**). A solution of 2,4-dibromo-4-methyl-3-heptanone (**38**) and 2,4,6-trimethylpyridine (1.5 equivalents) in THF was refluxed until starting material nearly disappeared as indicated by GC (11 hr). The reaction mixture was taken up in ether and washed with 1 N

HBr to remove trimethylpyridine. The organic layer was washed, dried ($MgSO_4$), and stripped of solvent by rotary evaporation leaving an oil of >90% purity (86% yield). A portion was purified of starting material and other impurities by column chromatography of silica gel (15:1 hexane-ether). The product was distilled bulb to bulb (bp 68°, 2mm). Configuration about the C=C bond was assigned by analogy to **19**. NMR: δ1.10 (t, 3, C7-CH_3), 1.77 and 1.84 (d and s, 6, C1-H_3 and C4-CH_3), 2.30 (m, 2, C6-H_2), 5.11 (q, 1, C2-H), 6.73 (t, 1, vinyl). IR: 1660 (C=O), 1630 cm^{-1} (C=C). MS (EI, 20 eV): *m/e* 97 ($M-CH_3CHBr$), 69 (97−CO).

1-Ethyl-2-methylpentylamine (**23**). This was prepared by reductive amination of **1** with NH_4OCOCH_3 and $NaBH_3CN$ in methanol for 4 days at room temperature (Borch, 1971). The ethereal extract was dried first with $MgSO_4$ and then overnight with 3 Å molecular sieves. The ether was stripped by rotary evaporation and the product factionally distilled (bp 59°, 18 mm). Yield 60%. NMR: δ0.88 (m, 9, C1-H_3, C4-H_3 and C7-H_3), 1.05 (s, 2, NH_2), 1.31 (m, 7, all CH_2 and C4-H), 2.50 (m, 1, C3-H). IR: 3315 and 3375 cm^{-1} (w, NH). MS (EI, 70 eV): *m/e* 100 ($M-C_2H_5$), 58 ($M-C_5H_{11}$); (CI, pos., NH_3) 147 (M+18).

3-Isothiocyanato-4-methylheptane (**24**). Compound **24** was prepared from the amine **23** and thiophosgene in the presence of $NaHCO_3$ using the procedure of Rice et al. (1979). The crude product after evaporation of solvent was fractionally distilled to a pale yellow liquid (bp 76–77°, 2 mm) in 75% yield. NMR showed approximately equal amounts of two diastereoisomers: δ0.95 and 1.02 (m and two t, 9, C4-CH_3 plus C7-H_3, C1-H_3), 1.34 and 1.63 (m and m, 7, all CH_2 and C4-H), 3.48 (m, 1, C3-H). IR: 2100 cm^{-1} (s, NCS). MS (EI, 70 eV): *m/e* 171 (M^+).

3-Azido-4-methylheptane (**25**). A solution of 3-bromo-4-methylheptane (**32**) (5 mmol) in methanol (3 ml) was refluxed over solid NaN_3 (15 mmol) for 76 hr until all starting material had been consumed. The reaction mixture was diluted with water and extracted with ether in the usual manner. The crude product, after drying and then evaporating the ether, was placed on a column of silica gel and eluted with hexane. The desired fraction was stripped of solvent and distilled bulb-to-bulb under vacuum to give a colorless liquid in 88% yield. The diastereoisomeric composition could not be determined. NMR: δ0.90 (m, 6, C4-CH_3 and C7-H_3), 1.00 (t, 3, C1-H_3), 3.13 (m, 1, C3-H). IR: 2081 (s) and 1272 cm^{-1} (ms) (—N_3). MS (CI, pos., NH_3): *m/e* 128 ($M-N_2+H$).

3-(4-Methylheptyl) acetate (**26**). 4-Methyl-3-heptanol (**1**) was heated at 130° with an equal volume of acetic anhydride containing a trace of $ZnCl_2$ for 4 hr. The mixture was washed with water until pH > 5, then 10% K_2CO_3, dried overnight with Na_2SO_4, and distilled (bp 113–114°, 105 mm). Yield 64%. A 1:1 mixture of diastereoisomers is assumed on the basis of starting material composition. NMR: δ0.87 (m, 9, $C\underline{H}_3CH_2$— and $C\underline{H}_3CH$), 2.06 (s, 3,

CH_3CO), 4.75 (m, 1, —CH—O). IR: 1740 (C=O), 1245 cm^{-1} (C—O). MS (EI, 20 eV): *m/e* 112 ($M^+-CH_3CO_2H$), 43 (CH_3CO^+); (CI, pos., NH_3) 190 (M+18).

3-(4-Methylheptyl)formate (**27**). 4-Methyl-3-heptanol (**1**) was refluxed in formic acid for 0.5 hr. The mixture was partitioned between ether and water and worked up in the usual manner. The crude product after rotary evaporation of the ether was distilled under vacuum (bp 64°, 11 mm). Yield 86%. A 1 : 1 diastereoisomeric composition is inferred from the composition of **1**. NMR: δ0.89 (m, 9, all CH_3), 4.81 (m, 1, C3-H), 8.15 (s, 1, —CHO). IR: 1731 (C=O) and 1184 cm^{-1} (s, C—O). MS (CI, pos., NH_3): *m/e* 176 (M+18).

4-Methyl-3,4-epoxyheptane (**28**). A solution of *m*-chloroperbenzoic acid (0.09 mol) in benzene (250 ml) was added at a moderate rate to a solution of 4-methyl-3-heptene (**34**, 58% *Z*, 0.07 mol) in benzene (50 ml) and the solution stirred an additional 1.5 hr. The reaction mixture was then shaken with portions of 10% K_2SO_3 solution until neutral to starch–iodine paper, washed with 10% $NaHCO_3$ until neutral, washed with saturated NaCl, and dried with $CaCl_2$. The solvent was removed by fractional distillation at atmospheric pressure up to 81° and the product distilled under vacuum (bp 72°, 60 mm). Yield 78% of a mixture of isomers (60% *Z*). NMR: δ0.97 (m, 6, C1-H_3 and C7-H_3), 1.16 and 1.19 (two s, 3, C4-CH_3, ratio 1 : 1.5), 1.4 (m, 6, all —CH_2—), 2.46 (broad t, 1, C3-H). IR: 890 and 805 cm^{-1} (w, C—O—C). MS (EI, 70 eV): *m/e* 128 (M^+).

Methyl 2-methylvalerate (**29**). A solution of 2-methylvaleric acid (**30**, 0.3 mol) in methanol (200 ml) containing 3 Å molecular sieves and H_2SO_4 (2 ml) was refluxed for 1.5 hr. The reaction mixture was filtered and then worked up in the usual manner with ether. The product after rotary evaporation of the ether was distilled under vacuum (bp 54°, 35 mm). Yield 93%. NMR: δ0.90 (t, 3, —$CH_2C\underline{H}$), 1.14 (d, 3, —$CHC\underline{H}_3$), 1.32 and 1.63 (m and m, 4, —CH_2CH_2—), 2.45 (m, 1, CH), 3.66 (s, 3, CH_3O—). IR: 1741 (C=O) and 1145–1195 cm^{-1} (s, 3, max, C—O). MS (CI, pos., NH_3): *m/e* 148 (M+18).

3-Bromo-4-methylheptane (**32**). To a degassed solution of triphenylphosphine (0.178 mol) and 4-methyl-3-heptanol (0.17 mol) in dry DMF (175 ml) was added, with mechanical stirring, Br_2 (0.178 mol) dropwise over 45 min, maintaining room temperature with a water bath. After stirring an additional hour, all the volatiles were transferred under vacuum with gentle heating to a flask at −78°. This was diluted in a separatory funnel with 750 ml water, causing the product to separate as an upper layer which was dried ($MgSO_4$) and fractionally distilled to the desired product in 43% yield (bp 52–53°, 4 mm) and 4-methyl-3-heptene (58% *E*) in 8% yield. The alkyl halide is unstable above 100°. The diastereoisomeric composition was not determined. NMR: δ0.92 and 1.02 (m and m, 9, all CH_3), 4.00 (m, 1, CHBr). MS (EI, 70 eV): *m/e* 149, 151 ($M-C_3H_7$), 113 (M−Br).

1-(1-Fluoroethyl)-2-methylpentylamine (**36**), *and 1-(1,1-Difluoroethyl)-*

2-methylpentylamine (**37**). These compounds were prepared by reductive amination of ketones **12** and **13** in 84 and 76% yields, respectively, by the same procedure that was used for synthesis of **23**.

36 (bp 60°, 22 mm): The NMR is consistent with a nearly equal distribution of four diastereoisomers. NMR: δ0.92 (m, $CHC\underline{H}_3$ and $CH_2C\underline{H}_3$), 1.322, 1.335, 1.347, and 1.359 (all d of d, J_{FH} = 24.3–25.0 Hz, $C\underline{H}_3CHF$), 2.42–2.81 (m, 1, $C\underline{H}—NH_2$), 4.43–4.79 (m, 1, $—C\underline{H}F$). MS (EI, 20 eV): *m/e* 146 (M−1), 100 (M−CH_3CHF), 76 (M−C_5H_{11}).

37 (bp 65–66°, 30 mm). The diastereoisomeric composition was undetermined. NMR: δ0.91 (m, $CH_2C\underline{H}_3$), 1.01 (d, $CHC\underline{H}_3$), 1.62 (t, 3, J_{HF} = 19.1 Hz, $C\underline{H}_3CF_2$), 2.82 (m, 1, $C\underline{H}NH_2$). MS (EI, 20 eV): *m/e* 164 (M−1), 100 (M−CH_3CF_2), 94 (M−C_5H_{11}).

2,4-Dibromo-4-methyl-3-heptanone (**38**). A solution of 4-methyl-3-heptanone (**11**) (0.2 mol) in acetic acid (125 ml) was treated with bromine in portions until the orange color of bromine persisted (0.4–0.5 mol). Water was added and the product extracted with hexanes. After washing the organic layer with water, $NaHCO_3$ solution, and water, drying ($MgSO_4$) and stripping the solvent, the resulting oil was distilled under vacuum (bp 77–79°, 2 mm). Yield 80%. NMR showed equal amounts of two diastereoisomers: δ0.99 (two t, 3, C7-H_3), 1.45 (m, 2, C6-H_2), 1.82 and 2.01 (s and s, C4-CH_3), 1.88 and 1.86 (d and d, C1-H_3), 2.06 (m, C5-H_2), 5.16 (two q, 1, C2-H). IR 1721 cm^{-1} (C=O). MS (CI, pos., NH_3): *m/e* 302, 304, 306 (M+18).

RESULTS AND DISCUSSION

The results of the walking-beetle assay are given in Table 1. The standard dose of **1** was 50 beetle-hour-equivalents (BH) (Lanier, 1977), corresponding to about 4×10^{-13} mol of compound spotted on the filter paper. The analogs were tested at the same dose. All compounds were tested in the presence of 10 BH α-multistriatin and α-cubebene. The activity of each analog is graded on a 4-point scale (0 to +++). This scale is based on a percentage response which is corrected for the response of the control, α-multistriatin plus α-cubebene, and normalized to the response of the full natural pheromone (+++, defined as 100):

$$\text{Activity of analog} = \frac{\%\text{ response of analog} - \%\text{ response of control}}{\%\text{ response of } \mathbf{1} - \%\text{ response of control}} \times 100\%$$

The grades are thus:

++, 50–75%; +, 21–49%; 0/+, 10–20%; and 0, <10%.

The most active analogs (**2** and **19**) were tested additionally in the absence of α-multistriatin and α-cubebene and found to be inactive. Compound **1** itself is also inactive under these conditions. This suggests that the active

analogs are indeed interacting with the receptors of **1** and are able to substitute for **1** in the pheromone to give the desired response. A number of analogs were also tested at a dose of 5000 BH. These results are included in Table 1. Most of the compounds that were inactive at the 50-BH level were also inactive even at this 100-fold increase in concentration, except **22** and **25** which became weakly active. Activities of compounds that were positive at the 50-BH dose were either unchanged or modestly enhanced at the higher level. In no case did activity exceed the level observed for **1**. These experiments indicate that little is gained by swamping animals with inferior stimuli. These results also suggest that volatility differences among the compounds are not a problem in this study.

The results of the EAG experiments are given in Table 2 as maximum depolarization amplitudes expressed as a percent of **1**, which was presented at a nominal concentration of 3.6 μl/liter. This concentration was chosen as the standard dose because it represents a point lying in the linear portion of a plot of EAG amplitude vs. log [**1**] that is just below the point at which response begins to level off. The analogs were tested at this same dose.

A GC assay was used to determine the actual concentrations in the air of the dose flask, as prepared by the method described in Methods and Materials, for a few representative compounds (**1**, **4**, **20**, **26**, and **35** of Table 1). This assay showed that only a fraction of the calculated amount was present in the air; the remainder was presumably adsorbed on the surface of the glass. Adsorption of organic compounds from the vapor phase onto glass surfaces is commonplace (Jednacak-Biščan, 1980; Grob, 1980). The uncertainty that this introduces to the EAG experiment for all compounds is mitigated by the following observations: (1) The extent of adsorption was nearly the same for all of the representative compounds examined, 79–88%, except for hydrocarbon **35** (41%). (2) The EAG amplitude for compounds **5**, **20**, and **6** was essentially unchanged when the glass surface area in the flask was increased several-fold by increasing the number of glass beads in the flask. (3) The EAG response for **1**, at least, appears to be relatively insensitive to moderate concentration uncertainties because the slope of a plot of EAG response vs. log [**1**] reveals that a 2.3-fold change in concentration is necessary to cause a 10% change in EAG response.

Comparison of the average EAG scores in Table 2 at the standard dose with the walking-beetle test results of Table 1 leads to some useful correlations: (1) Analogs that give a positive behavioral response (0/+ or higher) generally give high EAG amplitudes (above ~72%). (2) There are qualitative correlations among members of certain series, particularly among and between the halogenated alcohols and ketones. (3) Analogs that show no behavioral activity in most cases produce low EAG amplitudes (below ~72%).

TABLE 1. LABORATORY OLFACTOMETER ACTIVITY OF 4-METHYL-3-HEPTANOL AND ANALOGS[a]

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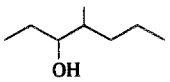
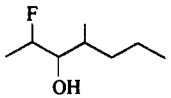
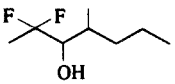
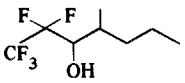
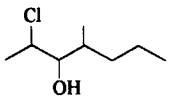
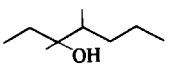
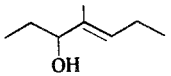
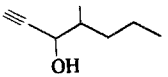
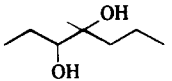
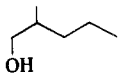
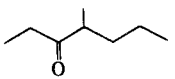
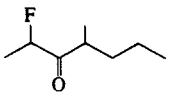
1[c]		+++	
2[c]		++ (7)[b]	++
3[c]		+ (7)	
4[c]		0 (2)	
5[c]		+ (7)	
6[c]		0/+ (6)	
7[d]		0 (6)	
8[c]		0 (4)	0
9[c]		+ (5)	++
10		0 (3)	0
11		+ (6)	
12[c]		0/+ (4)	

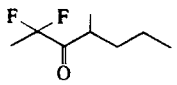
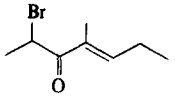
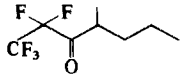
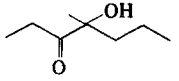
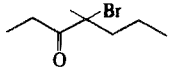
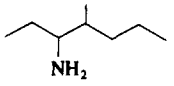
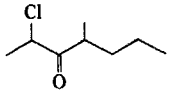
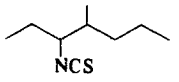
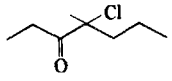
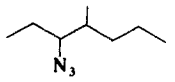
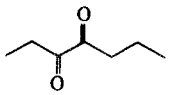
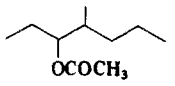
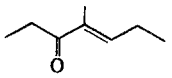
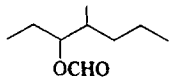
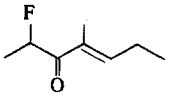
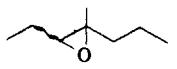
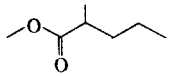
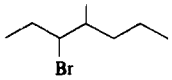
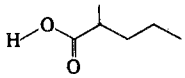
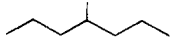
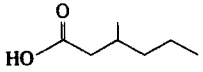
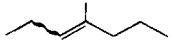
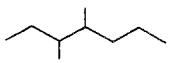
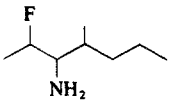
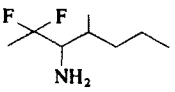
13		0 (2)		**21**[d]		0 (3)	
14		0 (2)		**22**		0 (2)	0/+
15		0 (2)		**23**[c]		0 (3)	0
16[c]		0 (3)		**24**[c]		0 (4)	
17[e]	 + **16**	0 (2)		**25**[c]		0 (3)	0/+
18		0 (2)		**26**[c]		0 (3)	
19[d]		++ (7)	++	**27**[c]		0/+ (3)	
20[d]		0 (3)		**28**[f]		+ (5)	+

TABLE 1. Continued[a]

Compound		Dose[b] 50 BH	Dose[b] 5000 BH	Compound		Dose[b] 50 BH	Dose[b] 5000 BH
29		+ (3)		**32**[c]		0 (6)	0
30		0/+ (4)	+	**33**		0 (2)	0
31		0 (2)		**34**[f]		0 (2)	0

35[c]		0 (2)
36[c]		0 (4)
37[c]		+ (4)

[a]Activity as defined in text: +++, 100% activity; ++, 50–75%; +, 21–49%; 0/+, 10–20%; 0, < 10%. Standard deviation 11–17 percentage points where determinable.
[b]BH = beetle-hour-equivalents (see text). Analog or **1** at stated dose plus 10 BH each of α-multistriatin and α-cubebene. Control composed of 10 BH each α-multistriatin and α-cubebene. Number in parentheses is number of replicates. 50 beetles per replicate.
[c]Mixture of diastereomers; see Methods and Materials.
[d]>95% *E* isomer.
[e]2:1 mixture of **16** and 4-Cl isomer.
[f]58% *Z* isomer.

TABLE 2. NORMALIZED EAG SCORES FOR ANALOGS[a]

	3.6 μg/cm³ dose													0.36 μg/cm³ dose	
	Experiment No.														
Analog	1	2	3	4	5	6	7	8	9	10	11	Average	Recovery index[b]	A	B
1	←					100					→	100	1.0	72	69
2						100	98			105	100	101	1.2	93	90
3						82	77			80	73	78	1.1		
4	63	53	77	72		53	45			47	47	57	1.3		
5						83	77			63	62	71	2.8		
6					107							107	1.0		
7					98	95	98					97	1.1	82	82
11			108	110	87			82	83			94	1.1	80	86
12								70	78			74	0.4		
13								55	48			59	0.5		
14			53	50				43	30			44	0.6		
17			92	90								91	0.7		
18								73	55			64	0.5		
19					87			82	77			82	0.9	83	88
20					77			78	63			73	0.6		
21								67	58			63	1.9		
23	117											117	1.2		
24	81	60										71	2.6		
26	78	68										72	1.0		
29	90	78										84	0.5		
38			57		57							57	2.4		
Air blank	12	5	6	4	2	28	22	24	13	18	16			7	8

[a] Analogs numbered as in Table 1. Values in percent response normalized to **1** = 100% at 3.6 μg/cm³. Numbers are averages of at least 3 measurements (0.15 mV average deviation). Numbers separated by ≤5 percentage points are not significantly different. Response to **1** varied individually from 4.1 to 6.0 mV. Experiments 1, 3, 5, 7, 8, 10, and B were on females.

[b] Average time (sec) from peak depolarization to half way to resting potential normalized to **1** = 1.0.

Compounds **2**, **7**, **11**, and **19** were also assayed at a 10-fold lower concentration, at which they produced considerably higher EAG amplitudes than **1** at the same concentration. This is in contrast to the situation at the higher concentration where their responses are less than or equal to that of **1**. Thus these compounds become more stimulating to the antenna than **1** as the concentration is lowered. Also, **6** and **23** surpass **1** at the higher dose. But since all of these analogs are inferior to **1** in the behavioral tests, these observations imply that analogs are interacting with more than one receptor site type. Thus, **1** may also be interacting with several receptor types. Additional evidence for this has been found in single-cell electrophysiological measurements; cell-to-cell variability in the order of response among a set of odorants including **1** has been observed (R. O'Connell and A. Grant, unpublished).

The EAG recovery index is listed in Table 2 for each analog. The index is a relative measure (compared to **1**) of the time required to return from peak depolarization to halfway to the resting potential. EAG recovery rates have been proposed to be a function of molecular fit to receptors (Roelofs, 1971) or differences in the time required for deactivation of the molecules (i.e., passive diffusion away from the sites) (Kaissling, 1974). The substituted ketones **12–18** and **20** have considerably shorter recovery times than **1** or their parent ketones **11** and **19**. Ester **29** also has a shorter recovery time. In contrast, the brominated ketones **21** and **38** and the isothiocyanato compound **24** have much longer recovery times. These results merit further study, particularly in view of the observation that isothiocyanates can bind covalently to nucleophilic groups at membrane receptor sites (Rice, 1979). All behaviorally active compounds except ester **29** have recovery times similar to **1**, but recovery time appears not to be a good predictor of behavioral activity for this insect.

The results of field trials using baited sticky traps are shown in Table 3. The analogs tested were those that scored 0/+ or greater in the walking-beetle assay or that gave a very high EAG response (e.g., **7** and **23**). Trap catches for analog + multistriatin + cubebene are much lower than the full natural pheromone and are not significantly different from control alone (multistriatin + cubebene) in any instance.

Structure–Activity Correlations. The results of the field tests show that they are not useful for establishing a structure–activity relationship (SAR) in this case. The lack of field activity for analogs that otherwise attract beetles in the laboratory could be due to the following: (1) The flight response of beetles to reach traps in the field probably involves more individual kinds of behavior steps than the simple turning response of walking beetles in the laboratory. If **1** is necessary to trigger each of these steps and if an analog is inferior to **1** for each step, the overall activity can diminish to a low value when a multiplicity of steps exists. (2) Analog may promote certain behavior, in addition to the desired behavior, that diverts beetles from their flight to the traps. (3) The concentration of chemical in the field may be much lower than that used in the

TABLE 3. RESULTS OF FIELD TRIALS FOR ANALOGS USING BAITED STICKY TRAPS[a]

Analog	Location[b]-year	Trap catch[c] Relative to 1 + M + C = 100	
		Analog + M + C	M + C
2	SYR-78	3.5 (4) ± 5.9[d]	0.7 ± 0.4[d]
5	SLP-81	1.8 (3)	1.1 ± 0.4
6	SLP-81	1.6 ± 1.0	1.1 ± 0.4
7	SLP-80	4.1 (1)	3.0
9	SLP-81	0.7 ± 0.5	1.3 ± 0.6
11	SLP-81	2.4 ± 1.4	1.3 ± 0.6
19	SYR-78	1.1 ± 0.7	0.7 ± 0.4
23	SLP-81	1.5 ± 0.8	1.1 ± 0.4
24	SYR-80	6.0 ± 5.2	8.5 ± 8.2
28	SLP-81	1.2 ± 0.7	1.3 ± 0.6
29	SLP-81	1.6 ± 0.7	1.3 ± 0.6
30	SLP-81	0.8 ± 0.3	1.1 ± 0.4
36	SLP-81	11.3 ± 8.8	7.3 ± 5.2
37	SLP-81	3.5 ± 2.4	7.3 ± 5.2

[a]Analogs numbered as in Table 1. M = α-multistriatin, C = α-cubebene. Dose: 10^7 BH per trap [20 μl **1** or analog, 2 μl M, 14 μl of cubeb oil (70% C)]. Duration 15–29 days. Average trap catch for 1 + M + C for each experiment varied from 165 to 1883 beetles.
[b]SYR = Syracuse, New York; SLP = St. Louis Park, Minnesota.
[c]Five replicates unless indicated otherwise in parentheses.
[d]Standard deviation.

laboratory test. This, together with the assumption that biological activity decreases more rapidly with decreasing concentration for analogs than for **1**, could lead to the observed results. However, the EAG tests show that the EAG amplitude for some of the behaviorally active compounds drops off less rapidly with concentration than does **1**.

The usefulness of the EAG test for establishing a SAR is impaired by the fact that the receptor neurons are likely to be of the "generalist" type that is found for other bark beetles (Priesner, 1979). Nevertheless, a rough correlation can be seen between the EAG amplitude and the activity in the walking-beetle assay for most compounds that were assayed by both techniques. It is possible that the high EAG activities of some analogs that do not respond in the walking-beetle assay (**7**, **17**, and **23**) implicates their importance in some other type of behavior.

A SAR may be examined on the basis of the walking-beetle bioassay. This assay demonstrates the ability of some analogs to substitute for **1** in triggering the positive anemotactic response that is associated with **1**. The EAG results show that these same active compounds interact strongly with

antennal receptors. One way to visualize the mechanism of olfaction is to consider the behavioral response to be directly related to the binding interaction of stimulant to receptors, although it may ultimately be found that the chemoreceptive process leading to a certain behavior is more complicated than this. Since it is presumed that there are multiple receptors for **1**, we must consider the SAR to be a composite view of receptors. A structure–activity correlation may be discernible if certain physicochemical parameters are more important than others for all receptor types or if certain receptor types dominate in generation of the stimulation pattern that leads to the response. These points are kept in mind in the following discussion.

Studies of biological SAR, principally in relation to pharmacological drug evaluations, have recognized the following intermolecular forces in substrate–receptor interactions: electrostatic attraction between charges or partial charges, steric repulsion, hydrophobic bonding, hydrogen bonding, and short range or van der Waals attractions (dispersion, orientation, dipole–dipole, etc.) (Hansch, 1973, 1979). In addition, intramolecular forces in the substrate that affect its conformation may be important, depending on the conformational requirements of the receptor site (Hopfinger, 1980).

A parameter that has been found to be of great, if not primary, importance (Hansch, 1973) in drug–receptor interactions is the hydrophobic bonding parameter Π, the log of the partition coefficient between *n*-octanol and water relative to a standard compound (**1** in this case, for which $\Pi = 0$) (Leo, 1971). In the oriental fruit fly, Π has been shown to be an important descriptor of activity in chemoreception of analogs of the feeding stimulant methyl eugenol (Metcalf, 1981). Figure 1 shows the Π value as a function of activity in the walking-beetle assay. A positive value indicates that the analog is more hydrophobic than **1**. It would appear from these data that the hydrophobic interaction is not a major determinant of activity in this case. Compounds showing significant activity span a wide range of Π values (+0.2 to −1.2) and inactive compounds are represented throughout the range from −1.4 to +1.8. Some dependence on Π is not ruled out but cannot be evaluated without a more quantitative measure of analog activity.

Dependence on the hydrophobic parameter in biological SARs reflects energy changes in the transport of substrates from an aqueous phase (e.g., tissue fluids) to the relatively nonpolar phase of receptor macromolecules. The energy of the "hydrophobic bond" arises principally from the negative entropy associated with ordering water molecules around the nonpolar portions of molecules in the aqueous phase, and not on any special attractive force between nonpolar molecules (Leo, 1971, and references therein). A lack of correlation with the partition coefficient parameter may therefore imply that **1** and analogs do not pass through an aqueous phase during transport to receptor sites. There has been speculation that odorants pass through a "sensillum liquor" or fluid in pore tubules before reaching receptors on the

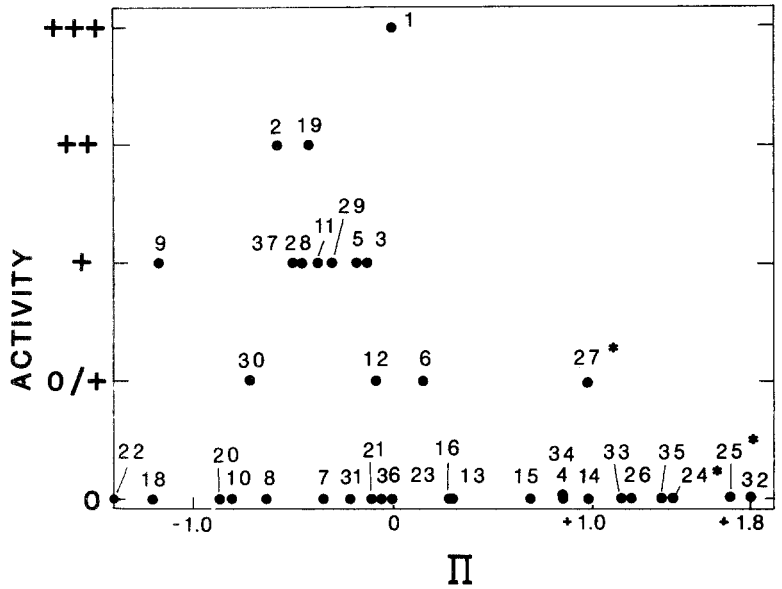


FIG. 1. Activity according to the walking-beetle assay vs. the hydrophobic parameter Π for analogs (numbered as in Table 1). Starred numbers indicate minimum values for Π. Π for **9**, **15**, **21**, **22**, **23**, **31**, and **33–35** are calculated according to substituent additivity rules (Leo, 1971). Uncertainty in experimental Π is less than ±0.07 for values <0.5 and ±0.10 for values >0.5.

dendrites (reviewed by Seabrook, 1977, and Kaissling, 1971). In the case of *S. multistriatus*, the dendrites are in very close proximity or in contact with the pore tubules (Borg, 1971). Our results support direct contact of stimulant in the gas phase with receptors on the membrane surface or contact via a lipid phase only.

Figure 2 shows a plot of activity in the walking-beetle assay vs. the molar refractivity parameter MR, which has been used as a measure of the importance of short range (van der Waals) forces in chemoreception (Hansch, 1979; Goldblum, 1981). MR is proportional to ionization potential and polarizability and can be calculated from molecular fragment values using additivity rules (Hansch, 1979). The active compounds (0/+ or higher) span a wide range of MR values. Most strongly active compounds (+ or higher) are distributed over a relatively narrow portion of the range (−2 to +2). However, there are more inactive compounds in this range than active ones. Therefore, it must be concluded that this parameter also does not dominate activity.

Receptor macromolecules are thought to be membrane proteins of the nerve cell dendrites. Some indirect evidence exists to support this (Beets, 1973; Kaissling, 1971, 1977). If true, one might expect the hydroxyl group of **1** to

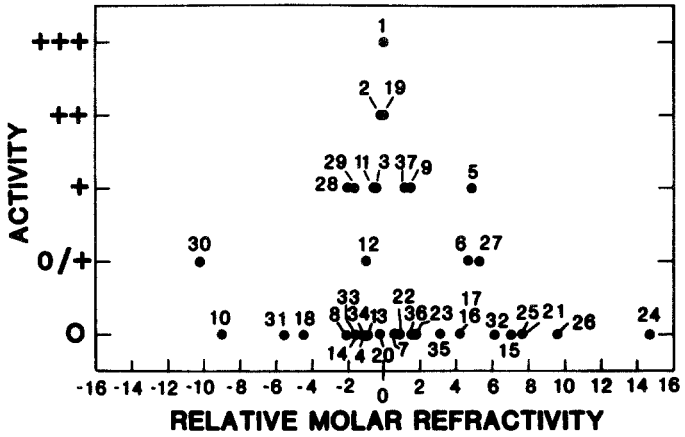


FIG. 2. Activity according to the walking-beetle assay vs. molar refractivity (MR) for analogs (numbered as in Table 1) calculated according to substituent additivity rules (Hansch, 1979). Epoxide **28** was regarded as **1** minus two hydrogen atoms.

interact electrostatically with one of the numerous polar functional groups available in a protein. Examination of Table 1 shows that activity decreases with increasing fluorine substitution for alcohols (**1–4**), ketones (**11–14**), and α,β-unsaturated ketones (**19, 20**). Activity also declines with substitution of Cl (**1** > **5**, **11** > **16**), Br (**11** > **15**, **19** > **21**), and OH (**1** > **9**, **11** > **22**), as well. These substituents may act by decreasing electron density on the 3-oxygen atom, thereby diminishing interactions of the stimulant with receptors that depend on this property. The electron-withdrawing inductive property of F, Cl, Br, and OH is apparent from their σ^* values of 1.1, 1.05, 1.00, and 0.55, respectively (σ^* is derived from hydrolysis of esters $X—CH_2CO_2C_2H_5$; Taft, 1956).

Superimposed on this electronic substituent effect may be a steric effect, since activity is also correlated with the Taft steric parameter, E_s (Hansch, 1973; Taft, 1956), i.e., F > OH (**2** vs. **9**) and F > Cl (**2** vs. **5**) for alcohols; F > OH, Cl, Br (**12** vs. **22**, **16**, **15**) for ketones; and F > F_2 > F_5 for alcohols and ketones. The relative steric size of these groups based on the E_s parameter is F < OH << Cl < Br. The EAG scores also show correlation with both σ^* and E_s; relative EAG amplitude decreases with increasing fluorine substitution and F > Cl > Br, for all individual beetles tested, without exception (Table 2).

A possible interpretation of the electronic substituent effects, if real, is that the oxygen at the 3-position of the alcohols is participating in a hydrogen bond as a proton acceptor and that this interaction is important in one or more receptor-stimulant interactions that govern or dominate the behavioral

response. In support of that interpretation, electron-withdrawing substituents near the hydroxyl group are known to decrease the basicity and consequently the hydrogen bond strength to a given proton donor. This has not been confirmed experimentally for alcohols because of complications due to the ambident nature of the hydroxyl group. However, it is in keeping with a theoretical study of fluorinated ethanols with water (Kollman, 1973) and experimentally with substituent effects in ethers (reviewed by Murthy, 1968, and Vinogradov, 1971), which can be expected to behave similar to alcohols.

Stimulant hydrogen bonding as proton acceptor can also explain the results for the ketones. The results of the walking-beetle tests (Table 1) show that the alcohols are more active than the corresponding ketones (i.e., **1** > **11**, **2** > **12**, **3** > **13**, **5** > **16**, **9** > **22**). This trend is also observed in the EAG experiments (Table 2). The hydrogen-bond strengths of ketones are usually lower than alcohols to a common proton donor, but not greatly so—normally about 0–2 kcal/mol less, upon examination of collected data (Murthy, 1968); cf., ΔH of phenol dimer = 5.1 ($\pm$0.1) and ΔH of phenol plus *p*-benzoquinone = 5.05 ($\pm$0.55) kcal/mol (CCl_4, 30° C); ΔH of *t*-butanol dimer = 4.8 ($\pm$1.1) and ΔH of *t*-butanol plus acetone = 2.94 kcal/mol (CCl_4). Also, there is extensive documentation of the weakening effects of electron-withdrawing groups on the carbonyl oxygen-to-proton hydrogen bond. It seems unlikely that steric interference to fit at receptor sites is responsible for the diminished activity of the ketones, because the C=O group is slightly smaller than CH—OH.

The ability to form a hydrogen bond may also explain the positive activity of analogs with other functional groups. Epoxide **28** can be expected to form a hydrogen bond that is somewhat weaker than a comparable open chain ether and, thus, somewhat weaker than **1**; cf., ΔH for phenol with 1,2-epoxy-2-methylpropane = 5.26, and phenol with diethyl ether = 5.41 kcal/mol (West, 1964). Its activity (+) is consistent with this. The activity of ester **29** (+) is consistent with the tendency of esters to form hydrogen bonds that are similar in strength to the analogous ketone ($\Delta\Delta H$ = 0.4 for $CH_3C(O)—X—C_2H_5$ and 0.8 kcal/mol for $CH_3C(O)—X—CH_3$ with phenol, where X = CH_2, O; Murthy, 1968). The activity of carboxylic acid **30** (0/+) is in line with the expectation that it forms a weaker hydrogen bond than the corresponding ester **29**. The strength of a hydrogen bond to a carboxylic acid from a proton donor is unknown because of experimental complications due to self-association of the acid. The difluoroamine **37** will be discussed in connection with the other amines (see below).

The absence of activity in other analogs listed in Table 1 may be explained in steric and/or hydrogen bonding terms by: (1) absence of a hetero atom at the 3-position (corresponding to **1**) that possesses an unshared electron pair for participation in a hydrogen bond, (2) insufficient availability

of an electron pair on the hetero atom at the 3-position for participation in a hydrogen bond, or (3) steric interference to fit in receptor sites near position 3.

Hydrocarbons **33–35** and acid **31** fall under category (1). Bromide **32** falls under category (2). The electron pairs of the 3-nitrogen or oxygen atoms of **24–27** are extensively delocalized [category (2)] and also steric interference at the 3-position may be important [category (3)]. Alcohol **6**, which has borderline activity, may be constrained sterically. Alcohol **10** is lacking a C_2 unit in the carbon chain. Chain-shortening has been found to have a deleterious effect on activity in several other pheromone SAR studies (see Introduction). Alchols **7**, and **8** are more difficult to place in these categories. They may be restricted to poorly fitting configurations by the double and triple bonds, respectively. It is, however, difficult to reconcile the inactivity of **7** with the high activity of **19**. In addition, **7**, **8**, and **10** can be expected to have a lower electron density on the oxygen atom than **1** due to the electron-withdrawing effects of the unsaturated groups (**7** and **8**) or the absence of the electron-donating C_2H_5 group (**10**).

The trend in the amine series **23**, **36**, and **37** can also be explained in the context of the hydrogen-bonding proposal. Since amines form stronger hydrogen bonds than ethers [ΔH for phenol to di-*n*-alkyl ethers = 5.2–5.7 (West, 1964) and ΔH for phenol to tri-*n*-alkyl amines = 5.9–9.1 kcal/mol, Murthy, 1968], amine **23** is likely to be a stronger hydrogen bonder than **1**. Successive fluorination of **23** to the difluoro compound **37** may be required to reduce the nitrogen electron density enough to bring it in range for proper interaction with receptors.

One example of a situation which could be very sensitive to the basicity of the stimulant is when the pK_a of the stimulant is close to the pK_a of the proton donor group on the receptor. As stimulant basicity approaches and exceeds that of the receptor donor, there will occur a transition from a hydrogen bond where the proton remains essentially on the donor group (STIM—X $\cdots$ H—REC) to one where the proton is transferred to the stimulant (STIM—$\overset{+}{\text{X}}$H $\cdots$ $\bar{\text{R}}$EC). Internuclear distances in the receptor and/or the resulting charge on the receptor, either of which could be important in chemoreception, would change significantly during this transition. The pK_as of **23**, **36**, and **37** are close to the values for the corresponding ethyl amines: 10.8, 8.8, and 7.1, respectively (Love, 1968). Donor groups that have pK_as of about 9 in a proteinaceous receptor include the hydroxyl of tyrosine (pK_a = 10) and a variety of protonated amino groups (pK_as 9–10). With these donors, the difluoro amine will form a hydrogen bond qualitatively similar to that of **1** while **23** and **36** may not.

How does the efficacy of a substrate relate to the strength of its hydrogen bond to receptors? The mechanism of cell excitation is unknown but the evidence favors a stimulant-induced change in conformation of a receptor

molecule leading to ion-channel opening and a flux of ions across the membrane (Kaissling, 1977). The strength of a stimulant–receptor hydrogen bond could affect the length of time a molecule spends at a receptor site (i.e., binding constant) or the energy required for the stimulant–receptor complex to attain an "active state," which could be proper conformation to open ion channels. In principle, either of these factors can affect the elementary receptor potential of a receptor site and, consequently, the impulse frequency of a cell (Kaissling, 1971, 1974).

The length of time a stimulant spends at a receptor site is related to the binding constant, and this mechanism predicts that increasing the concentration of an inferior stimulant in the external medium (air) can compensate for a reduced binding constant. Our experiments with walking-beetles show that activity is not much affected by a 100-fold increase in concentration.

The case requiring attainment of an active state after initial binding of the stimulant in the receptor cavity can more closely conform to our results. In this scheme, chemoreception is viewed as a two-step mechanism. Binding is dictated largely by molecular size and shape factors. The results reported here indicate that reception is sensitive to steric effects. Also, the fact that only one of four possible stereoisomers of **1** is active is further evidence that molecular shape is important. The active state is then reached by an additional, reversible transformation within the stimulant–receptor complex. This transformation is governed by the strength of a hydrogen bond between the 3-heteroatom of a stimulant and a proton donor on the receptor. This mechanism may explain why increasing the dose of inferior substrates does not increase attractiveness; activity depends on the ability to attain the active state, not solely on the number of receptor sites occupied.

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IDENTIFICATION OF A SEX PHEROMONE PRODUCED BY FEMALE VELVETBEAN CATERPILLAR MOTH[1,2]

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Abstract—A sex pheromone produced by female velvetbean caterpillar moths, *Anticarsia gemmatalis* Hübner, that attracts conspecific males was isolated and identified as a blend of (*Z,Z,Z*)-3,6,9-eicosatriene and (*Z,Z,Z*)-3,6,9-heneicosatriene in a ratio of ca. 5:3, respectively, when combined. The synthesized compounds elicited responses by velvetbean caterpillar moth males equivalent to those elicited by females in both laboratory wind tunnel bioassays and field trapping experiments.

Key Words—Sex pheromone, 3,6,9-heneicosatriene, 3,6,9-eicosatriene, velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, Lepidoptera, Noctuidae, attractant, hydrocarbons.

INTRODUCTION

The velvetbean caterpillar moth (VBC), *Anticarsia gemmatalis* Hübner, is a major pest of soybeans in North and South America. It is presently classified as an underwing moth in the noctuid family, subfamily Catocalinae, Group 5 (Crumb, 1956), or Erebiinae, Group 2 (Forbes, 1954).

[1]Lepidoptera: Noctuidae.

[2]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

[3]Employed through a cooperative agreement between the Department of Entomology & Nematology, University of Florida, and the Insect Attractants, Behavior, and Basic Biology Research Laboratory, Gainesville, Florida 32604.

The response of VBC males to calling conspecific females in screen cages placed over soybean plants was first observed by Greene et al. (1973). Subsequently, Johnson et al. (1981) reported that calling females and ether extracts of calling females elicited upwind taxis and occasional clasper extension by males in laboratory assays. They also found that electric grid traps baited with virgin female VBC captured significantly more feral VBC males than those baited with mated VBC females in tests conducted in soybean fields. Thus there is substantial evidence for pheromone emission by virgin female VBC that attracts conspecific males.

We report here the isolation and identification of a sex pheromone from the female VBC and the response of conspecific males to the synthesized pheromone in the laboratory and the field. The pheromone consists of (*Z*,*Z*,*Z*)-3,6,9-eicosatriene and (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene.

METHODS AND MATERIALS

Pheromone Extraction and Bioassay. Insects were obtained from a colony maintained at the Insect Attractants, Behavior and Basic Biology Research Laboratory (Greene et al., 1976) originally established with larvae obtained from Florida and infused annually with eggs from adults collected near Gainesville. Daily batches of pupae were sexed; the males and females were held for emergence in separate 16 × 16 × 16-cm Plexiglas cages housed in different controlled-temperature rooms. About 50 moths were maintained in each cage and provided with liquid food (50 g sucrose + 0.1 g ascorbic acid + 5 ml unprocessed honey dissolved in 1000 ml H_2O and dispensed in 60-ml cotton-filled paper cups). Conditions were 26° ± 2°C and 65 ± 5% relative humidity with a 14 ± 0.2-hr photophase (lights on at 1900 hr EST, 4-F40 CWX and 4-F40 GRO lamps, 310–750 nm, ca. 500 lux). Everyday at 0830 the pupae accumulated from successive batches were transferred to new cages, leaving the moths that had emerged during the previous 24 hr. At 4 ± 1 day after emergence, the females were extracted to provide crude pheromone and the males were used for bioassay.

Typically, 50–100 virgin female moths were placed in a jar and sufficient ether was added to cover the moths. After 1 hr the bodies and other solid materials were removed, and washed with an additional 50 ml of ether. The combined ether rinses were filtered through a Whatman No. 1 filter paper and then concentrated to ca. 10 ml by distillation at atmospheric pressure. The concentrated crude extract was stored at −60° C.

A laboratory bioassay was used to monitor all extracts and chromatographic fractions. Bioassays were conducted daily from 1300 to 1600 hr, 4–7 hr after the beginning of scotophase. A 2.4-m × 43.7-cm-diam Plexiglas observation tunnel was used to confine the moths. Conditioned and filtered

air was drawn through the tunnel from an upwind plenum at a rate of 0.75 m/sec (measured in the center of the tunnel at the male release point). The plenum produced a central cone-shaped plume from exposed $TiCl_4$ that extended to the sides of the tunnel at a point about 1 m from its origin. A red lamp placed above the upwind end of the tunnel produced a light intensity gradient ranging from 54 lux at the plenum to 2.7 lux at the downwind screen. The red light alone did not induce taxis.

Samples [2 female equivalents (FE) in 0.25 ml of hexane] were tested by placing them on a triangular target made of black construction paper, cut to resemble the silhouette of a VBC moth, and stapled to the center of a white 5.5-cm-diam Whatman No. 1 filter paper. This pheromone source was suspended in the center of the upwind end of the tunnel on a wire attached to a pedicel. After allowing 5 min for the solvent to evaporate and be exhausted, an individual male was collected in a 38-ml plastic tube with aluminum screen covering one end and released manually by means of a port located in the bottom of the tunnel ca. 1 m from the target. The elapsed times of male response were recorded from exposure to leaving the tube and from first flight to hovering in front of the target. Extension of the genitalia, with associated hairpencils, and darting back and forth in front of the pheromone source confirmed male courtship behavior. Moths that flew to the source and exhibited male courtship behavior within 2 min of exposure were scored positive; any other behavior was considered negative. Each moth was vacuumed from the tunnel prior to testing another; 10 males were exposed in rapid succession to each sample. The tunnel was rinsed with 70% EtOH and allowed to dry after a pheromone source was removed and 3 moths were exposed to an untreated target before the next test.

Field bioassays were conducted in August–October 1981 in soybean fields using cone traps (Hartstack et al., 1979), each baited with a wire mesh cage containing three 2-day-old virgin female moths or with 1/2 of a rubber septum (A.H. Thomas #8753-D22, Philadelphia, Pennsylvania, 5 × 9 mm, split lengthwise and extracted with methylene chloride for 1 hr) impregnated with synthetic pheromone. A randomized complete block experimental design with two blocks was used. Each block consisted of a row of traps, 30 m apart, containing randomly assigned baits. Blocks were at least 150 m apart. The baits in each block were systematically rotated in the originally assigned order through the trapping locations by moving them each time the traps were checked (daily, Monday through Friday). Each collection date constituted a replication.

Isolation. The concentrated crude female wash was first chromatographed on a gravity flow glass column (20 × 5 cm ID) prepared by slurry packing 100 g of 60–100 mesh silica (J.T. Baker Chemical Co., Phillipsburg, New Jersey) in hexane. The active fraction from the gravity-flow column was concentrated and injected onto a high-performance liquid chromatography

(HPLC) column (25 × 1.25 cm OD) packed with BioSil A, containing 2–10 μm silica (Heath et al., 1978). Hexane was used as the mobile phase at a flow rate of 2.5 ml/min. The active material from the BioSil A HPLC column was rechromatographed on a second 25-cm × 1.25-cm-OD silica column containing 5 μm silica gel (Lichrosorb). The material was eluted with hexane at 2.5 ml/min. The fraction from the Lichrosorb HPLC column was then chromatographed on a 25-cm × 1.25-cm-OD HPLC column packed with 5% $AgNO_3$ on 5 μm silica (Heath et al., 1977). Toluene was used as the mobile phase at a flow rate of 2.5 ml/min.

All micropreparative gas–liquid chromatography (GLC) was performed with a Varian model 1400 gas chromatograph (GC) equipped with a flame-ionization detector. Stainless-steel columns were used, and the effluent from the packed columns was split with 2% of the effluent routed to the detector, and 98% collected in a cooled, 30-cm glass capillary tube (Brownlee and Silverstein, 1968). The active fraction from the $AgNO_3$ HPLC column was concentrated and further purified by GLC on a 2-m × 2.3-mm-ID column packed with 4.4% OV-101 on 80–100 mesh Chromosorb G-HP; the column temperature was programmed from 150 to 220°C at 10°/min. Helium was used as the carrier gas at 20 ml/min. The active fraction from the OV-101 column was then chromatographed on a 2-m × 2.3-mm-ID column packed with 3.2% Carbowax 20 M on 100–200 mesh Chromosorb G-HP. The column temperature was programmed from 110 to 190°C at 4°/min. Helium (20 ml/min) was used as the carrier gas. Two active fractions from the Carbowax 20 M were individually purified on a 2-m × 2.3-mm-ID column column packed with 5.3% cholesterol *para*-chlorocinnamate (liquid crystal phase) on 80–100 mesh Chromosorb G-HP operated isothermally at 175°C, with a He carrier gas at a flow of 20 ml/min (Heath et al., 1979).

The active fractions collected from packed GLC columns were analyzed to determine their purity on a 32-m × 0.20-mm-ID cholesterol *para*-cholorcinnamate (Heath et al., 1979) and a 54-m × 0.2-mm-ID OV-101 glass capillary column with He carrier gas (linear flow velocity of 18 cm/sec).

Identification. The active components of the pheromone were identified by infrared, proton nuclear magnetic resonance (NMR), and chemical ionization mass spectrometry (CI-MS). Additional structural information on the compounds was derived from the mass spectra obtained on the products of microhydrogenation and microozonolysis.

All mass spectra were obtained with a Finnigan model 1015C chemical ionization mass spectrometer equipped with a gas chromatographic inlet. The total effluent of a 2-m × 2.3-mm OD column packed with 3% OV-101 on 100–120 Chromosorb W was introduced directly into the ionization source. Methane was the GLC carrier and reagent gas. Data acquisition and reduction were accomplished with a System Industries 150 computer interfaced to the mass spectrometer.

The proton magnetic resonance spectra were obtained with a Nicolet 300-MHz spectrometer. Approximately 5 μg of a pure component was placed in a 25-mm capillary NMR tube (Wilmad, Buena, New Jersey) containing 15 μl of deuterated benzene. Over a 2-hr period, 2000 transients were acquired with a 90° tip angle.

The infrared spectra were obtained with a Nicolet 7190 Fourier transform interferometer equipped with a 4X beam condenser and a mercury cadmium telluride liquid N_2-cooled detector. Spectra of the individual components were obtained on ca. 400 ng of material which was deposited as a thin film on micro KBr pellets. The spectra obtained were ratioed against an equal number of transients obtained on a KBr blank.

The individual pheromone components were reduced in the inlet of the gas chromatograph with hydrogen and neutral palladium catalyst (Beroza and Sarmiento, 1966), equipped with a 2-m × 2.3-mm Carbowax 20 M column. Microozonolysis of each pheromone component was carried out in CS_2 at −70° C, and the ozonide was reduced with triphenylphosphine (Beroza and Bierl, 1967). The ozonolysis and hydrogenation products were analyzed by CIMS. When possible, synthetic material was used to confirm the identity of microdegradative products and also to verify spectral data.

Synthesis. The preparations of (*Z*, *Z*, *Z*)-3,6,9-eicosatriene (III) and (*Z*, *Z*, *Z*)-3,6,9-heneicosatriene (IV) were accomplished by treatment of the tosylate of linolenyl alcohol (II) with the appropriate lithium dialkylcuprate (Figure 1) (Conner et al., 1980). The linolenyl alcohol (I) (Sigma Chemical Co.) was stirred with 1.1 equivalents of *p*-toluenesulfonyl chloride in pyridine at 4° C for 24 hr. Aqueous work-up followed by silica gel chromatograph (10% ether–hexane) afforded pure (*Z*, *Z*, *Z*)-9,12,15-octadecatrien-1-ol *p*-toluenesulfonate (II). The tosylate was treated with 2.5 equivalents of lithium diethylcuprate or lithium di-*n*-propylcuprate, in ethyl ether at −20° C for 5 hr. The reactions were quenched with saturated aqueous ammonium choride, and the hexane extracts were concentrated and chromatographed over silica gel (hexane) to afford 3,6,9-eicosatriene (III), or 3,6,9-heneicosatriene (IV), respectively. The synthetic materials were purified on a 20% $AgNO_3$ silica gel HPLC column, 25 cm × 1.25 cm OD, and were eluted with toluene at a flow rate of 4.0 ml/min.

RESULTS AND DISCUSSION

The crude extract consistently elicited a positive response from 80% or more of the moths in the laboratory bioassay. All of the activity of the crude extract was recovered in the hexane eluant (450 ml) of the gravity-flow silica column. Attractiveness was not increased by the addition of the more polar components obtained by further elution with 20% ether–hexane. The active

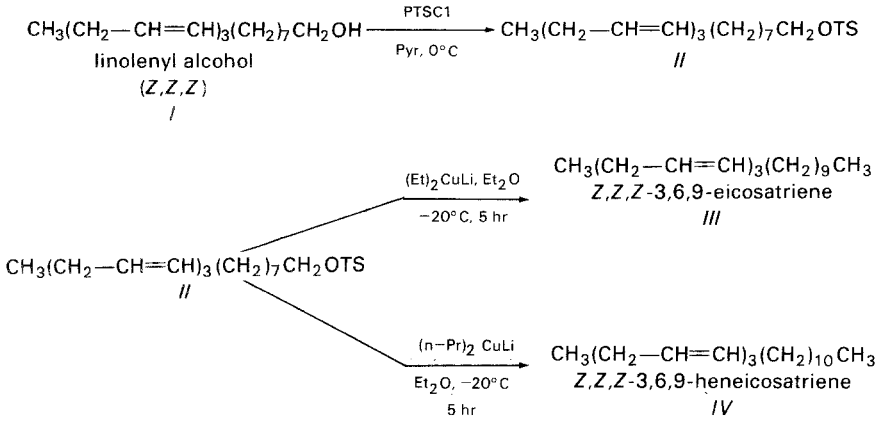

FIG. 1. Synthesis of (*Z*,*Z*,*Z*)-3,6,9-eicosatriene and (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene.

fraction from the gravity flow column was subsequently purified on two 1/2 in. HPLC silica columns. This two-step purification process was required to reduce the overloading caused by the bulk of the associated inactive crude material, which was also hydrocarbon in nature. Total pheromone activity was obtained from the first silica HPLC column in the fraction that eluted between a column capacity ratio (k') of 0 and 2. When this partially purified material was chromatographed on a second more efficient silica HPLC column, the active material eluted between a k' of 0 and 1. After the hydrocarbon content was reduced without loss of biological activity, the pheromone was further purified on a 5% $AgNO_3$ silica HPLC column.

All pheromone activity of the original crude extract was retained in the 20- to 48-ml ($k' = 1$–6) fractions collected from this column. The active material obtained from sequential liquid chromatographic fractionations was further purified by GLC on a packed OV-101 column. All the activity was contained in a 4-min fraction with Kovats index (KI) = 1914–2080. Further purification on a Carbowax 20 M column yielded two distinct peaks eluting at 9 and 11 min. Recombination of these two components was required for full activity in the laboratory bioassay. Individually, these fractions did occasionally show erratic activity. The 9-min peak from the Carbowax 20 M column appeared as a single peak with a KI of 1938 when chromatographed on a packed cholesterol *para*-chlorocinnamate column. The 11-min peak from the Carbowax 20 M column was resolved into two peaks on the liquid crystal column; a minor peak (KI = 2009) and a major peak (KI = 2036). Only the peak with a KI of 2036 when combined with the peak having a KI of 1938 produced a blend with full activity. The minor impurity did not increase or decrease the biological attractiveness when added to the other two components.

The two active peaks collected from the packed liquid crystal column were each found to be >99.9% pure when analyzed on the cholesterol *para*-chlorocinnamate and OV-101 capillary columns.

Methane ionization mass spectra were obtained on the purified active components. A molecular weight of 276 was established for the first eluting peak (KI = 1959) from the OV-101 column which was used as the inlet to the mass spectrometer. The mass spectra contained ions at *m/e* 275 (M−1), 277 (M+1), 305 (M+29), and 317 (M+41) as shown in Figure 2A. The fragmentation pattern for the second eluting component from the OV-101 column (KI = 2062) was similar except that the M−1, M+1, M+29, and M+41 were 14 mass units higher, resulting in a larger molecular weight assignment of 290 (Figure 2B). Molecular formulae of $C_{20}H_{36}$ and $C_{21}H_{38}$ were assigned to the two pheromone components.

Hydrogenation of the compounds in the inlet of the GC leading to the mass spectrometer resulted in mass spectra that were identical to those obtained with *n*-eicosane (*n*-$C_{20}H_{42}$) and *n*-heneicosane (*n*-$C_{21}H_{44}$). These data suggested that both pheromone components were triunsaturated hydrocarbons. Ozonolysis of the pheromone components resulted in only one detectable aldehyde for each compound. Based on coelution on capillary columns and mass spectral data, the detected ozonolysis product from the C_{20} triene compound was determined to be 1-undecanal, and that from the C_{21} triene to be 1-dodecanal. A literature search of the mass spectra of trienes (Conner et al., 1980, and references therein) suggested that the pheromone components were methylene interrupted polyenes. A diagnostic fragment in the mass spectra at *m/e* 108, the base peak in both compounds, corresponding to $[CH_3CH_2(CH{=}CH)_3H]^+$ is characteristic of methylene interrupted systems with a double bond in the carbon-9 position. Additionally, the *m/e* fragments at 220 (22% of the base peak) and 234 (18% of the base peak) for the C_{20} and C_{21} trienes, respectively, are consistent with ions corresponding to $[H(CH{=}CH)_3(CH_2)_n CH_3]^+$ (where $n = 9$ or 10). The fragmentation pattern we obtained is identical to that reported by Conner et al. (1980) for (*Z*, *Z*, *Z*)-3,6,9-heneicosatriene.

The high-field proton NMR spectra were consistent with the postulated methylene interrupted triene structures (Figure 3). The spectra consisted of downfield olefinic absorptions $\delta = 5.59$ (6H); doubly allylic methylenes $\delta = 2.88$ (4H); allylic methylene $\delta = 2.16$ (4H); methylene envelope at 1.33; and a triplet corresponding to two terminal methyl groups at 1.02 ppm. Infrared spectra were nondescript, showing only olefinic absorption at 3010 cm^{-1} and no absorption in the 970 cm^{-1} region. This suggested the absence of *trans* double bonds for both pheromone components. Thus, the analytical data support the structure of (*Z*, *Z*, *Z*)-3,6,9-eicosatriene and (*Z*, *Z*, *Z*)-3,6,9-heneicosatriene for the two components of the VBC sex pheromone.

All synthetic materials were purified on a 20% $AgNO_3$ silica gel HPLC

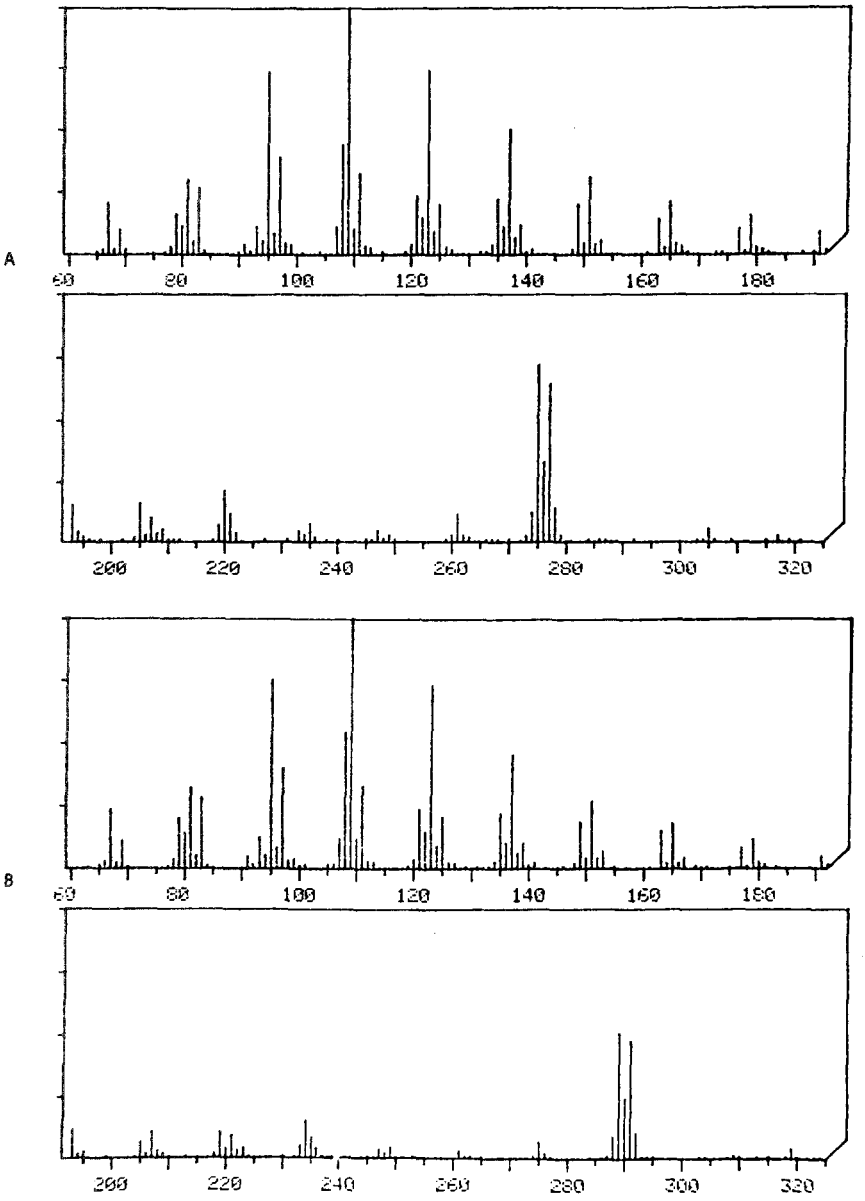

FIG. 2. Methane ionization mass spectra of VBC pheromone components.

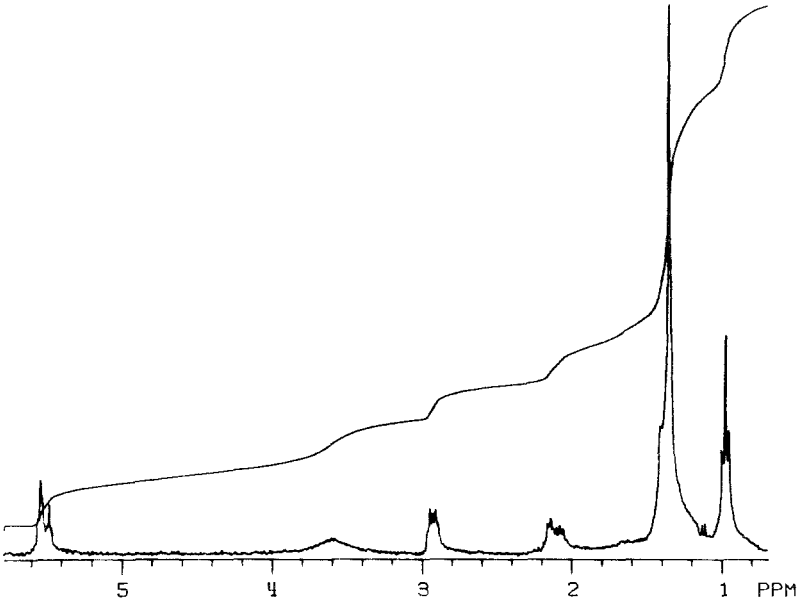

FIG. 3. Three-hundred-MHz proton NMR of ca. 5 μg of the 20-carbon triene, 2000 transients, benzene-d_6 used as solvent. Broad signal at 3.6 due to residual H_2O in probe.

column. The retention of the *Z, Z, Z* isomers on this column was well beyond that of the seven other isomeric impurities. The *k'*s of the (*Z, Z, Z*)-3,6,9-eicosatriene and the (*Z, Z, Z*)-3,6,9-heneicosatriene on the $AgNO_3$ silica gel column were both 0.52. The purified eicosatriene and heneicosatriene compounds each produced a single peak when analyzed on a 54 M OV-101 glass capillary column. The KI of the eicosatriene was 1963 and that of the heneicosatriene was 2071. Analysis of the purified products on the cholesterol *para*-chlorocinnamate capillary column also produced single peaks with KI of 1958 and 2062 for the eicosatriene and the heneicosatriene, respectively.

The synthesized compounds, III and IV, were identical in chromatographic and spectral characteristics with the two pheromone components isolated from the insect. The geometry of the natural compounds was confirmed as *Z, Z, Z* by isomerizing the synthesized compounds (Conner et al., 1980) to produce all eight possible isomers of each compound. The isomers of each compound were resolved by a combination of an HPLC 20% $AgNO_3$ silica gel column and capillary GLC on the cholesterol *para*-chlorocinnamate liquid crystal column. The bond geometry of the isomers were identified using carbon magnetic resonance spectroscopy. All assignments of the olefinic carbon resonances were assigned, and thus the geometry of the synthetic

pheromone materials was established as being *Z, Z, Z* (Heath et al., in preparation). The components of the pheromone had retention times identical with the respective synthetic *Z, Z, Z* isomer on the liquid crystal capillary column.

Based on the liquid crystal capillary column analysis, 1 FE of the crude extract yielded ca. 5 and 3 ng of the C_{20} and C_{21} trienes, respectively. We cannot be assured of the exact ratio in the insect as some discrimination may have occurred during the isolation process. Field test data (see below) suggest the exact ratio is not crucial to trap captures.

Four field trapping experiments were conducted. The first compared rubber septa impregnated with 0.1 mg of a 5:3 (C_{20} triene–C_{21} triene) ratio of synthetic pheromone with three virgin females. The second evaluated 5:3 ratio baits of 0.03 mg, 0.1 mg, 0.3 mg, 1.0 mg, and 3 mg. A third test compared captures in traps baited with 1, 3, or 5 rubber septa, each impregnated with 0.1 mg of the 5:3 ratio of synthetic pheromone. The last test compared 1.0-mg baits of pure (*Z, Z, Z*)-3,6,9-eicosatriene, pure (*Z, Z, Z*)-3,6,9-heneicosatriene, and five ratios of the two compounds.

The two traps containing 0.1 mg of 5:3 (C_{20}–C_{21}) synthetic pheromone captured a total of 479 males in 11 nights (nine replications), while the two virgin female-baited traps captured 107 males ($F = 88.4$).

No overall dose–response relationship was expressed in the capture of males in traps baited with 0.1–3.0 mg of 5:3 (C_{20}–C_{21}) pheromone impregnated in a rubber septum (Table 1, Experiment A); however, a doubling of captures occurred between 0.03 and 0.1 mg. A strong relationship between

TABLE 1. CAPTURE OF MALES IN TRAPS BAITED WITH VARIOUS CONCENTRATIONS OF 5:3 RATIO (C_{20}–C_{21}) OF VBC PHEROMONE COMPOUNDS AND PLACED IN SOYBEAN FIELD

Concentration (mg)	Number of septa/trap	Mean males per trap/night
Experiment A (August 22–26, 1981), $N = 10$		
0.03	1	5.1
0.1	1	10.8
0.3	1	10.2
1.0	1	14.0
3.0	1	12.7
Blank	1	0.5
Experiment B[a] (August 28–31), $N = 8$		
0.1	1	4.1
0.3	3	8.5
0.5	5	19.7

[a] Each septum treated with 0.1 mg. $y = 2.72\,e^{3.92x}$; $R^2 = 0.99$.

dose and male captures was found when traps were baited with 1, 3, or 5 septa, each impregnated with 0.1 mg of 5:3 pheromone (Table 1, Experiment B). This suggests that the release of these hydrocarbons was limited by the half-septa formulation and that it reached a maximum at ca. the 1.0-mg load.

The VBC males responded to a wide range of C_{20}–C_{21} ratio (Table 2) from 4:1 to 1:4. Captures were reduced by the 9:1 (C_{20}–C_{21}) ratio. Pure C_{21} or C_{20} and the 1:9 (C_{21}–C_{20}) baits captured only a few more males than the blank.

Our present knowledge of pheromone chemistry and insect behavior suggests that any compound or combination of compounds isolated and identified as a pheromone from a particular species cannot be designated as "the" pheromone of that particular species. There is still the possibility that other compounds emitted by female VBC may have subtle undetected effects on the behavior of the conspecific males. Further behavioral analyses are being conducted to study subtle variations in male response to different component ratios and to explore the possibility that the female-produced pheromone may contain additional components. However, our chemical and biological data strongly support the conclusion that a blend of (*Z,Z,Z*)-3,6,9-eicosatriene and (*Z,Z,Z*)-3,6,9-heneicosatriene in an approximate ratio of 5:3, respectively, is emitted by the female, attracts male VBC, and releases male sexual behavior. Methylene interrupted triene hydrocarbons have only recently been discovered as insect pheromones. Conner et al. (1980) identified (*Z,Z,Z*)-3,6,9-heneicosatriene in volatiles collected from females of an arctiid moth, *Utetheisa ornatrix bella* (L.), and concluded that it is a sex pheromone

TABLE 2. CAPTURE OF MALES IN TRAPS BAITED WITH VARIOUS RATIOS OF VBC SEX PHEROMONE COMPONENTS APPLIED IN SOYBEAN FIELD (SEPTEMBER 29–OCTOBER 9, 1981)

Concentration (μg)		Mean per trap/replication (N =18)[a]
C_{21}	C_{20}	
800	200	38.0 a
400	600	33.4 ab
200	800	26.5 ab
900	100	23.9 b
1000	0	5.3 c
100	900	3.5 c
0	1000	1.9 c
0	0	1.0 c

[a]Means with the same letter of not significantly different, Duncan's multiple-range test, α level = 0.05.

from electroantennogram and field bioassay data. They also suggested that additional, chemically related components may be present. Underhill et al. (1981) reported, at the National Meeting of the Entomological Society of America, that these same two compounds have been found in another noctuid of the subfamily Catocalinae, *Caenurgina erechtea* (Cramer), and function as the sex pheromone. Thus a new group of pheromonal compounds has been discovered in two different families of Lepidoptera by three independent research groups within a relatively short period of time. It will be interesting to see if further identifications suggest any taxonomic significance for this type of compound.

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EFFECT OF SOYBEAN PHYTOALEXINS ON THE HERBIVOROUS INSECTS MEXICAN BEAN BEETLE AND SOYBEAN LOOPER[1,2]

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Abstract—Effects of soybean phytoalexins on the feeding of the soybean looper and Mexican bean beetle were investigated to test the hypothesis that phytoalexins might be a defense mechanism of plants against insects as well as against pathogens. Short-term behavioral responses to the phytoalexins were analyzed using dual-choice tests with phytoalexin-rich and phytoalexin-poor (control) tissues. Phytoalexin production was elicited with ultraviolet radiation. Results from the dual-choice tests indicated that 6th instar soybean looper larvae fed equally on the control and phytoalexin-rich tissues. Feeding by adult and 4th instar Mexican bean beetles, however, was strongly deterred by the phytoalexins as evidenced by "single-bite" mandible scars on the phytoalexin-rich cotyledon discs. Nutritional effects of the isoflavonoid phytoalexin glyceollin on early instar soybean looper larvae were tested by incorporating the phytoalexin into an artificial medium at a level of 1% dry weight (0.15% fresh weight). The larvae were reared for 7 days from emergence on diets of control and glyceollin-containing media. Although survival on the glyceollin diets was initially less than on the control diets, under the experimental conditions glyceollin had no significant effect on the growth, development, or subsequent survival of the larvae. Efficiency of food utilization (ECI) was reduced, indicating that the phytoalexins may be a mild digestibility-

[1]Mexican bean beetle = *Epilachna varivestis* Mulsant, (Coleoptera: Coccinellidae); soybean looper = *Pseudoplusia includens* (Walker), (Lepidoptera: Noctuidae).

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reducing factor for the loopers. Implications of the results for host-plant resistance are discussed.

Key Words—*Pseudoplusia includens*, soybean looper, Lepidoptera, Noctuidae, *Epilachna varivestis*, Mexican bean beetle, Coleoptera, Coccinellidae, feeding preferences, nutrition, food utilization, host-plant resistance, induced resistance, glyceollin, isoflavonoids, soybean, phytoalexins.

INTRODUCTION

The literature on phytochemical defense against insect herbivores abounds with reports on secondary plant products that are present at potent levels prior to insect herbivory (Whittaker and Feeny, 1971; Levin, 1976; Kogan, 1977; Swain, 1977; Hedin, 1977; Rosenthal and Janzen, 1979; Schoonhoven, 1981). There are comparatively few examples of allelochemicals induced by herbivory (Ryan and Green, 1974; Ryan, 1978; Carroll and Hoffman, 1980), although induced substances, such as phytoalexins, are commonly considered to be involved in defenses of higher plants to microbes (Cruikshank, 1963; Kuc, 1972; Deverall, 1976; Swain, 1977). Phytoalexins may be defined as antibiotic metabolites which undergo enhanced or de novo synthesis and accumulate in plants following exposure to microorganisms (Van Etten and Pueppke, 1976; Paxton, 1980). Physiological stress from mechanical injury, UV radiation, and chemicals—including some pesticides—may also elicit phytoalexin production (Kuc et al., 1976). In some cases herbivores may be involved in eliciting phytoalexin production (Akazawa et al., 1960; Loper, 1968; Uritani et al., 1975). Recent work suggests that phytoalexins may be a causal factor in plant resistance to nematodes (Rich et al., 1977; Kaplan et al., 1980) and insects (Russell et al., 1978; Sutherland et al., 1980; McIntyre et al., 1981).

Given the theoretical and practical importance of knowing whether phytoalexins play a multiple role in plant defense, we studied their involvement in resistance of soybean to two herbivorous insects: the Mexican bean beetle (MBB), *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae), and the soybean looper (SBL), *Pseudoplusia includens* (Walker) (Lepidoptera: Noctuidae). The MBB is an oligophagous insect with a host range restricted to a few genera of Leguminosae (Kogan, 1972). The species causes significant damage to soybean in the Atlantic coastal states and certain regional pockets in the Midwest, but there is considerable temporal variation in its pest status on soybean (Turnipseed and Shepard, 1980). The SBL, a comparatively polyphagous insect (Herzog, 1980), is a serious pest of soybean in the southern United States (Burleigh, 1972).

With soybean, as with many plants, more than one phytoalexin accumulates after infection (Kuc et al., 1976). Glyceollin, an isoflavonoid with four isomeric forms (Figure 1) (Lyne et al., 1976; Lyne and Mulheirn, 1978),

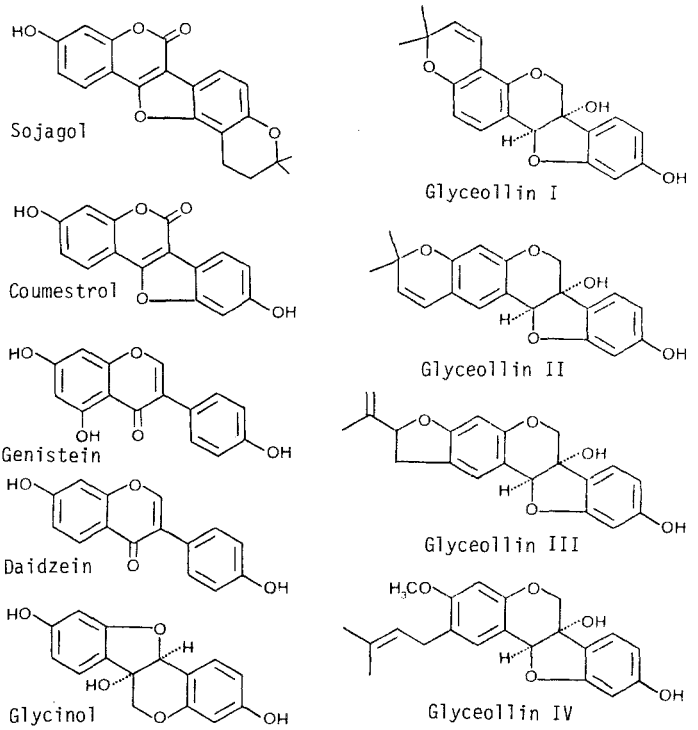

FIG. 1. Isoflavonoids commonly associated with the phytoalexin response in soybean plants.

has been the most-studied phytoalexin of soybean (Keen et al., 1972; Keen and Kennedy, 1974; Keen and Paxton, 1975). Until 1975 glyceollin was misnamed hydroxyphaseollin (Burden and Bailey, 1975). Recently another isoflavonoid phytoalexin, glycinol, was named (Figure 1) (Weinstein et al., 1981).

Chemical resistance of plants to insects either disrupts the behavioral sequence of host selection (antixenosis) or renders plants unfit for insect nutrition through antibiosis or both (Kogan, 1977). Hence we tested the effects of soybean phytoalexins on short-term host selection by the two insects using dual choice tests and assayed chronic effects of glyceollin on early instars of the soybean looper. The ecological implications of antiherbivory effects of soybean phytoalexins are discussed.

METHODS AND MATERIALS

Dual-Choice Tests. The two cotyledons from each 8- to 12-day-old soybean seedling, cultivar Clark 63, were excised and kept in two separate

sets. After all cotyledons were surface-sterilized for 5 min in 3% H_2O_2 and rinsed with sterile deionized water, 1-cm-diam. disks were cut from their centers. The first set of cotyledon disks was irradiated with a UV microbicidal lamp for 20 min at a distance of ca. 17 cm. UV light induces phytoalexin production, with significant accumulation occurring 12–24 hr after irradiation (Bridge and Klarman, 1973). Apparently, phytoalexin production mechanisms by UV stimulus are similar to those induced by biotic elicitors (Moesta and Grisebach, 1980).

The irradiated disks (designated hereafter as phytoalexin disks) were incubated in darkness at 24–25°C for 2 days to permit accumulation of phytoalexins. The other set of disks (designated hereafter as control disks) were kept under the same conditions as the phytoalexin disks and were irradiated for 20 min at the end of the 2-day incubation period.

Phytoalexin production may be elicited by physical injury such as cutting (Kuc et al., 1976). Thus, aliquots of control disks were analyzed to ascertain that the levels of glyceollin were very low relative to those in the phytoalexin disks.

For each replicate of the choice test, a control and a phytoalexin disk derived from the same seedling were placed in a 5-cm-diam. feeding arena (Figure 2). Tests were conducted with 4th instar MBB, 6-day-old adult MBB, and 6th instar SBL larvae. One insect was introduced into each dual-choice feeding arena within 2–4 hr after irradiation of the control disks. The MBB larvae were ca. the 3rd greenhouse-reared generation of beetles field-collected in North Carolina. The MBB adults were the 2nd greenhouse-reared generation of beetles collected in Tifton, Georgia. Larval and adult beetles were reared in the greenhouse on snap bean, *Phaseolus vulgaris*, foliage. The larvae, supplied with water from a cotton wick, were starved 1 day prior to the test. Adults were starved 2 days before testing. The SBL larvae came from a laboratory culture reared on an alfalfa meal–wheat germ medium adapted from Henneberry and Kishaba (1966). The genetic diversity and vigor of the stock culture were maintained by an annual addition of field-collected individuals. The SBL larvae were not starved prior to testing.

The MBB larvae ($N = 22$) were permitted to feed for 18 hr and the adults ($N = 30$), for 21 hr, at 27°C. Although mean feeding on the most-consumed disk in each replicate was only 35% (visual estimate) in the larval test and 20% in the adult test, the tests had to be terminated before phytoalexins reached significant levels in the controls. The tests were conducted in darkness to elimi te visual responses to the reddish phytoalexin disks or the green control disks. Feeding was measured as mean No. of feeding ridges/disk (see Figure 3).

The SBL larvae ($N = 40$) were allowed to feed 5 hr at 27°C in darkness. After 5 hr, mean feeding on the most consumed disk in each replicate was

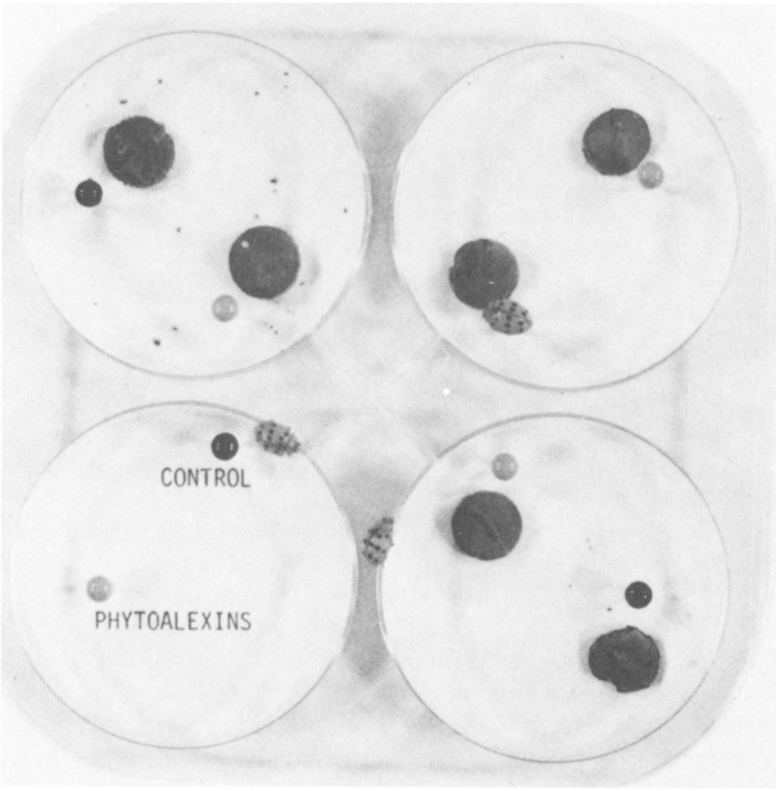

FIG. 2. Clustered plastic Petri dishes used as feeding arenas in dual-choice experiments. Each of the four round cells is a separate arena and contains a control and a phytoalexin cotyledon disk. For this photo the lid was removed and 3 adult Mexican bean beetles are standing on a cotyledon disk or on the rim of the arena.

ca. 50% (visual estimate). The mean fresh weight (FW) and mean dry weight (DW) of the disks consumed during the test were evaluated. An initial DW for the disks was obtained by multiplying their initial FW by a DW:FW ratio. This ratio (pooled mean) was calculated from the initial FW and final DW of 16 randomly selected pairs of phytoalexin and control disks. Weight loss due to evaporation was similar in control and in phytoalexin disks ($P < 0.05$).

For both the MBB and SBL choice tests, differences between feeding on control and phytoalexin disks were analyzed with a paired t test ($P < 0.05$). The preference index, C, was used to show the direction and magnitude of feeding preference on a scale of 0 to +2 (Kogan and Goeden 1970). Computation is based on the relation $C = 2P/(S + P)$, where S is the amount of

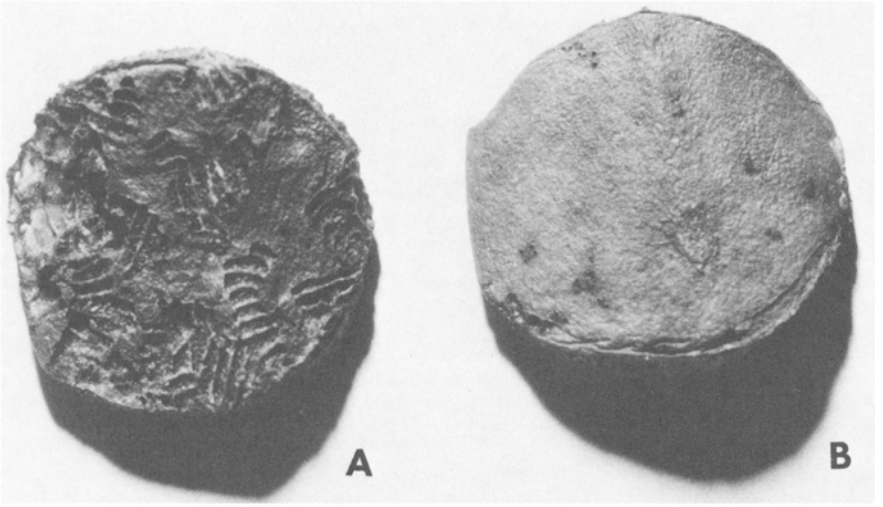

FIG. 3. Feeding patterns on cotyledon disks from Mexican bean beetle dual-choice tests. (A) Feeding ridges observed on the control disks. (B) Mandible holes (probing) observed on the phytoalexin disks.

feeding on the standard control disk and P denotes feeding on the corresponding phytoalexin disk. $C = 1$ indicates no preference for the control disk relative to the phytoalexin disk in an arena; $C < 1$ indicates a preference for the control disk vis-à-vis the phytoalexin disk. The hypothesis that the mean values of C were less than 1 was tested with a one-tailed t test ($P < 0.05$).

Random samples of 6–10 phytoalexin and control disks were frozen in 80% EtOH at the onset of the test for glyceollin analysis. Three of these samples, one from each of the choice tests, were later extracted in hexane and ethyl acetate using the first extraction procedure in Keen et al. (1971). The EtOAc fractions and glyceollin standards were applied to TCL plates (250 μm thick) of silica gel GF_{254} (E. Merck). The plates were developed in 60 hexane–40 EtOAc–1 MeOH (v/v). Bands at the R_f values of the glyceollin standards were eluted in EtOH. The identity of the eluates was confirmed by their UV absorption spectra. Glyceollin concentration was measured at the absorption maximum of 287 nm. The concentrations of glyceollin were adjusted for a mean ($N = 3$) recovery from the TLC plates (250 μm thick) of 44% ($s = 8.1$). No attempt was made to measure total recovery of glyceollin during tissue extraction. Concentrations of induced compounds other than glyceollin were not determined.

Nutrition Test. Using an artificial medium, the chronic effects of glyceollin were tested at the high but physiological level of 1% DW (0.15 FW)

(Keen and Kennedy, 1974). Glyceollin was obtained by excising soybean cotyledons from week-old seedlings (cv. Clark 63) and inoculating them with an aqueous extract of *Phytophthora megasperma* var. *sojae*, race 1, as described by Frank and Paxton (1971).

The inoculated cotyledons were incubated at 25°C for 2 days, permitting high levels of glyceollin to accumulate. The cotyledons and diffusates were subsequently freeze-dried and ground, yielding 5.6 g of powder from which 11.5 mg of glyceollin was extracted. Extraction followed the same procedure described for the analysis of the choice-test cotyledons, but silica gel $60F_{254}$ TLC plates, 2 mm thick (EM Reagents®), were used. For the control diets, an eluate (EtOH) was prepared using a quantity of blank silica gel equivalent (by weight) to that providing the glyceollin.

Since the quantity of glyceollin available was relatively small, we devised a method for making small volumes of diets. Five mg of glyceollin in 6 ml EtOH was added to the alphacel component of the alfalfa meal–wheat germ medium (AM) in each of two 5-cm-diam. Petri dishes. The EtOH was evaporated from the alphacel under vacuum. Media containing 1% glyceollin (DW) were prepared by pipetting 3.5 g of warm AM (15% DW) minus alphacel into each Petri dish. While a warm water bath prevented solidification of the media, the alphacel and extract residues were thoroughly mixed into the AM. The media without glyceollin were prepared using the same procedures, but the solution of glyceollin was replaced with a 6-ml solution of the control eluate.

Disks of media, 9 mm in diam. and 1–3 mm thick, were placed at the bottom of 9 × 30-mm shell vials (¼ dr). The vials were sealed with Saran® film. Thirty of the diets were randomly selected from each treatment and inoculated with 2 newly emerged SBL larvae. The larvae were derived from the same stock as that used in the choice tests. The diets were incubated for 7 days at 27 ± 2°C, 80 ± 5% relative humidity, under a 15:9 hr light–dark photoperiod. At the start and end of the test, four diets were randomly selected from each treatment and analyzed to determine if glyceollin decomposed during the test.

The first day after inoculation of the diets (day 2), dead larvae were replaced with newly emerged larvae, and the Saran seals were pierced with an insect pin for ventilation. On day 3 the smaller larva was removed from each vial so that there remained one larva per vial. When both larvae appeared similar in length, removal was random.

Larval survival was recorded every other day. On day 7, the stadia of the larvae were determined using head capsule measurements (Jackai, 1978). Differences in survival were tested using contingency tables ($P < 0.05$). Insect growth and food utilization during the 7-day period were appraised by the mean DW gain of the larvae (μg), mean DW of the diet removed (μg), mean efficiency of conversion of ingested food (ECI), mean

consumption index (CI), and mean relative growth rate (RGR) (Waldbauer, 1968; Klein and Kogan, 1974). Larval weight gain was calculated by subtracting the initial from the final larval weight. Initial DW was obtained from the mean of a 15-larvae aliquot from the same original population. For the determination of DW diet removed, the initial fresh weights of the diets were multiplied by a DW:FW ratio to obtain an estimate of initial DW. This DW:FW ratio (pooled mean) was calculated from the initial DWs and FWs of an aliquot ($N = 20$) of diets from each treatment. The quantity of diet consumed by larvae through the 3rd day of the test was assumed to be negligible. Dry weights were measured on a Cahn 21/Ventron® automatic electrobalance. Significance of differences between means was tested using one-way analysis of variance ($P < 0.05$).

The diets analyzed for glyceollin were extracted with EtOAc that had been treated with 0.01 M K_2HPO_4 (10:1) to reduce acidity. Analytical TLC of glyceollin was similar to that described for the choice tests, but TLC plates spotted with final extractions of the diets were developed in 85 benzene–15 MeOH (v/v), and glyceollin was eluted in MeOH or EtOAc. The EtOAc eluates were dried under air and reconstituted with MeOH before spectrophotometric analysis.

RESULTS

Choice Tests. The UV absorption spectra of glyceollin extracts matched those reported by Burden and Bailey (1975). The mean ($N = 3$) concentration of glyceollin was determined to be 1.82 ($s = 1.031$) mg/g DW [0.20 ($s = 0.101$) mg/g FW] for the phytoalexin disks and 0.13 ($s = 0.119$) mg/g DW [0.01 ($s = 0.013$) mg/g FW] for the control disks. Glyceollin production in the control cotyledons may have been induced when the tissue was cut into disks (Kuc et al., 1976).

The mean number of feeding ridges (Figure 3) made by the MBB larvae and adults of both sexes was significantly greater on the control disks than on the phytoalexin disks (Table 1). The mean preference indices (C), 0.22 ± 0.05 for the MBB larvae and 0.02 ± 0.02 for the adults, were both significantly less than 1 ($P < 0.05$) showing that these insects ate little of the phytoalexin tissue compared to the control tissue (Figure 4). Only one of the 30 adult beetles fed on a phytoalexin disk. While there were few feeding ridges on phytoalexin disks in the MBB tests, numerous mandible holes were observed where the insects bit the phytoalexin tissue but did not proceed to feed (Figure 3). Sixty-eight percent (30/44) of the phytoalexin disks without feeding ridges had mandible holes. All but one of the control disks had feeding ridges.

For the SBL test in which feeding was measured as both fresh and dry

TABLE 1. MEAN DIFFERENCE (X_d) IN FEEDING ON PAIRED CONTROL PHYTOALEXIN DISKS[a]

Insect test	N	X_d	(SE)	
MBB				
Larvae	22	+33.1	(5.41)	*[b]
Adults				
♀♀	16	+10.8	(2.35)	*
♂♂	14	+14.9	(3.02)	*
♀♀ +♂♂	30	+12.7	(1.89)	*
SBL larvae	40	+ 0.1	(0.87)	NS

[a]Feeding was measured as number of feeding ridges for the Mexican bean beetle (MBB) and in estimated mg dry weight eaten for the soybean looper (SBL). Positive values indicate greater consumption of control disks than of phytoalexin disks.
[b]The mean difference in feeding on pairs of control and phytoalexin disks in the same arena is significantly greater than 0 at the 0.05 level (paired t test).

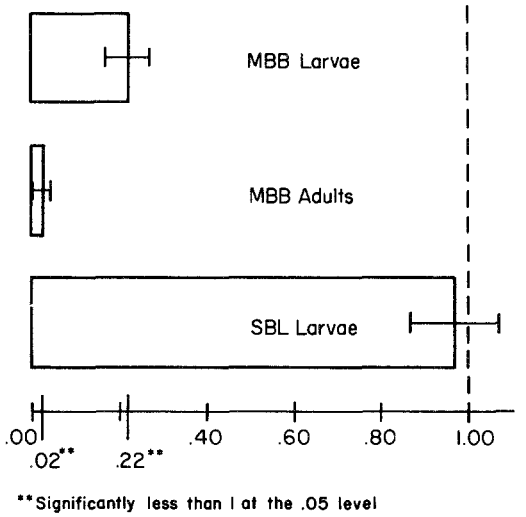

FIG. 4. Mean preference indices (C) for feeding on control disks (S) and on phytoalexin disks (P). MBB = Mexican bean beetle; SBL = soybean looper. Feeding was measured as number of feeding ridges for the MBB, and in estimated mean dry weight for the SBL.

TABLE 2. SURVIVAL OF NEONATE SOYBEAN LOOPER LARVAE REARED ON ARTIFICIAL DIETS OF 0% AND 1% GLYCEOLLIN (DRY WEIGHT) FOR 7 DAYS

Day of test	No. live larvae (% survival)	
	Control diet	1% Glyceollin diet
1	60	60
2 (before replacement of dead larvae)	55 (92%)	47 (78%)
3 (before thinning)	33 (51%)	40 (55%)
3 (after thinning)	30	30
7	19 (63%)	21 (70%)

weight of disk consumed, the differences between feeding on control disks and phytoalexin disks were not significant (Table 1). Likewise the mean preference indices (C), calculated from both the FW and DW data, were 1.00 ± 0.12 and 0.97 ± 0.10, respectively, and not significantly less than 1 (Figure 4). Hence at the test concentrations, glyceollin and other phytoalexins present did not affect the SBL larvae's choice of food.

Nutrition Test. Survival of SBL larvae on day 2 of the nutrition test was significantly greater ($P < 0.05$) on the control diets (92%) than on the glyceollin diets (78%) (Table 2). Such effect, however, was not observed among the batch of newly emerged larvae that replaced the dead larvae on

TABLE 3. GROWTH AND FOOD UTILIZATION OF SOYBEAN LOOPER LARVAE FED ARTIFICIAL DIETS OF 0% AND 1% GLYCEOLLIN (DRY WEIGHT) FOR 7 DAYS AFTER EMERGENCE

Food utilization index	Mean (SE)[a]	
	Control diet (N = 19)	1% Glyceollin diet (N = 20)
Larvae dry weight gain (μg) (estimate)	141 (26.8)	110 (21.0) NS
Dry weight of diet consumed (μg) (estimate)	827 (160.0)	933 (121.5) NS
Efficiency of conversion of ingested food	39 (11.9)	15 (3.3) *
Consumption index	2.0 (0.51)	2.3 (0.26) NS
Relative growth rate[b]	0.35 (0.022)	0.31 (0.022) NS
Relative growth rate[c]	0.23 (0.007)	0.22 (0.009) NS

[a]NS, differences between means not statistically significant; *, $P < 0.05$, $df = 37$, Student's t test.
[b]Klein and Kogan (1974).
[c]Waldbauer (1968).

day 2. On day 3, larval survival on those diets with replaced larvae was 60% (6/10) for the control diets and 79% (19/24) for the glyceollin diets. No differences were subsequently apparent between survival (Table 2), development, or growth of larvae reared on the control and glyceollin diets during the 7-day test. The mean DW gain of larvae fed the glyceollin diets was less, although not significantly different, than that of larvae on the control diets (Table 3). However, ECI was more than two times greater with the control than with the phytoalexin diet, although there were no significant differences in CI or RGR (Table 3). Hence the results of the nutrition study indicate that the survival, development, and growth of early SBL instars were not greatly affected by the 1% DW concentration of glyceollin, but food utilization was reduced. Glyceollin levels remained constant during the experiment; the mean concentration of glyceollin at the end of the test—6.1 μg/mg DW, $s = 4.02$ ($N = 4$)—was not significantly different from the mean concentration at the start of the test—5.2 μg/mg DW, $s = 2.45$ ($N = 4$).

DISCUSSION

Many of the isoflavonoids and related flavonoids possessing reactive free hydroxyl groups are biologically active (Harborne, 1979). Some of the flavonoids are attractant, deterrent, repellent, antibiotic, or toxic to insects (Shaver and Lukefahr, 1969; Todd et al., 1971; Harborne, 1979; Jones and Firn, 1979; Elliger et al., 1980). Many of the isoflavonoids are fungistatic and have estrogenic effects on mammals (Harborne, 1979; Mabry and Ulubelen, 1980). The insecticidal activity of the isoflavonoid rotenone is well-known (Fukami and Nakajima, 1971). Several isoflavonoid phytoalexins including coumestrol and genistein (Figure 1) deter feeding of two beetle species (Russell et al., 1978; Sutherland et al., 1980). Hence it was no surprise that cotyledons containing an isoflavonoid phytoalexin(s) had feeding-deterrent properties as defined by Dethier et al. (1960) and Beck (1965) towards the Mexican bean beetle. Deterrency is assumed to be due to the phytoalexins in the cotyledons, although other metabolites may also be involved.

At the physiological level of 1% DW (Keen and Kennedy, 1974), glyceollin had some effect on survival of 1-day-old SBL larvae. Subsequent survival and development were not markedly affected. The substantial reduction in ECI suggests that the isoflavonoid phytoalexins may be a digestibility-reduction factor even for a rather polyphagous species such as the SBL. No other chronic symptoms were detected, probably due to a compensatory increase in food consumption.

Plants may use substances for protection which have relatively subtle effects on attacking herbivores (Rosenthal and Janzen, 1979). Although

the differences in mean larval DW gain and mean DW of diet consumed for the SBL larvae on the control diets and on the 1% glyceollin diets were not statistically different, the effect on ECI was. There seem to be subtle differences between the two treatments that may have been obscured by several factors. The mean initial DW of control diet/vial as estimated from the FW was 15.5 mg. On the average only 0.50 mg DW or 3.3% of this initial quantity was consumed, whereas the mean absolute value of the differences between the estimated DW and the actual DW was 0.42 mg or 2.8% as determined from standard control diets. Hence the error in the estimate of initial DW was large relative to the DW quantity of diet eaten/vial. The relative size of the error in the estimate of DW of food eaten increases with the proportion of uneaten food when the initial dry weight of food is estimated from a DW:FW ratio (Waldbauer, 1968).

Error in measurements of weight gain may also have resulted because we did not account for the gut contents of the larvae. The amount of food in the gut of an insect varies within an instar, the gut being more or less empty just prior to and after a molt. However, on the basis of head capsule coloration, we detected no differences in within-stadium age of the 3rd instar larvae which represented over 80% of the larvae from both treatments.

Conditions of the miniature diets may have been stressful to the larvae, possibly masking effects of glyceollin. The DW gain and DW food consumed by the insects was only one tenth that of larvae reared on Harosoy soybean (Kogan and Cope, 1974), but the developmental rates of the larvae were similar to those reported by Kogan and Cope (1974) and to those for SBL reared on a lima bean–pinto bean artificial diet (Mitchell, 1967).

Although no large differences in glyceollin content were detected among diets analyzed for levels of glyceollin ($N = 8$), small variations in the distribution of the phytoalexin within diets may have contributed to variability in insect response on the glyceollin diets. The small larvae may have eaten the diet but avoided the points of greater glyceollin concentration.

Although glyceollin at 1% DW did not appear to affect the growth and development of young SBL larvae, perhaps glyceollin affects later stages of the insect. Another consideration is that the synthesis of glyceollin is accompanied by the accumulation of the isoflavonoids glycinol, coumestrol, sojagol, daidzein, and genistein (Keen et al., 1972; Keen and Kennedy, 1974; Keen and Paxton, 1975; Weinstein et al., 1981). The combined action of these phytoalexins may be needed to affect herbivory. In addition, since phytoalexin production in soybean appears to be a local phenomenon (Keen and Bruegger, 1977; Yoshikawa et al., 1978), the in situ concentration may exert effects not evident when the compounds are dispersed, such as in an artificial medium.

Our results show that the oligophagous MBB is clearly deterred from feeding on phytoalexin-rich tissue. MBB is not easily reared on an artificial

medium; therefore, we were unable to measure metabolic effects of glyceollin. The rather polyphagous SBL, however, readily accepted phytoalexin-rich soybean cotyledons. This insect is obviously endowed with the ability to handle a wide variety of plant defensive chemistry, as indicated by its broad host range (Herzog, 1980) Thus phytoalexin production by soybean does have an acute allomonal effect against the specialist MBB, but against the generalist SBL the effect is mildly chronic. The dual role as feeding deterrent to certain insects and as an antimicrobial factor reinforces the view that the postchallenge accumulation of phytoalexins is an important mechanism in plant strategies, one that may be more ubiquitous than has been reported so far.

Knowledge of the mode of action of phytoalexins against insects is important. In view of their microbiocidal properties, perhaps phytoalexins would be deleterious to the insect by harming microbial symbionts that aid nutrition or digestion (Mittler, 1971; Scriber and Slansky, 1981). On the other hand, phytoalexins might conceivably afford the insect some protection against entomopathogens (Shirata, 1978).

To determine whether phytoalexins increase a plant's fitness through a role in defense against insect herbivores, it is necessary to ascertain the level of contact of specific herbivores with potent concentrations of phytoalexins in a natural ecological context. Since phytoalexin production is most often a localized and delayed reaction, phytoalexins may only have a significant impact on sessile or confined herbivores, or perhaps phytoalexins cause feeding to be distributed over the total surface area of the plant in such a manner that the reduction in the plant's fitness is minimized (Janzen, 1979).

Requisite to a sound understanding of the interaction between phytoalexins and insects would be the identification of the insect-related elicitors of phytoalexin production—be they feeding by the herbivores themselves, secondary microbial infection of the insect-inflicted wounds, primary microbial infection, or chemicals.

If phytoalexins prove to be a facultative defense against herbivorous insects as well as against potential pathogens, the knowledge, adding a new dimension to our understanding of insect–plant–microbe interactions, would find valuable application in integrated pest management of crops. Unless yield is greatly reduced, crops might conceivably be temporarily or protractedly immunized by phytoalexin elicitors or nonpathogens when pest populations reach economic thresholds. More conventional applications might include breeding cultivars whose resistance to certain pathogens also may impart resistance to herbivores.

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FIELD EVALUATION OF CHIRAL ISOMERS OF THE SEX PHEROMONE OF THE EUROPEAN PINE SAWFLY, *Neodiprion sertifer*

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Abstract—Among optical isomers of 3,7-dimethylpentadecan-2-ol (diprionol) acetate or propionate tested as synthetic attractants, the 2*S*, 3*S*, and 7*S* isomers were most effective in attracting the males of *Neodiprion sertifer* in the field. The 2*S*, 3*S*, and 7*R* isomers showed weak activity, but the other optical isomers were not attractive. Capillary GC analysis showed that the natural pheromone from body extracts of females was identical with the synthetic acetate of diprionol in its GC behavior. However, the natural pheromone was about 100-fold stronger than the most purified synthetic acetate of 2*S*,3*S*,7*S*-diprionol in the field. As a result of various isomer combination studies, it was found that the acetate of 2*S*,3*R*,7*R*-diprionol, when added to 2*S*,3*S*,7*S*-diprionol preparation at a low concentration, increased the catch by the latter. It was therefore concluded that the above combination of the optical isomers could account for the major sex attractancy in this species.

Key Words—*Neodiprion sertifer*, European pine sawfly, Hymenoptera, Tenthredinidae, 3,7-dimethylpentadecan-2-yl acetate and propionate, optical isomers, enantiomers, esters.

INTRODUCTION

A female-produced sex pheromone component of the redheaded pine sawfly, *Neodiprion lecontei* (Fitch), has been isolated and identified as the acetate of 3,7-dimethylpentadecan-2-ol (Jewett et al., 1976). They observed,

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by using nuclear magnetic resonance spectroscopy, that the pheromone has an erythro configuration of the two adjacent optically active carbons (i.e., carbons 2 and 3). Electroantennographic studies have shown that males of many species of diprionid sawflies are responsive to either the acetate or propionate of diprionol isolated from *Neodiprion sertifer* (Geoffroy) or *N. lecontei* females. However, the responses of *Dipron similis* (Hartig) and *Gilpinia frutetorum* (Fabricius) males appeared different from those belonging to the *Neodiprion* genus (Jewett et al., 1976). Several optically active enantiomers of the pheromone were later synthesized by two groups; Dr. Tai's group at Osaka University (Tai et al., 1978) and Dr. Mori's group at Tokyo University (Mori et al., 1978). By using these preparations, we found that the chiral arrangement of the pheromone for *N. lecontei* was 2*S*,3*S* (Matsumura et al., 1978). The chirality of all the carbons of the sex attractant of *Neodiprion pinetum* (Norton) was also established as the acetate of 2*S*,3*S*,7*S*-diprionol. This was the only active erythro isomer for that species (Kraemer et al., 1979). In the present study, we have extended our work to *N. sertifer*, a common species in the Great Lakes Region.

METHODS AND MATERIALS

Chemicals. The acetate and propionate for four erythro isomers, 2*R*,3*R*,7*R*; 2*R*,3*R*,7*S*; 2*S*,3*S*,7*R*; and 2*S*,3*S*,7*S* were obtained from Dr. Mori (Mori et al., 1978). These were the same compounds used previously by us for *N. pinetum* (Kraemer et al., 1979). Two erythro isomers, 2*R*,3*R*, 7(*R*/*S*) and 2*S*,3*S*,7(*R*/*S*) were synthesized by us (Matsumura et al., 1979). NMR analyses of these compounds showed that each erythro isomer was free from contamination by the other. On the other hand, both preparations contained approximately 5% of threo isomer. It is likely that in the process of synthesis by Tai et al. (1978), the (+) erythro pheromone (2*R*,3*R*) had been contaminated by a trace of the 2*R*,3*S* isomer as the result of an epimerization reaction of the Wittig reaction at the 3 carbon. Similarly the (−) erythro pheromone (2*S*,3*S*) is contaminated by the 2*S*,3*R* isomer. Two threo isomers, 2*S*,3*R*,7(*R*/*S*) and 2*R*,3*S*,7(*R*/*S*) diprionol, were also synthesized by us (Kikukawa et al., 1981).

To study the effect of mixing 2*S*,3*R*,7*S*-A with 2*S*,3*S*,7*S*-A, it was necessary to synthesize the former. This was done by reacting (+)-2*S*,3*R*-3-tetrahydropyranoxy-2-methylbutan-1-ol with *R*-3-methyl-1-bromoundecan through the Wittig reaction. The details of synthesis of this 2S, 3R, 7R-A epimer are published elsewhere (Kikukawa et al., 1982). The coding system and the abbreviations used for these preparations are indicated in Table 1.

Field Tests. All chemicals were dissolved in 1 ml of *n*-hexane and

TABLE 1. ABBREVIATIONS, CODING SYSTEM, AND SOURCE OF SYNTHETIC PHEROMONES USED IN STUDY[a]

	Code name	Source
2*R*,3*R*,7*R*-A[b]	TA-1	Mori et al. (1978)
2*R*,3*R*,7*S*-A	TA-2	Mori et al. (1978)
2*S*,3*S*,7*R*-A	TA-3	Mori et al. (1978)
2*S*,3*S*,7*S*-A	TA-4	Mori et al. (1978)
2*R*,3*R*,7(*R*/*S*)-A	A-1	Tai et al. (1978)
2*S*,3*S*,7(*R*/*S*)-A	A-2	Tai et al. (1978)
2*S*,3*R*,7(*R*/*S*)-A	CA-1	Kikukawa et al. (1981)
2*R*,3*S*,7(*R*/*S*)-A	CA-2	Kikukawa et al. (1981)
2*S*,3*R*,7*R*-A	KA-1	Kikukawa et al. (1982)
2(*S*/*R*),3*R*,7*R*-A	TMA-1	Mori et al. (1978); chirality of carbon 2 has been
2(*S*/*R*),3*R*,7*S*-A	TMA-2	modified by Kikukawa et al. (1978)

[a]A stands for acetate and corresponding propionates are marked by P.
[b]Abbreviation of (2*R*,3*R*,7*R*)-3,7-dimethylpentadec-2yl acetate. The corresponding propionates, 2*R*,3*R*,7*R*-P, etc., have been coded as TP-1, TP-2, TP-3, etc.

stored in sealed glass ampules. In the field, the ampule was broken and its contents were poured onto a 2.5-cm cotton wick held with a pair of forceps. The wick was then attached to the inside roof of a Pherocon II trap (Zoecon Corp., Palo Alto, California). To avoid contamination, the forceps were washed with alcohol after each use.

Most tests were conducted from September to November, 1978 and 1981, in the arboretum and Rose Lake, near Lansing, Michigan. Some tests were also conducted at Kellogg Forest, Hickory Corners, Michigan, and Burlington, Wisconsin. Traps were hung on Scotch and red pine branches at a height of approximately 1.5–2 m.

Preparation of Pheromone from "Natural Volatiles" from Living Female N. sertifer. Each female was placed for one week in a 3 × 5-cm vial, along with a thin strip of Teflon. After the females were removed, the vials and the Teflon strips were washed three times with 3.0 ml ether. The ether wash from more than 100 vials was combined, and the solvent was removed by rotary evaporator. The oily residue was spotted on a TLC (Silica Gel $HF_{254+366}$) plate, and developed with 20% ether in hexane as the mobile phase. The zone ($R_f = 0.45$–0.60) corresponding to the synthetic acetate of diprionol ($R_f = 0.53$) was extracted with ether. This fraction was used for GC collection of the component corresponding to the synthetic acetate (10% SE-30 on Gaschrom Q, 220°C, $\frac{1}{4}$ in. × 6 ft).

Partial Purification of Natural Pheromone from KOH-Treated Body Extract. Two hundred *N. sertifer* virgin females were immersed in ether overnight (Figure 2). The solvent was decanted, and the insects were washed

two more times with the same solvent. The solvent was evaporated by a rotary evaporator under reduced pressure. The residue was hydrolyzed by the addition of 1 g KOH in 50 ml methanol. This mixture was refluxed for 5 hr. The methanol was removed by rotary evaporation. The organic material was separated in a separatory funnel (hexane–water). The hexane fraction was dried over Na_2SO_4 to give the crude alcohol fraction. The hexane was removed by rotary evaporation and the residue was esterified by adding 2 ml pyridine, and 1 g of acetic anhydride. The reaction was left overnight, in the dark and at room temperature. The ester obtained was designated as "modified natural ester." The crude ester fraction thus obtained was purified by thin-layer chromatography (TLC) [activated Silica Gel $HF_{254+366}$ with hexane–ether (4:1) mobile phase]. The most field-active fraction ($R_f = 0.65–0.39$) was further purified by a second TLC system using *n*-hexane first and benzene second as the mobile phases.

Partial Purification of Natural Pheromone from Untreated Body Extract. Sixteen hundred *N. sertifer* virgin females were immersed in 100 ml of ether. The solvent was collected by decantation, and the process was repeated twice. The combined ether phase was evaporated until about 5.0 ml of the residue remained. It was then distilled and collected up to 250–260 mm Hg, to obtain 2.0 ml of volatile mixture. The mixture was transferred to a silica gel column (2.5 × 60 cm) packed in *n*-hexane and eluted with 300 ml of 10% ether in hexane and then with 300 ml of ether. Six fractions (i.e., 100 ml each) were collected. All these fractions were checked for biological activity in the field. All fractions were then acetylated by acetic anhydride in pyridine and checked again for activity in the field. The most active fraction was further purified by column chromatography. Five-ml fractions were collected and analyzed by GC. A component with the same retention time as the standard synthetic pheromone component [2*S*,3*S*, 7(*R*/*S*)-A] was collected in fractions 20–26. After bioassay, active fractions were combined and subjected to TLC clean-up as shown in Figure 4. GC was used to identify the natural pheromone. Columns and other conditions used were summarized and shown in figure captions.

RESULTS

A comparison of the field attractiveness of the individual acetate or the propionate preparations showed that only 2*S*,3*S*,7*S*-A and 2*S*,3*S*,7*S*-P were highly active, catching 73 and 53 males, repsectively, per trap at 25 μg each in this series of experiments. 2*S*,3*S*,7*R*-A and 2*S*,3*S*,7*R*-P caught 1 each at the same dose. Inactive were acetate and propionate of the two erythro 2*R*,3*R*,7*R* and 2*R*,3*R*,7*S*, and two threo isomers, 2*S*,3*R*,7(*R*/*S*) and 2*R*,3*S*,7(*R*/*S*). Throughout the study blank traps without added pher-

omones were tested, but usually they did not catch sawfly males. Blank values are shown only when they caught males.

To study the possibility that there may be enantiomers having important roles synergizing the pheromone activity, various optical isomers of 2*S*,32*S*,7*S*-diprionol acetate were mixed with the acetate of 2*S*,3*S*,7*S*-diprionol (Table 2). In no instance was an increase in male catch observed. Also tried were *trans*- and *cis*-perillenal as Ahlgren et al. (1979) isolated *trans*-perillenal from glands of both male and female *N. sertifer*. No significant increase in trap catch was observed by the addition of either geometric isomer to the acetate of 2*S*,3*S*,7*S*-diprionol in this particular field test.

In the next series of experiments, the dose of acetate of 2*S*,3*S*,7*S*-diprionol was kept constant and that of the proposed synergist was varied (Table 3). With *trans*-perillenal the total field catch increased at the two high doses. Also, the corresponding 2*R*,3*R*,7*R* isomer, when added to 2*S*,3*S*,7*S* acetate, showed a slight increase in trap catch, although no definite conclusion may be made in this regard.

It has been observed that if the proper optical isomer is used for field tests, the increase in field catch becomes a function of the dose employed (Cardé et al., 1977). Where the doses of the synthetic attractant, either 2*S*,3*S*,7*S* acetate alone (Table 4) or in combination with 2*R*,3*R*,7*S* acetate were increased, the field catch increased proportionally without showing

TABLE 2. FIELD RESPONSE OF MALE *N. sertifer* BY COMBINATION OF SEVERAL SYNTHETIC PHEROMONES WITH 2*S*,3*S*,7*S*-A, SEPTEMBER 23–NOVEMBER 15, 1978 AND 1979, ROSE LAKE AND ARBORETUM, LANSING, MICHIGAN

Optical configuration	Amount (μg)	Average trap catch[a]	Active index (%)[b]
2*R*,3*R*,7*R*-A + 2*S*,3*S*,7*S*-A	25/25	47	81
2*R*,3*R*,7*S*-A + 2*S*,3*S*,7*S*-A	25/25	28	48
2*S*,3*S*,7*R*-A + 2*S*,3*S*,7*S*-A	25/25	37	64
2*S*,3*S*,7*S*-A	25	58	100
2*S*,3*S*,7*S*-P	25	44	76
t-per[c] + 2*S*,3*S*,7*S*-A	25/25	51	88
c-per[c] + 2*S*,3*S*,7*S*-A	25/25	36	62
2*S*,3*R*,7(*R*/*S*)-A + 2*S*,3*S*,7*S*-A	50/25	0.7	1
2*R*,2*S*,7(*R*/*S*)-A + 2*S*,3*S*,7*S*-A	50/25	28	48
2*S*,3*R*,7(*R*/*S*)-P + 2*S*,3*S*,7*S*-P	50/25	0.7	1
2*R*,3*S*,7(*R*/*S*)-P + 2*S*,3*S*,7*S*-P	50/25	12	20

[a]The average of 3 replicates.
[b]The maximum catch was 100.
[c]Abbreviation of *trans*- and *cis*-perillenal, respectively.

TABLE 3. TRAP CATCH OF MALE *N. sertifer* BY COMBINATION OF 25 μG OF 2*S*,3*S*,7*S*-3,7-DIMETHYLPENTADEC-2YL ACETATE AND VARIOUS AMOUNTS OF OTHER MATERIALS

Combinations of synthetic isomers[a]		Replicates (catch/trap) A	B	Total
Series I[b]				
2*R*,3*R*,7*R*-A + 2*S*,3*S*,7*S*-A				
0	25	6	7	13
12.5	25	14	6	20
25	25	20	5	25
50	25	5	12	17
Series II[b]				
2*R*,3*R*,7*S*-A + 2*S*,3*S*,7*S*-A				
0	25	23	8	31
12.5	25	8	3	11
25	25	8	7	15
50	25	6	7	13
Series III[c]				
trans-Perillenal + 2*S*,3*S*,7*S*-A				
0	25	20	19	39
25	25	17	19	36
50	25	49	42	91
100	25	35	26	61
Series IV[c]				
cis-Perillenal + 2*S*,3*S*,7*S*-A				
0	25	20	19	39
25	25	13	25	38
50	25	28	12	40
100	25	9	13	20

[a]The same as in Table 1.
[b]The test was conducted at Rose Lake, Lansing, Michigan from November 1 to November 14, 1978.
[c]The test was conducted from October 3 to November 3, 1978 at Upton Road, Ingham County, Michigan.

any sign of tapering. On the other hand, although higher doses of the acetate of 2*S*,3*S*,7(*R*/*S*) (Matsumura et al., 1978) increased the field catch of *N. lecontei*, this was not the case for *N. sertifer*, indicating that the males of the latter species are more discriminating towards the difference between the 2*S*,3*S*,7*S*-A and the 2*S*,3*S*,7(*R*/*S*)-A preparations. In this series the amount of 2*S*,3*S*,7(*R*/*S*)-A and the number of trap catches were: 0 at 900 μg, 5 at 300 μg, 3 at 100 μg, 0 at 10 and 0.1 μg and 5 at 1 μg/trap. At the same time a trap baited with 10 female equivalents of natural pheromone caught 300 males.

TABLE 4. TRAP CATCH OF MALE *N. sertifer* BY VARIOUS AMOUNTS OF 2*S*,3*S*,7*S*-3,7-DIMETHYLPENTADEC-2-YL ACETATE AND IN COMBINATION WITH 2*R*,3*R*,7*S*-A, OCTOBER 31–NOVEMBER 14, 1978, IN ROSE LAKE, MICHIGAN

Treatment	Replicates (catch/trap) A	B	Total
2*S*,3*S*,7*S*-A (μg)			
30	51	75	126
10	37	44	81
3.0	34	12	46
1.0	4	2	6
0.3	0	1	1
0.1	4	0	4
Blank	0	2	2
RRS-A + *SSS*-A (2:3) (μg)			
30	89	57	146
10	34	58	92
3	13	32	45
1	9	19	28
0.3	7	0	7
0.1	6	4	10
0.03	1	2	3
Blank	2	0	2

Ester Form of Natural Volatile Pheromone. The natural volatiles (i.e., cage wash) from virgin female *N. sertifer* and *Neodiprion nanulus nanulus* (Schedl) were collected from vials. As a result of a field test, it was found that only the volatiles of *N. sertifer* were attractive to *N. sertifer* males in the field (Table 5). The volatiles of *N. nanulus* appeared to reduce the catch of both the *N. sertifer* volatiles and the acetate 2*S*,3*S*,7*S* isomer. It must be noted here that these two species are taxonomically close and, furthermore, that they are sympatric.

The natural volatiles from the female *N. sertifer* were separated by TLC and the zone corresponding to $R_f = 0.39–0.60$ was injected into an SE 30 column. Effluent fractions were collected and field tested. Biological activity was found to align with the GC area corresponding to the synthetic acetate of 3,7-dimethylpentadecan-2-ol (Figure 1, Table 6). The results show that the female releases the acetate of diprionol as the sex attractant.

TLC Purification of Modified Natural Ester Fraction. Acetylation of the KOH-treated body extract fraction gave a higher response than either the propionylated crude pheromone or any of the other synthetic compounds (Table 7). After partial purification by two consecutive TLC systems (Figure 2), the biological activity was aligned with the same zone as the synthetic

TABLE 5. TRAP CATCH OF MALE *N. sertifer* BY 2*S*,3*S*,7*S*-A, THE "NATURAL VOLATILE" PHEROMONE (CAGE WASH) OF *N. sertifer*[a] AND *N. nanulus*[b] AND MIXTURES

Pheromone composition of the bait	Catch/total
4 female equivalents of *N. nanulus* natural volatiles	0[c]
4 female equivalents of *N. nanulus* natural volatiles + 4 female equivalent of *N. sertifer*	18[c]
4 female equivalent of *N. sertifer* natural volatiles	38[c]
4 female equivalent of *N. nanulus* natural volatiles	0[d]
4 female equivalent of *N. nanulus* natural volatiles + 2*S*,3*S*,7*S*-A (μg)	114[d]
2*S*,3*S*,7*S*-A (25 μg)	232[d]

[a]Volatile component obtained from living *N. sertifer* females.
[b]Volatile component obtained from living *N. nanulus* females.
[c]The experiment was conducted from October 2 to 21, 1979, in the arboretum, Michigan with 1 replicate.
[d]The experiments were conducted from September 19 to 25, 1979 in Rose Lake, Michigan with 2 replicates. The values shown are sum of both series.

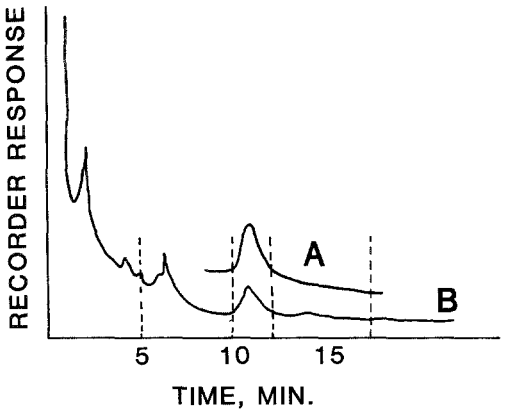

FIG. 1. Gas-liquid chromatographic (GC) pattern of partially purified "natural volatiles" (pattern B) from females of *N. sertifer*. This fraction has been precleaned by thin-layer chromatography (i.e., the ester fraction at R_f 0.39–0.60). The GC pattern of reference standard, 2*S*,3*S*,7(*R*/*S*)-A is also shown for comparison (see pattern A). The areas of collection are indicated by the dotted lines (i.e., zone I, II, etc.). The GC conditions are: 10% SE 30(1.8 × 6 mm) on Gaschrom at 30 ml/mm N_2 at 170°C.

TABLE 6. TRAP CATCH OF *N. sertifer* MALES TO GC FRACTIONS (SEE FIGURE 1) OF NATURAL VOLATILE (CAGE WASH) AT ROSE LAKE, LANSING, MICHIGAN IN 1979

GC fraction (female equivalent)	Dates of replicates (catch/trap)			Total
	10/21 to 10/24	10/25 to 10/29	10/29 to 11/20	
I (10 females)	0	0	0	0
II (10 females)	0	0	0	0
III[a] (10 females)	7	2	1	10
IV[b] (10 females)	1	0	0	1
V (10 females)	2	0	0	2
2*S*,3*S*,7*S*-A (100 ng)	0	1	1	2
2*S*,3*S*,7*S*-A (1 μg)	3	1	2	6

[a]Synthetic diprionol acetate (*SSS*-A) comes at fraction III, see Figure 1.
[b]Synthetic diprionol propionate (*SSS*-P) comes at fraction IV.

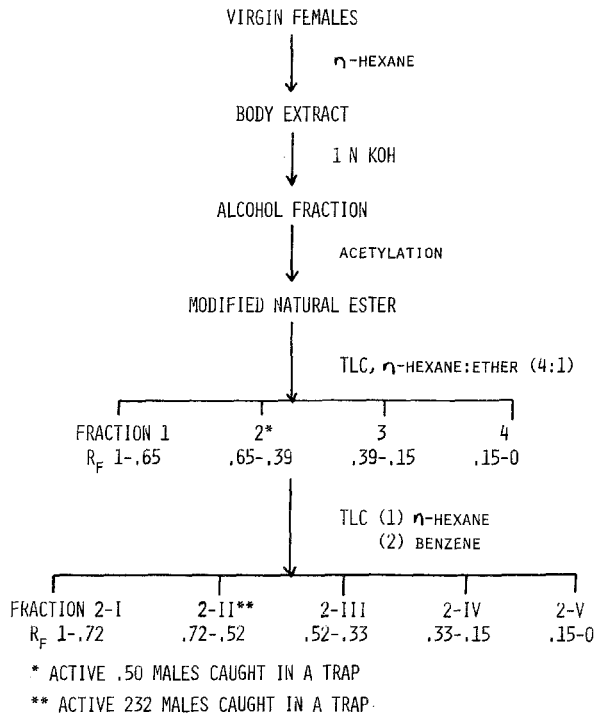

FIG. 2. Schematic diagram of purification procedure of the major sex pheromone (modified natural ester) from the KOH-treated body extract of the females of *N. sertifer*. The fractions were tested for their activity at Rose Lake, Michigan (9/19–9/30/79) after readjusting the quantity per trap to 4 female equivalents.

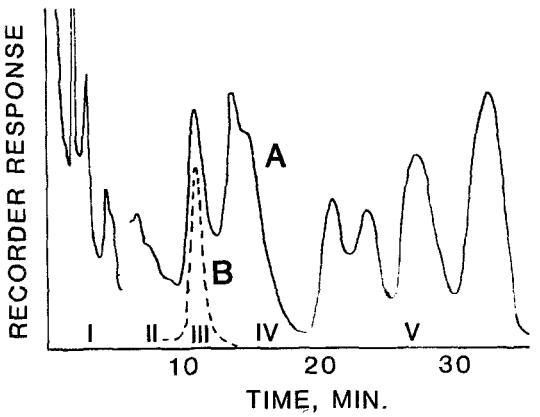

FIG. 3. GC pattern of a partially purified pheromone fraction from the KOH treated body extract (pattern A). For this purpose 5 female equivalents of fraction 2-II (see Figure 2) was used. Effluent zones collected are shown as in Figure 1. The reference standard (150 ng of 2*R*, 3*R*, 7(*R*/*S*)-A) is shown as pattern B. The GC condition was identical to that shown in Figure 1.

attractant. The active ester fraction from the TLC purification was then subjected to GC purification (Figure 3). Fractions were collected and checked for biological activities. The area that had biological activity was completely aligned with the position of the synthetic acetate of diprionol. Therefore, a comparison of the activity of the natural pheromone component and the synthetic acetate of 2*S*,3*S*,7*S*-diprionol was made (Table 7). The natural

TABLE 7. COMPARISON OF FIELD CATCH OF *N. sertifer* MALES TO SYNTHETIC SEX ATTRACTANTS AND CRUDE MODIFIED PHEROMONE[a]

Treatment (isomer)	Amount	Replicates (catch/trap) A[b]	B[c]	Total
2*S*,3*S*,7(*R*/*S*)-A	100 μg	44	3	47
2*S*,3*S*,7(*R*/*S*)-P	100 μg	45	25	70
2*S*,3*S*,7*S*-A	25 μg	125	12	137
2*S*,3*S*,7*S*-P	25 μg	88	41	129
N. sertifer acetate[a]	2 females[d]	315	—	315
N. sertifer propionate[a]	2 females[d]	42	—	42

[a]Obtained by treating first with KOH followed by reesterification of the extract from female *N. sertifer*. Only one trap was used for each series.
[b]Tested from September 26 to October 30, 1978, in the Arboretum, Michigan.
[c]Tested from September 26 to November 1, 1978, in Rose Lake, Lansing, Michigan.
[d]Two female equivalents contained approximately 20 ng of natural pheromone.

pheromone component, although present in lower quantities (20 ng), was more attractive than any of the synthetic attractants (25–100 μg). One possibility for this difference may be a complex interaction among several volatile compounds. To study possibility various GC fractions were mixed and tested for field effectiveness. In the first series of experiments, GC fractions I, II, and IV were mixed in the 2*S*,3*S*,7*S*-A (25 μg) and their field effectiveness was tested at Rose Lake by using duplicate traps. These combinations caught the totals of 30, 42, and 46 males, respectively, while the corresponding traps baited with 2*S*,3*S*,7*S*-A alone caught 102 males. In the next series of experiments, these GC fractions themselves were combined. The results (Table 8) clearly show that there is no significant effect of any mixing.

Partial Purification of Diprionol from Untreated Body Extract. Fraction 4, which was obtained by column chromatography (Figure 4) of untreated body extract from female *N. sertifer*, is not attractive to the males by itself, but the acetylation product from this fraction showed a strong biological activity as described by Jewett et al. (1976). This fraction (Figure 4) was further purified by silica gel column and thin-layer chromatography. Fraction 4.A.I.4 aligned with the synthetic pheromone [2*S*,3*S*,7(*R*/*S*)-A]. This fraction had potent biological activity. The result of GC analysis showed that the retention time of the peak showing the biological activity (B in Figure 5) was identical with the synthetic acetate. The coinjection of synthetic acetate with natural pheromone component showed that they come at the same R_t on the capillary SE 30 column (15 m). In addition, these materials were analyzed by two more columns, 3% OV-101 (3 m) and 3% OV-1 (15 m). The result showed that all R_ts were identical for the synthetic and natural pheromone component as judged by both independent and coinjection methods. Also, it was possible to quantify the natural pheromone component on GC by using an SE 30 capillary column (Figure 5). On an average, each female was found to have approximately 10 ng of diprionol. The most active fraction (4.A.I.4) was further fractionated by GC on 10% SE 30, on Gaschrom Q, or 3% on Supelcoport. The active fraction aligned with synthetic pheromone on both columns.

Although 2*S*,3*S*,7*S*-A showed no evidence of threo contamination by GC analysis (Mori et al., 1978), it is possible a trace amount of threo isomer could reduce catch. Therefore, the above preparation obtained from Dr. Mori was first purified on TLC to eliminate a trace of alcohol contaminant (TLC purified 2*S*,3*S*,7*S*-A) and passed through an active charcoal column. The resulting "purified" preparation showed a higher field effectiveness than the original 2*S*,3*S*,7*S*-A preparation (experiment I, Table 9). The original 2*S*,3*S*,7*S*-A preparation was also subjected to GC with a Carbowax 20 M (4 m) column on Chromosorb AW. In this system erythro isomers come slightly ahead of threo isomers (Mori et al., 1978; unpublished data). To achieve a partial purification, the front, middle, and tail parts of the peak due to 2*S*,3*S*,7*S*-A were separately collected (Figure 6). The central portion

TABLE 8. TRAP CATCH OF MALE *N. sertifer* USING GC-COLLECTED FRACTIONS FROM FIGURE 3; OCTOBER 29–31, 1979, AT ROSE LAKE, MICHIGAN

Fraction no. in Figure 3[a]	Trap location[b]	Catch/trap	Fraction no. combined	Trap location	Catch/trap	Fraction no. plus *SSS*-A (100 ng)	Trap location	Catch/trap
I + II	A	0	III + II	A	9	I + *SSS*-A[c]	A	8
	B	0		B	5		B	1
III	A	0		C	11	I + II + *SSS*-A	A	6
	B	10		D	4		B	3
	C	14	III + IV	A	19	IV + *SSS*-A	A	5
	D	6		B	1		B	1
IV	A	1		C	26	V + *SSS*-A	A	3
	B	0		D	0		B	0
V	A	0	I + II + III + IV + V	A	24	IV + V + *SSS*-A	A	10
	B	0		B	11		B	3
IV + V	A	0				*SSS*-A	A	8
	B	0					B	5

[a]Tested at 5 female equivalents.
[b]Traps were set in four different locations (sites A, B, C, and D) at Rose Lake.
[c]Abbreviation of 2*S*,3*S*,7*S*-A.

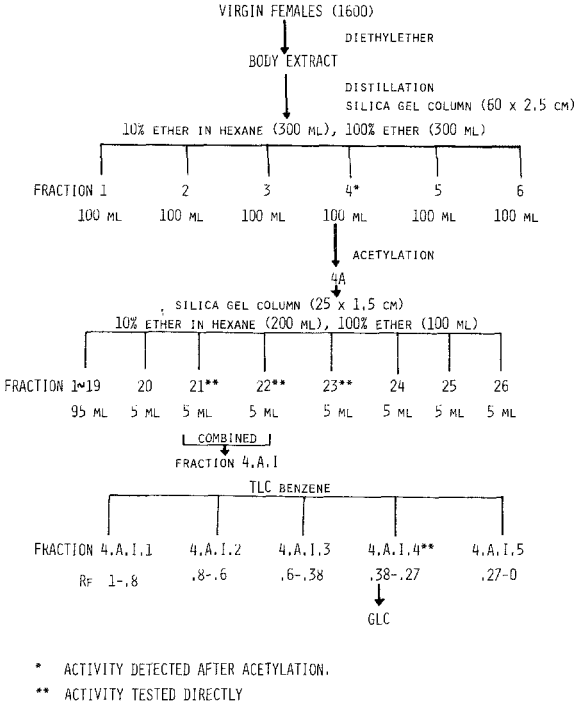

FIG. 4. Schematic diagram of purification method employed to purify the naturally occurring diprionol (alcohol component) from the untreated body extracts of the females.

was most active, and the other two portions, the front and the tail portions showed a lower activity (Table 9). However, this purified 2*S*,3*S*,7*S*-A preparation was less active than the natural pheromone in a side-by-side comparison test (Table 10).

Chiral Combinations Most Inhibitory to Effectiveness of the 2S,*3*S,*7*S *Isomer.* Two key pieces of information obtained by the previous experiment are: (1) the purified natural pheromone is much more effective in attracting *N. sertifer* males than the original 2*S*,3*S*,7*S*-A preparation, and (2) the 2*S*,3*S*,7*S*-A preparation may be further purified to increase its field effectiveness. One possible explanation for these observations is that the original 2*S*,3*S*,7*S*-A preparation contains a trace of a contaminant(s) which inhibits its effectiveness. Earlier it was shown that the presence of a 2*S*,3*R* configuration is detrimental to the effectiveness of 2*S*,3*S*,7*S*-A. To ascertain the above tendency and to determine the inhibitory configuration at the 7th position, various threo isomers were mixed with 2*S*,3*S*,7*S*-A and their relative inhibitory potencies were measured.

The results (Table 11) clearly show that all isomers containing a 2*S*,3*R*

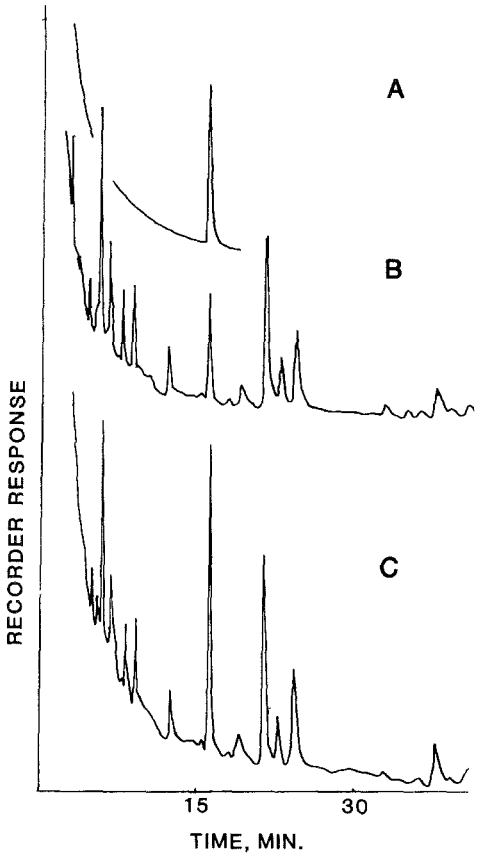

FIG. 5. Capillary GC comparison of acetylated, naturally occurring diprionol with a synthetic pheromone. (A) Synthetic pheromone 2*S*,3*S*,7*S*-A; (B) acetylated natural diprionol (fraction 4.A.1.4 Figure 4); (c) coinjection of A and B. GC conditions are 15 m SE 30 capillary column at 170° C at He flow rate of 1 ml/min.

configuration are inhibitory and that 2(*S*/*R*),3*R*,7*R*-A and 2(*S*/*R*),3*R*,7*R*-P which contain 2*S*,3*R*,7*R* configuration are most potent inhibitors of 2*S*,3*S*, 7*S*-A. 2(*S*/*R*),3*R*,7*R*-P was more inhibitory than 2(*S*/*R*),3*R*,7*S*-P, implying that configuration 2*S*,3*R*,7*R* is more inhibitory than 2*S*,3*R*,7*S*. The 2*S*,3*R*,7*R* configuration is identical to one of the most active chiral isomers for *D. similis* (Kikukawa et al., 1981). To confirm its inhibitory potency, a partially purified natural pheromone of *D. similis* was added to 2*S*,3*S*,7*S*-A. This amount (approximately 40 ng) of the pheromone was enough to suppress the activity of 2*S*,3*S*,7*S*-A. As to the relative importance of *R* contamination between the 3rd and 7th position, we tested several combinations of isomers (Table 12). The combinations giving no *R* contamination at the

TABLE 9. COMPARISON OF *N. sertifer* TRAP CATCH BETWEEN ORIGINAL 2*S*,3*S*,7*S*-A PREPARATION AND THAT PURIFIED BY TLC CHARCOAL AND BY GC; SEPTEMBER TO OCTOBER, 1980 AND 1981, AT ROSE LAKE, LANSING, MICHIGAN

Preparation	Replicates (catch/trap) A	B	C	D	E	Total
Experiment I						
SSS-A original[a] (10 μg)	11				15	25
SSS-A purified[b] (10 μg)	84				26	110
Experiment II	A	B	C	D	E	
SSS-A GC[c] peak frong (0.5 μg)	7	7	6	3	2	25
SSS-A GC[c] peak center (0.5 μg)	3	7	10	1	12	33
SSS-A GC[c] peak tail (0.5 μg)	0	8	7	2	2	19

[a]Abbreviation of 2*S*,3*S*,7*S*-A contaminated by a trace of 2,7-dimethylpentadecan-2-ol and other impurities (see Figure 6).
[b]Purified by TLC and then charcoal column.
[c]The front, middle, and tail portions of the 2*S*,3*S*,7*S*-A peak were collected separately as shown in Figure 6.

3 position (e.g., 2*S*,3*S*,7*R*-A + 2(*S*/*R*),3*S*,7*S*-A) did not give a significant inhibitory activity, while those having some degree of *R* contamination at this position did inhibit the activity.

Stimulatory Effect of Trace Amount of 2S,3R,7R-*A Isomer on Effectiveness of* 2S,3S,7S-*A Isomer.* In this series of experiments we have explored a possibility that the presence of a trace amount of a threo isomer in the 2*S*,3*S*,7*S*-A preparation could increase the effectiveness of the trap catch. The experiments were conducted in the fall of 1981 when a new preparation of 2*S*,3*R*,7*R*-A isomer became available. It was shown by three independent tests (Table 13) that the presence of 0.01–0.003 μg of 2*S*,3*R*,7*R*-A in 5 μg of 2*S*, 3*S*, 7*S*-A was advantageous in terms of effective catching of *N. sertifer* males. In the next test the synergistic effectiveness of several threo preparations containing some 2*S*,3*R*,7*R*-A component was compared at a fixed ratio of 5:0.003 μg. Among them 2*S*,3*R*,7*R*-A and 2(*R*/*S*),3*R*,7*R*-A were most synergistic followed by 2*S*,3*R*,7(*R*/*S*)-A and 2(*S*/*R*),3*R*,7*S*-A; the sum of three traps for each compound being 13, 17, 12, and 8, respectively.

In view of the increased effectiveness of these mixtures an attempt was made to compare the potency of naturally occurring diprionol which was purified through TLC and its acetate prepared as before (Figure 4). The second TLC gave the fraction with the natural pheromone. The amount of the pheromone present with this preparation was determined through GC with an FID detector (Figure 5).

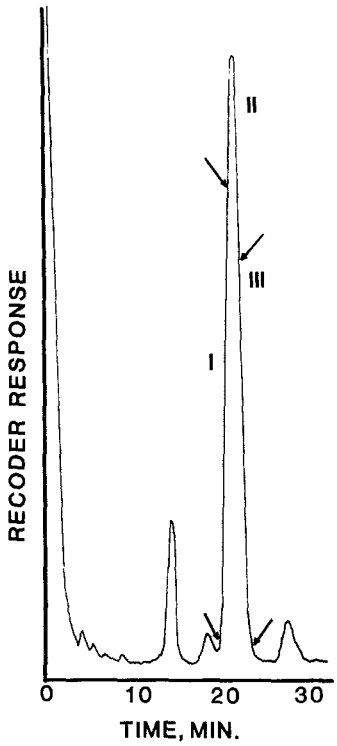

FIG. 6. GC pattern of the original 2*S*, 3*S*, 7*S*-A on 5% Carbowax 20 M column. The fractionation pattern adopted is shown by arrows and the number (I, II, and III). Other GC conditions are column 4 m × 3 mm, stainless steel, at 160°C, 30 ml/min N_2, Carbowax on AW.

As a result of a comparison test (Table 14), it was established that the synergized preparation, containing 2*S*,3*S*,7*S*-A and a trace of 2*S*,3*R*,7*R*-A at a ratio of 5:0.003, was roughly as active as the naturally occurring pheromone preparation.

DISCUSSION

That the chiral configuration of the major pheromone of *N. sertifer* is 2*S*,3*S*,7*S*-A was established by this work. Throughout the purification processes, the pheromone activity was closely aligned with the synthetic enantiomer. In fact, there was no other active component in any of the chromatographic fractions. Also, none of the other enantiomers tested were active for the *N. sertifer* males except 2*S*,3*S*,7*S*-P and some racemic preparations that contained an ester of 2*S*,3*S*,7*S*-diprionol.

TABLE 10. COMPARISON OF *N. sertifer* TRAP CATCH OF TLC–CHARCOAL COLUMN PURIFIED 2*S*,3*S*,7*S*-A PREPARATION AND PURIFIED NATURAL PHEROMONE OF *N. sertifer*, TESTED AT ROSE LAKE, MICHIGAN[a]

Preparation	Amount (ng)	Replicates (catch/trap) A	B	Total
SSS-A purified[b]	300	5	6	11
	100	19	0	19
	30	4	1	5
Natural pheromone purified[c]	30	68	40	108
	10	8	9	17
	3	10	5	15

[a]Blank caught no males.
[b]TLC-charcoal purification as described in Method and Materials.
[c]GC purified as shown in Figure 3.

TABLE 11. ANTAGONISTIC ACTIONS OF SEVERAL THREO ISOMERS AGAINST 2*S*,3*S*,7*S*-A TRAP CATCH OF *N. sertifer* MALES; KELLOG FOREST, IN TRIPLICATE, SEPTEMBER 20–OCTOBER 4, 1980

Preparations (isomer)	Amount (μg)	Replicates (trap/catch) A	B	C	Total
(*R*/*S*)*RR*-P + *SSS*-A	10/5	0	0	0	0[b]
(*R*/*S*)*RS*-P + *SSS*-A	10/5	0	2	2	4[b]
SSS-A	5	6	7	6	19
(*R*/*S*)*RR*-P	10	0	0	0	0
(*R*/*S*)*RS*-P	10	0	0	0	0[b]
(*R*/*S*)*RR*-A + *SSS*-A	10/5	0	0	0	0[b]
(*R*/*S*)*RR*-A	10	0	0	0	0[b]
(*R*/*S*)-*RS*-A	10	0	0	0	0[b]
D. similis pheromone[a] + *SSS*-A	4 ♀/5	0	2	1	3[b]
RRR-P + *SSS*-A	5/5	1	2	6	9
RRS-P + *SSS*-A	5/5	4	15	3	22
RRS-A + *SSS*-A	5/5	2	5	19	26
SR(*R*/*S*)-P + *SSS*-A	10/5	0	0	0	0[b]
SR(*R*/*S*)-A + *SSS*-A	10/5	0	0	0	0[b]

[a]Partially purified pheromone prepared from the body extract from virgin females of *Diprion similis* by using purification methods shown in Figure 4. The fraction used here is the ester fraction from the first TLC. This fraction when tested alone showed no attractiveness to *N. sertifer* males.
[b]Significant reduction as compared to the data for 2*S*,3*S*,7*S*-A (compared by mean trap catch).

TABLE 12. EFFECTS OF PRESENCE OF OTHER ISOMERS WITH TA-4 ON FIELD TRAP CATCH OF *N. sertifer* MALES; SEPTEMBER 6–OCTOBER 3, 1980, AT FENWAY ARBORETUM, LANSING, MICHIGAN

Preparations[a]	Amount (μg)	Replicates (catch/trap) A	B	C	Total
SSS-A	(25)	26	11	4	41
SSS-A + (*S*/*R*)*RR*-A + *RRS*-A[b]	(50/25/25)	0	2	0	2[d]
SSS-A + (*S*/*R*)*RS*-A + *RRR*-A[b]	(50/25/25)	1	2	0	3[d]
SSS-A + (*R*/*S*)*SR*-A[c]	(25/50)	7	7	14	28
SSS-A + (*R*/*S*)*SS*-A[c]	(25/50)	21	0	7	28

[a]See Tables 1 and 2 for coding of each preparation.
[b]In these preparations the portions of *R* in relation to *S* for each position were kept constant at $2R(\frac{1}{2})$, $3R(\frac{3}{4})$, $7R(\frac{1}{2})$.
[c]The first combination gave $2R(\frac{1}{3})$, $3R(0)$, $7R(\frac{2}{3})$, and the second combination gave $2R(\frac{1}{3})$, $3R(0)$, $7R(\frac{1}{3})$.
[d]Significant reduction at $P < 0.05$.

TABLE 13. STIMULATORY EFFECT OF TRACE AMOUNT OF 2*S*,3*S*,7*R*-A (KA-1) ON EFFECTIVENESS OF TRAP CATCH BY 2*S*,3*S*,7*S*-A; ROSE LAKE, MICHIGAN, SEPTEMBER 19–NOVEMBER 9, 1981

SSS-A + *SRR*-A (μg/trap)	Series A[a] 9/19–11/9 ($\bar{X} \pm$ SE)	Series B[a] 9/27–11/9 ($\bar{X} \pm$ SE)	Series C[a] 10/9–11/9 ($\bar{X} \pm$ SE)
5/1	0.7 ± 0.7	0.7 ± 0.7	0.7 ± 0.7
5/0.3	1.7 ± 0.3	1.3 ± 0.3	0.0 ± 0.0
5/0.1	22.3 ± 4.3	14.3 ± 5.0[b]	2.7 ± 0.9
5/0.03	7.7 ± 4.7	4.7 ± 2.7	3.0 ± 1.0
5/0.01	43.7 ± 16.8[b]	24.3 ± 13.9[b]	12.7 ± 4.8[b]
5/0.003	87.7 ± 39.1[b]	13.7 ± 1.5[b]	7.0 ± 3.2[b]
5/0.001	[c]	4.0 ± 1.0	5.0 ± 0.6
5/0.0005	[c]	4.7 ± 0.9	2.3 ± 0.9
5/0	[c]	5.0 ± 1.2	2.7 ± 2.2

[a]The result of catches by three sets of traps for each concentration.
[b]All means significantly different from others by Duncan's multiple range test at 5% level.
[c]These traps were inadvertently left out of series A. During the same period and at the same location a trap baited for 20 μg of *SSS*-A alone caught 37 males.

TABLE 14. COMPARISON OF FIELD EFFECTIVENESS OF NATURAL AND SYNTHETIC PHEROMONE OF *N. sertifer;* ROSE LAKE, MICHGAN, OCTOBER 9–NOVEMBER 9, 1981

Pheromone	Amount (ng/trap)	Replicates A	B	C	Total	Mean/trap
Synthetic mixture TA-4/KA-1 (5:0.003)	30	0	0	4	4	1.3
	10	0	7	5	12	4.0[a]
	3	2	3	10	15	5.0[a]
	1	0	3	5	8	2.7
Natural Pheromone (acetate)	3 (=0.3 FE)	4	6	15	25	8.3[a]
	1 (=0.1 FE)	1	0	8	9	3.0[a]
	0.3 (=0.03 FE)	0	0	0	0	0

[a]Means significantly different by Duncan's multiple range test at 5% level from other values.

On the other hand, there is no question about the superiority of the naturally occurring pheromone in catching the males in the field over the synthetic attractant 2*S*,3*S*,7*S*-A. Therefore, a question must be raised why such a discrepancy should exist, if these two are expected to be identical. There could be three major possibilities: first, there is a synergistic component or components in the sawfly extracts that make the natural pheromone very active; second, the synthetic attractant used in this study was not completely pure, containing contaminants that greatly reduced the field effectiveness of the attractant; and third, the natural pheromone consists of a precise mixture of enantiomers or derivatives which could not be separated even by capillary column GC.

Initially the first possibility was pursued in detail in this work. The major volatile component from the female and the male *N. sertifer*, *trans*-perillenal (Ahlgren et al., 1979) appeared to have a modest synergistic action, when it was added to 2*S*,3*S*,7*S*-A at a ratio of 2:1. However, it is not likely that this component alone can explain the difference in potency between the natural and the synthetic pheromone found in this work. The main reason for this conclusion is that *trans*-perillenal is a rather labile and volatile compound and, therefore, is destroyed by KOH; even if it is not, it should be easily separated from the major pheromone in any of the chromatographic processes. Yet, the difference was observed in the most purified fraction. To test the presence of other synergistic components, various fractions obtained during the purification processes were combined with the synthetic attractant, 2*S*,3*S*,7*S*-A. In none of the cases was it possible to increase the activity of the synthetic attractant by this approach.

Under the circumstances, the above possibility was ruled unlikely, al-

though a complete dismissal was not possible. The second possibility, that the synthetic attractant preparation 2*S*,3*S*,7*S*-A contains some contaminants which reduce its field effectiveness, has been shown to be partially true according to the data obtained in the current work. In the data shown in Table 14, we could demonstrate that purification of the original 2*S*,3*S*,7*S*-A through TLC, charcoal column, and by GC resulted in a fourfold increase in the activity of the original preparation. It was also possible to demonstrate that the activity of 2*S*,3*S*,7*S*-A preparations could be drastically reduced by a small amount of enantiomers containing a 2*S*,3*R* configuration. The third possibility is the one which would most likely explain the phenomenon. The addition of a trace of threo acetate containing 2*S*,3*R*,7*R*-A configuration certainly increases the field effectiveness of 2*S*, 3*S*, 7*S*-A.

Since it has been shown in this work that mixing of a small amount of optical isomers could drastically affect the effectiveness of the synthetic attractant 2*S*,3*S*,7*S*-A, a question must be raised as to the optical purity of these synthetic epimers themselves. At this stage there is no convenient method to measure the optical purity of each active carbon in the final synthetic product per se. NMR spectroscopic analysis, however, gives information as to the diastereometric arrangement on carbons 2 and 3 (Jewett et al., 1976). As judged by this approach, none of the erythro isomers provided by Dr. Mori's group is contaminated with threo isomers. The limit of detection is approximately 1%. On the other hand, all threo compounds synthesized via Wittig reaction (Tai et al., 1978; Kikukawa et al., 1981) contain approximately 5% of epimerization product at carbon 3. A study is in progress to develop a methodology to assess the extent of chiral contamination at each carbon position. Meanwhile, it appears from our data that the contamination of the 3*S* position of 2*S*,3*S*,7*S*-A with 3*R* is the crucial point in the case of field attraction of *N. sertifer* males, as mixing of a small portion (0.06%) of *SR* (*R*/*S*)-A, (*S*/*R*) *RR*-A, and *SRR*-A to *SSS*-A gave an equally stimulatory effect. If such is the case the maximum possible 3*R* contamination of 2*S*,3*S*,7*S*-A is 1%, whereby the additional increase of 0.2–0.06% of the 3*R* component must be recognized by *N. sertifer* males.

In conclusion, the current work clearly established that the natural pheromone aligns with the acetate of diprionol in all chromatographic behavior. It was also found that 2*S*,3*S*,7*S* is the most preferred chiral configuration by the males of *N. sertifer*. The most intriguing aspect of our finding is that small amounts of 2*S*,3*R*,7*R*-A stimulates the field effectiveness of the 2*S*,3*S*,7*S* preparation.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS XIV. Response of *Telenomus remus*[1] to Abdominal Tips of *Spodoptera frugiperda*,[2] (*Z*)-9-Tetradecene-1-ol Acetate and (*Z*)-9-Dodecene-1-ol Acetate[3,4]

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Abstract—*Telenomus remus* Nixon is a parasitoid that attacks egg masses of *Spodoptera frugiperda* (J.E. Smith). Increased host-seeking behavior was elicited from *T. remus* females in Y-tubes, Petri dish, and greenhouse bioassays by *S. frugiperda* female abdominal tips as well as (*Z*)-9-tetradecene-1-ol acetate and (*Z*)-9-dodecene-1-ol acetate.

Key Words—Kairomone, pheromone, parasitoid, *Telenomus remus*, Hymenoptera, Scelionidae, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, fall armyworm, (*Z*)-9-tetradecene-1-ol acetate, (*Z*)-9-dodecene-1-ol.

INTRODUCTION

Telenomus remus Nixon, a scelionid egg parasitoid indigenous to Sarawak and New Guinea, was introduced into Israel for control of *Spodoptera littoralis* Boisduval (Gerling, 1972) and has been successfully established in Barbados and Montserrat on *Spodoptera frugiperda* (J.E. Smith), *Spo-*

[1]Hymenoptera: Scelionidae.
[2]Lepidoptera: Noctuidae.
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doptera eridania (Cramer), and *Spodoptera sunia* (Guénee) (Wojcik et al., 1976). *T. remus* was introduced into Florida (near Homestead) from May 1975 to May 1977 but apparently was not established (Waddill and Whitcomb, 1982).

Sekul and Sparks (1967) identified (*Z*)-9-tetradecen-1-ol acetate (*Z*-9-TDA) as the sex pheromone of *S. frugiperda.* However, *Z*-9-TDA proved to be ineffective as a sex attractant in the field, although it does elicit wing fanning and copulatory behavior in the laboratory (Mitchell and Doolittle, 1976; Sparks, 1980) and was referred to as a secondary sex pheromone by Jones and Sparks (1979). Sekul and Sparks (1976) identified a second compound, (*Z*)-9-dodecen-1-ol acetate (*Z*-9-DDA), from *S. frugiperda* female abdominal tips, and this compound is highly attractive in the field to *S. frugiperda* males (Mitchell, 1979; Sparks, 1980).

The involvement of kairomones in the host-location behavior of parasitoids has been demonstrated on numerous occasions (Weseloh, 1981; Arthur, 1981; Jones, 1981). There have also been several demonstrations of compounds that function as pheromones in intraspecific interactions and as kairomones in interspecific interactions (Rice, 1969; Vité and Williamson, 1970; Sternlicht, 1973; Kennedy, 1979; Corbet, 1971; Lewis et al., 1982). As an example, Lewis et al. (1982) demonstrated that the compounds comprising the sex pheromone of *Heliothis zea* (Boddie) (Klun et al., 1980) increased rates of parasitization by *Trichogramma* spp. in the laboratory and field.

The study reported here was designed to determine if chemicals emanating from the abdominal tips of *S. frugiperda,* and particularly *Z*-9-TDA and *Z*-9-DDA, have any effect on the host-finding behavior of *T. remus.*

METHODS AND MATERIALS

The *T. remus* used in this study were reared on *H. zea* eggs that had been processed with a sodium hypochlorite wash as described by Burton (1969) and irradiated with ca. 25 krad (^{60}CO source) when 8–36 hr old, at ca. 70% relative humidity and 26°C using the method described by Lewis and Redlinger (1969) for *Trichogramma.* Female parasitoids were used in the experiments when 1–2 days old.

The *S. frugiperda* moths and egg masses used in this study were obtained from a laboratory culture maintained according to the procedure of Perkins (1979). The *H. zea* eggs used were also obtained from a laboratory culture, processed as stated above, and stored at ca. 10°C.

The abdominal tip extract used in this study was obtained from the tips excised from 2-day-old virgin *S. frugiperda* moths. The moths were

anesthetized with CO_2, their abdomens squeezed, and the tips cut with scissors. The excised tips were washed twice in hexane and twice in anhydrous ether; the solvent extracts were combined and filtered through a fine-fritted Buchner funnel over sodium sulfate and then concentrated to 10 female equivalents (FE)/ml.

The *Z*-9-TDA used in these studies was supplied by Dr. U.E. Brady (Department of Entomology, University of Georgia, Athens, Georgia 30602; ENT33474a) and the *Z*-9-DDA used was obtained from Midwest Research Institute (425 Volker Blvd., Kansas City, Missouri, 64110; MRI No. 71-68-9).

Statistical analysis was done by analysis of variance. Arcsin transformations were conducted on all percentages prior to analysis.

RESULTS

Y-Tube Olfactometer Experiments. The Y-tube olfactometer used in these studies consisted of a basic Y tube, each arm of which was 20 cm long and a leg 33 cm long made of 2-cm-ID glass tubing. Air that had been passed through an activated charcoal filter was introduced at ca. 20 ml/min through each arm with the flow controlled by separate flow meters. These experiments were run at room temperature in a laboratory lighted with fluorescent lights and with an incandescent bulb (150 watt) at ca. 50 cm directly over the Y tube.

During the experiment, the treated side of the olfactometer was alternated so that effects of irregularities in the Y tube itself or room lighting would be eliminated. The treatment was applied to Whatman No. 1 filter paper (ca. 6 cm^2), and a piece of untreated paper was placed in the control arm of the olfactometer. *T. remus* females were released from 2-dr shell vials into the end of the leg of the olfactometer (10/rep), and at the end of 10 min the number of parasitoids in each arm of the Y tube was counted. At the end of each replication, the olfactometer was thoroughly washed with soap and water, rinsed with acetone, and dried in an oven.

The results are given in Table 1 and indicate that *T. remus* females are attracted to extracts of *S. frugiperda* female abdominal tips as well as to *Z*-9-TDA and *Z*-9-DDA.

Petri Dish Bioassays. Comparative evaluations of parasitism rates by *T. remus* on *H. zea* eggs were made in 150 × 15-mm Petri dishes on a laboratory table. A pipet was used to apply the treatment solution to the bottom of the treated dishes at the rate of 1 ml/dish. Control dishes received no such treatment. *H. zea* eggs were applied to the bottom surface with three groups of five eggs. The groups were evenly distributed around the dish, ca. 30 mm from the outside edge. The eggs in each group were within 1 mm of

TABLE 1. RESPONSE OF *Telenomus remus* FEMALES TO EXTRACT OF *Spodoptera frugiperda* FEMALE ABDOMINAL TIPS AND TO SPECIFIC CHEMICALS OCCURRING IN TIPS[a]

Replications	Treated	Control
	Abdominal tip extract (0.05 FE/run)	
24	3.4 (±0.4)	0.6 (±0.1)
	(*Z*)-9-DDA (0.1 μg/run)	
24	3.5 (±0.3)	1.8 (±0.3)
	(*Z*)-9-TDA (0.1 μg/run)	
24	4.6 (±0.3)	1.9 (±0.2)

[a]Means significantly different ($P < 0.05$) as determined by ANOVA.

each other. Two female *T. remus* were introduced and allowed to search and oviposit for 1 hr. Percent parasitization was determined by the method described by Lewis and Redlinger (1969) for determining parasitization by *Trichogramma*.

The results of these experiments, given in Table 2, demonstrate that the abdominal tip extract as well as the *Z*-9-TDA and *Z*-9-DDA elicit increased rates of parasitization in Petri dishes.

Greenhouse Experiments. In these experiments, pans of pink-eyed purple hull cowpeas were arranged on greenhouse tables in groups of three (close enough together that the foliage touched) with ca. 0.75 m between groups. *S. frugiperda* egg masses, on paper, were pinned to the leaves in the pans, 3 egg masses/pan. The treatment was applied to two cotton rolls (No. 2 medium, Uni-Disco, Inc., P.O. Box 4450, Detroit, Michigan 48228). The two rolls were placed on the greenhouse table in the center of each group of treated pans. The control groups received no such treatment. Two

TABLE 2. PERCENT PARASITIZATION OF *Heliothis zea* EGGS BY FEMALE *Telenomus remus* IN A LABORATORY PETRI DISH BIOASSAY OF FEMALE *Spodoptera frugiperda* ABDOMINAL TIP EXTRACT AND SPECIFIC CHEMICALS OCCURRING IN TIPS[a]

Replications	Treated	Control
	Abdominal tip extract (1 FE/ml)	
60	27.3 (±5.1)	7.0 (±2.0)
	(*Z*)-9-TDA (1 μg/ml)	
80	14.0 (±2.0)	6.6 (±1.2)
	(*Z*)-9-DDA (1 μg/ml)	
80	21.2 (±2.3)	12.5 (±2.0)

[a]Means significantly different ($P < 0.05$) as determined by ANOVA.

TABLE 3. MEAN PERCENTAGE PARASITIZATION OF *Spodoptera frugiperda* EGG MASSES ON PANS OF PINK-EYED PURPLE HULL COWPEAS IN A GREENHOUSE, BY FEMALE *Telenomus remus* IN RESPONSE TO TREATMENTS OF *S. frugiperda* FEMALE ABDOMINAL TIP EXTRACT OR SPECIFIC CHEMICALS FOUND IN TIPS[a]

Replications	Treated	Control
	Abdominal tip extract (1 FE/ml)	
30	75.5 (±2.1)	62.2 (±2.5)
	(*Z*)-9-TDA (0.5 μg/cotton roll)	
20	43.3 (±2.3)	28.9 (±1.9)
	(*Z*)-9-DDA (0.5 μg/cotton roll)	
15	49.6 (±2.8)	35.6 (±2.7)

[a]Means significantly different ($P < 0.05$) as determined by ANOVA.

vials of 6 female *T. remus* each were released under the foliage, on opposite sides of each group. The parasitoids were allowed to search for ca. 5 hr. The egg masses were then collected and stored at ca. 70% relative humidity and 26°C until the parasitized eggs turned black. Each egg mass that had parasitized eggs was counted as a parasitized egg mass, even though not all the eggs in the mass were parasitized. A pair of one treated and one control group of pans constituted a replication.

The results of this series of experiments, given in Table 3, again demonstrated that the abdominal tip extract, Z-9-TDA, and Z-9-DDA elicit increased rates of parasitization in the greenhouse. It should be noted that although it is possible that the parasitoids made direct contact with the cotton rolls containing the test materials, the response of increased host search and the resulting increased rates of parasitization occurred some distance from the rolls.

DISCUSSION

The data presented in this study clearly demonstrate that *T. remus* females respond to chemicals emanating from the abdominal tips of *S. frugiperda* and to Z-9-TDA and Z-9-DDA, in particular, two compounds that have been identified as sex pheromones of *S. frugiperda* (Sekul and Sparks, 1967, 1976). The presence of these compounds stimulates increased rates of parasitization by *T. remus* females and can apparently function without direct contact.

Thus, we have an additional example of pheromones also functioning as a kairomone. The possibility of integrating augmentation of entomophagous insects and mating disruption techniques into a control strategy

for *S. frugiperda*, should *T. remus* be established in the United States or used in augmentative releases, is evident. It may also be possible that these materials could be used in traps to monitor establishment and dispersion of the parasitoid in any future release efforts.

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SYNTHESIS OF δ-LACTONIC PHEROMONES OF *Xylocopa hirsutissima* AND *Vespa orientalis* AND AN ALLOMONE OF SOME ANTS OF GENUS *Camponotus*

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Abstract—Simple preparations of *cis*-3,6-dimethyltetrahydro-2-pyrone, VII, 6-*n*-undecyltetrahydro-2-pyrone, XVIIb, and 6-*n*-pentyl-5,6-dihydro-2-pyrone, XVIa, have been achieved. Products VII and XVIIb, respectively, are the major constituent of the pheromonal blend of a carpenter bee (*Xylocopa hirsutissima*) and the pheromone of the queens of the Oriental hornet (*Vespa orientalis*). The lactone XVIa is a suspected defensive allomone in two species of formicine ants of the genus *Camponotus*. All three compounds have been prepared from dehydroacetic acid, I, a cheap and industrially available starting material.

Key Words—*Xylocopa hirsutissima, Vespa orientalis, Camponotus*, Hymenoptera, Xylocopidae, Vespidae, Formicidae, pheromone, allomone, chemical preparation, *cis*-3,6-dimethyltetrahydro-2-pyrone, 6-*n*-undecyltetrahydro-2-pyrone, 6-*n*-pentyl-5,6-dihydro-2-pyrone, lactones.

INTRODUCTION

δ-Lactones are widely spread in nature; some of them are important as flavoring substances in plants (Ohloff, 1978) and others play a role in insect behavior (Brand et al., 1979). The simple triketide, 4-hydroxy-6-methyl-2-pyrone (triacetic acid lactone, II), a well-known natural product (Bentley et al., 1967 a and b) could be an appropriate starting material for the synthesis of natural δ-lactones. Moreover, II is easily accessible in one synthetic step by deacetylation of the industrially available 3-acetyl-4-hydroxy-6-methyl-2-pyrone (dehydroacetic acid, I), (Collie, 1891, 1907) (Scheme 1).

We describe preparations of the racemic forms of *cis*-3,6-dimethyltetrahydro-2-pyrone, VII, the major constituent of the pheromonal blend of a carpenter bee (*Xylocopa hirsutissima*) (Wheeler et al., 1976); 6-*n*-undecyl-

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tetrahydro-2-pyrone,XVIIb,pheromone of the queens of the Oriental hornet (*Vespa orientalis*) (Ikan et al., 1969), also found in the plant *Cistus ladaniferus L.* (De Pascual et al., 1979); and 6-*n*-pentyl-5,6-dihydro-2-pyrone,-XVIa, called massoia lactone, a suspected defensive substance in two species of formicine ants of the genus *Camponotus* (Cavill et al., 1968). The lactone XVIa is also present in *Cryptocaria massoia* (Fam. Lauraceae) (Crombie, 1955a; Benoni et al., 1968; and references cited in these papers) and in *Polianthes tuberosa L.* (Fam. Amaryllidaceae) (Kaiser et al., 1976).

Although this work has been published in preliminary form (Bacardit et al., 1980, 1982), full experimental details were not disclosed; the interest very recently drawn by our target molecules (see below) prompts us to describe those details.

One enantiomer of VII is the major volatile component of the *Xylocopa hirsutissima* pheromonal blend. Syntheses have been described for the racemic form (Wheeler et al., 1976; Pyysalo et al., 1975), for the enantiomeric pure forms (Pirkle et al., 1978, 1979), and for the 3*R*,6*R* isomer (*trans*) (Hanessian et al., 1981). However the aforementioned synthesis produce large amounts of the *trans* isomers VIII.

Vespa orientalis is the most serious enemy of the honeybee in the Near East (Ikan et al., 1969), and therefore some effort has been devoted to the preparation of the lactone XVIIb. Syntheses have been previously described for the racemic form (Ikan et al., 1969; Robinson, 1930) and for the pure enantiomers (Pirkle et al., 1979; Coke et al., 1976; Solladié et al., 1982). All of them start from open-chain materials.

Similarly, massoia lactone, XVIa, has been also prepared in racemic form (Crombie, 1955b, and papers cited therein; Carlson et al., 1975; Chmielewski et al., 1981; Fehr et al., 1981). Its *R* enantioisomer is the natural one. Syntheses of both *R* and *S* isomers (Pirkle et al., 1980) and of the *S* antipode (Mori, 1976) have been described.

METHODS AND MATERIALS

IR spectra were recorded on a Perkin-Elmer Infracord 720 spectrophotometer. [^{1}H]NMR and [^{13}C]NMR spectra were recorded on a Perkin-Elmer R-12 and Varian CFT-20 spectrometer, respectively, and mass spectra were run on a Hewlett-Packard 5930-A spectrometer. Melting points are uncorrected.

Preparation of 4-Hydroxy-6-methyl-5,6-dihydro-2-pyrone, III. Triacetic acid lactone, II (1.1 g, 8.7 mmol) was hydrogenated at room temperature and 1 atm. in absolute ethanol (40 ml) with 10% Pd-C (0.2 g). After 20 min, uptake of one equivalent of hydrogen was observed. The catalyst was filtered off and the solution was evaporated to afford III (92% yield), mp 123–124° (from ethanol), lit. mp 123° (Nedjar et al., 1978).

Preparation of 4-Hydroxy-3,6-dimethyl-5,6-dihydro-2-pyrone, IV. The lactone III (9.78 g, 0.076 mol) was added to a solution (75 ml) of lithium isopropoxide (from lithium, 535 mg, 0.0764 mol in isopropanol). After stirring at room temperature for 1 hr, methyl iodide (40.0 g, 0.281 mol) was added. The mixture was stirred for 24 hr, acidified with hydrogen chloride in methanol and evaporated to afford a dark oil which was dissolved in chloroform. The chloroform solution was washed with aqueous sodium thiosulfate, dried, and evaporated to yield a solid residue from which upon recrystallization (chloroform–pentane) IV (2.2 g) was obtained, mp 152–153°, lit. mp 148–149° (Jones et al., 1949). The liquor from the above recrystallization was evaporated and chromatographed through silica gel. The following compounds were isolated: *3,3,6-Trimethyl-4-oxotetrahydro-2-pyrone, XII,* (2.2 g, eluted with chloroform), bp 65–68° (oven temp.)/0.5 mm Hg, IR ($CHCl_3$): 3000, 2950, 1740, 1720 cm^{-1} [^{1}H]NMR ($CDCl_3$): δ 1.40 (s, 3 H), 1.45 (s, 3 H), 1.50 (d, $J = 6$ Hz, 3 H), 2.4–3.0 (part AB of an ABX system. J_{AB}, J_{AX}, and J_{BX} ca. 14, 6, and 0 Hz, 2 H), 4.55–5.10 (m, 1 H). MS: 156(M^+, 1), 70(78), 69(47), 45(10), 43(58), 42(100). *4-Methoxy-6-methyl-5,6-dihydro-2-pyrone, XI,* (1.05 g, eluted with chloroform–ethyl acetate, 95/5), mp 63–65° (from ether–pentane), lit. mp 68° (Hänsel et al., 1966). Pyrone IV, (2.65 g, eluted with chloroform–ethyl acetate, 90/10). The overall yield of IV was 45%.

Preparation of 4-Hydroxy-3,6-dimethyltetrahydro-2-pyrone, V. The lactone IV (213 mg, 1.5 mmol) was hydrogenated at room temperature and 1 atm. in ethyl acetate (10 ml) with PtO_2 (50 mg). After 24 hr the catalyst was filtered off, and the solution was evaporated to afford V in 95% yield. IR ($CHCl_3$): 3620, 3500 (broad), 1730 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 1.1–1.5 (complex, 6 H), 1.5–2.8 (m, 3 H), 3.2–3.6 (broad, interexchanges with D_2O, 1 H), 3.6–4.6 (m, 2 H). MS: 145 ($M^+ + 1$, 7), 85(22), 74(21), 73(17), 72(34), 71(62), 70(31), 69(19), 58(100), 57(89), 56(86), 55(29), 45(29), 43(96).

Preparation of 3,6-Dimethyl-5,6-dihydro-2-pyrone, VI. The hydroxylactones V (mixture of stereoisomers, 200 mg, 1.38 mmol) were refluxed for 15 hr in benzene with a catalytic amount of *p*-toluenesulfonic acid. The solution was washed with aqueous potassium carbonate, dried and evaporated to afford VI (83% yield), mp 31–33° (from methylene chloride–pentane) and bp 55–60° (oven temp./0.2 mm Hg). IR ($CHCl_3$): 3000, 2950, 1720 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 1.42 (d, $J = 7$ Hz, 3 H), 1.91 (broad s, 3 H), 2.1–2.5 (m, 2 H), 4.2–4.8 (m, 1 H), 6.5–6.75 (m, 1 H). MS: 126 (M^+, 37), 111(24), 82(100), 67(11), 55(26), 54(59), 43(24). Analysis: Calc. for $C_7H_{10}O_2$: C, 66.65; H, 7.99. Found: C, 66.51; H, 7.87.

Preparation of cis*-3,6-Dimethyltetrahydro-2-pyrone, VII.* The unsaturated lactone VI (500 mg, 3.96 mmol) was hydrogenated at room temperature and 1 atm. in ethyl acetate (3 ml) with 10% Pd-C (50 mg) for 3 hours. After about 20 min, uptake of one equivalent of hydrogen was observed. The

catalyst was filtered off and the solution was evaporated to afford products VII and VIII (bp 60–61° (oven temp.)/0.7 mm Hg) as a 9:1 mixture (GC in a DEGS column (2 m) at 150° and in a Carbowax column (1 m) at 150°). Spectroscopic data were as previously described (Wheeler et al., 1976; Pyysalo et al., 1975; Pirkle et al., 1979). [^{13}C]NMR ($CDCl_3$) for VII: δ 16.21, 21.09, 25.68, 28.49, 33.00, 74.49, and 176.26. Small peaks belonging to VIII were observed at 17.37, 22.16, 28.49, 31.04, 35.82, and 78.22. Analysis: Calc. for $C_7H_{12}O_2$: C, 65.60; H, 9.44. Found: C, 65.54; H, 9.40.

*Preparation of 3-acetyl-4-hydroxy-6-*n*-pentyl-2-pyrone, XIIIa.* Dehydroacetic acid, I, (4.25 g, 25.3 mmol) was added to a suspension of sodium amide [from sodium (2.1 g, 0.091 mol) and a catalytic amount of iron(III) nitrate] in liquid ammonia (500 ml). The mixture was mechanically stirred for 30 min until a yellowish color had developed. 1-Bromobutane (4.0 g, 29.2 mmol) in anhydrous ether was added dropwise and the stirring was continued for 45 min, the mixture turned green. Ammonium chloride (5.5 g, 0.103 mol) was added, and the ammonia was allowed to evaporate overnight. A mixture of ice water (100 ml) and ether (150 ml) was added to the residue and some hydrochloric acid was also added until the mixture was acidic. The organic layer was separated, dried, and evaporated to afford an oil from which crystallized 2.25 g of the pyrone XIIIa, mp 42–43° (from pentane). IR ($CHCl_3$): 2950, 2900, 1725, 1640, 1550 (broad) cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.8–1.15 (broad t, 3 H), 1.15–1.9 (m, 6 H), 2.50 (t, $J = 8$ Hz, 2 H), 2.67 (t, 3 H), 5.9 (s, 1 H), 16.7 (s, 1 H). MS: 224 (M^+, 13), 209(10), 206(10), 182(12), 181(12), 168(83), 153(100), 126(18), 111(42), 85(13), 69(45), 55(18), 43(58). Analysis: Calc. for $C_{12}H_{16}O_4$: C, 64.27; H, 7.19. Found: C, 64.18; H, 7.37. The mother liquor of the crystallization was chromatographed through silica gel, and the following products were eluted: *3-Acetyl-5,5-di-*n*-butyl-4-hydroxy-6-methylene-5,6-dihydro-2-pyrone*, XIXa (0.32 g, with hexane–ether (98/2), bp 105° (oven temp.)/0.15 mm Hg. IR ($CHCl_3$): 2960, 2900, 1720 cm^{-1} [^{1}H]NMR ($CDCl_3$): δ 0.7–1.05 (broad t, 6 H), 1.05–2.10 (m, 12 H), 2.65 (s, 3 H), 4.45 (d, $J = 2.5$ Hz, 1 H), 5.00 (d, $J = 2.5$ Hz, 1 H), 18.3 (broad s, 1 H). MS: 280 (M^+, 6), 237(14), 224(25), 209(39), 195(50), 191(17), 182(100), 181(61), 153(47), 151(44), 139(72), 111(50), 98(42), 69(50), 55(53), 43(83). *3-Acetyl-4-hydroxy-6-*n*-pentyl-2-pyrone, XIIIa* (1.82 g, with hexane–ether, 96:4). The overall yield of XIIIa was 72%. *3-Acetyl-5-*n*-butyl-4-hydroxy-6-methyl-2-pyrone, XXIa* (0.47 g, with hexane–ether 94:6), bp 90–93° (oven temp.)/0.25 mm Hg. IR ($CHCl_3$): 3000, 2950, 2900, 1720, 1640, 1550 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.75–1.10 (broad t, 3 H), 1.10–1.85 (m, 4 H), 2.30 (s, 3H), 2.30–2.65 (m, 2 H), 2.65 (s, 3 H), 17.25 (s, 1 H). MS: 224 (M^+, 22), 209(22), 206(10), 195(13), 191(13), 182(67), 181(52), 168(28), 153(29), 140(28), 139(100), 111(22), 97(52), 69(18), 55(20), 43(48). *Dehydroacetic acid*, I (0.62 g, with hexane–ether, 80:20).

*Preparation of 4-Hydroxy-6-*n*-pentyl-2-pyrone, XIVa.* A solution of XIIIa (1.9 g, 8.5 mmol) in 90% H_2SO_4 (7.6 g) was heated at 130° for 18 min. After cooling to room temperature, the solution was poured into ice water (100 ml). The precipitated oil was extracted with chloroform. The organic layer was dried and evaporated to afford a residue which was recrystallized to afford XIVa (75% yield), mp 53–54° (from ether–pentane), lit. mp 46–47° (Kögl et al., 1952). IR ($CHCl_3$): 3500–2600 (broad), 2990, 2970, 1690, 1610, 1570 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.7–1.1 (broad t, 3 H), 1.1–1.9 (m, 6 H), 2.50 (t, $J = 8$ Hz, 2 H), 5.60 (d, $J = 2$ Hz, 1 H), 6.02 (d, $J = 2$ Hz, 1 H), 10.35 (broad s, 1 H). MS: 182 (M^+, 20), 140(14), 139(11), 127(76), 111(69), 98(51), 97(18), 84(61), 69(100), 56(20), 55(39), 43(61). Analysis: Calc. for $C_{10}H_{14}O_3$: C, 65.92; H, 7.74. Found: C, 65.61; H, 7.62.

*Preparation of cis-4-Hydroxy-6-*n*-pentyltetrahydro-2-pyrone, XVa.* The lactone XIVa (400 mg, 2.2 mmol) was hydrogenated in ethanol (10 ml) at room temperature and 1 atm with Ra-Ni (75 mg). After about 6 hr, uptake of two equivalents of hydrogen was observed. The catalyst was filtered off and the solution was evaporated to afford XVa (95%). This product could not be distilled without partial dehydration to XVIa (see below). IR ($CHCl_3$): 3640, 3500–2900 (broad), 1730 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.7–1.1 (broad t, 3 H), 1.1–1.8 (m, 8 H), 1.8–3.1 (m, 4 H), 3.85–4.5 (m, 3 H, one proton is deuterated with D_2O). MS: 187 ($M^+ + 1$, 0.6), 115(56), 97(78), 73(100), 69(40), 55(38), 43(88).

*Preparation of 6-*n*-pentyl-5,6-Dihydro-2-pyrone (Massoia lactone, XVIa).* As above for the transformation V → VI, from XVa (200 mg, 1.07 mmol). Product XVIa (91% yield) had bp 100–105° (oven temp.)/0.2 mm Hg. Its IR spectrum was identical to that in the literature (Benoni et al., 1969).

*Preparation of 6-*n*-Pentyltetrahydro-2-pyrone, XVIIa.* The unsaturated lactone XVIa (100 mg, 0.6 mmol) was hydrogenated in ethyl acetate (5 ml) at room temperature and 1 atm with 10% Pd-C. After 20 min, uptake of one equivalent of hydrogen was observed. The catalyst was filtered off and the solution was evaporated to afford product XVIIa (96% yield) (Cavill et al., 1968), bp 100–105° (oven temp.)/0.2 mm Hg which was compared with an authentic sample.

*Preparation of 3-Acetyl-4-hydroxy-6-*n*-undecyl-2-pyrone, XIIIb.* This was carried out as for the transformation I → XIIIa, from I (4.25 g, 25.3 mmol), sodium (2.1 g, 91.0 mmol), liquid ammonia (500 ml), and 1-bromodecane (6.5 g, 29.2 mmol). The pyrone XIIIb (74% overall yield) had mp 70–71° (from ether). IR ($CHCl_3$): 2960, 1720, 1640, 1560 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.75–1.05 (broad t, 3 H), 1.05–1.9 (m, 18 H), 2.5 (broad t, 2 H), 2.65 (s, 3 H), 5.9 (s, 1 H), 16.65 (s, 1 H). MS: 308 (M^+, 4), 181(74), 168(100), 153(94), 129(40), 111(43), 85(23), 69(29), 43(29). Analysis: Calc. for $C_{18}H_{28}O_4$: C, 70.10; H, 9.15. Found: C, 69.76; H, 9.04. From the chroma-

tography the following products were eluted: *3-Acetyl-6-(1-*n*-decyl-*n*-undecyl)-4-hydroxy-2-pyrone*, *XVIII* (0.15 g, with hexane–ether, 98.5:1.5), bp 198–201° (oven temp.)/0.2 mm Hg. IR ($CHCl_3$): 2960, 1725, 1630 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.7–1.1 (broad t, 6 H), 1.1–1.95 (m, 36 H), 2.2–2.6 (m, 1 H), 2.75 (s, 3 H), 6.1 (s, 1 H), 16.65 (s, 1 H). MS: 448 (M^+, 1), 97(24), 88(24), 85(100), 84(40), 83(68), 71(60), 69(40), 57(80), 56(40), 55(60), 43(96). *3-Acetyl-5,5-di-*n*-decyl-4-hydroxy-6-methylene-5,6-dihydro-2-pyrone*, *XIXb* (0.062 g, with hexane–ether, 98:2), mp 73–77° (from pentane). IR ($CHCl_3$): 2980, 1725, 1630 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.65–1.05 (broad t, 6 H), 1.05–1.95 (m, 36 H), 2.7 (s, 3 H), 4.5 (d, $J = 2.5$ Hz, 1 H), 5.05 (d, $J = 2.5$ Hz, 1 H), 18.15 (s, 1 H). MS: 448 (M^+, 14), 308(21), 293(46), 182(100), 181(68), 153(36), 140(25), 139(39), 129(21), 111(29), 110(25), 109(21), 100(36), 97(41), 95(39), 85(79), 83(79), 73(29), 71(29), 70(25), 69(46), 57(39), 55(39), 43(89). *3-Acetyl-5-*n*-decyl-4-hydroxy-6-*n*-undecyl-2-pyrone*, *XX* (1.27 g, with hexane–ether 96:4), bp 200–203° (oven temp.)/0.1 mm Hg. IR ($CHCl_3$): 2980, 1725, 1610 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.7–1.05 (broad t, 6 H), 1.05–2.0 (m, 34 H), 2.15–2.7 (m, 4 H), 2.7 (s, 3 H), 17.20 (s, 1 H). MS: 448 (M^+, 4), 293(37), 182(57), 181(29), 139(43), 97(29), 95(17), 85(28), 83(23), 81(23), 71(28), 69(31), 58(31), 57(31), 55(37), 43(100). Pyrone XIIIb (0.76 g, with hexane–ether, 93:7), mp 70–71°. The overall yield of XIIIb was 74%. *3-Acetyl-5-*n*-decyl-4-hydroxy-6-methyl-2-pyrone*, *XXIb* (0.25 g, with hexane–ether, 9:1), bp. 149–145° (oven temp.)/0.2 mm Hg. IR ($CHCl_3$): 2980, 1720, 1630 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.7–1.1 (broad t, 3 H), 1.1–1.8 (m, 16 H), 2.20 (s, 3 H), 2.2–2.5 (m, 2 H), 2.62 (s, 3 H), 17.25 (s, 1 H). MS: 308 (M^+, 8), 293(25), 275(10), 195(15), 182(100), 181(55), 168(10), 164(18), 140(16), 139(65), 97(30), 69(10), 43(55). *Dehydroacetic acid*, I (0.29 g, with hexane–ether, 86:14).

*Preparation of 4-Hydroxy-6-*n*-undecyl-2-pyrone*, *XIVb*. This was carried out as for the transformation XIIIa → XIVa, from XIIIb (3.0 g, 9.7 mmol), and 90% H_2SO_4 (12 g). The pyrone XIVb (86% yield) had mp 87–88° (from chloroform–ether), lit. mp 80° (Kögl et al., 1952). IR ($CHCl_3$): 3500–2600 (broad), 2970, 1690 cm^{-1}. [^{1}H]NMR ($CDCl_3$): δ 0.7–1.1 (broad t, 3 H), 1.1–1.9 (m, 18 H), 2.3–2.7 (m, 2 H), 5.65 (d, $J = 2$ Hz, 1 H), 6.05 (d, $J = 2$ Hz, 1 H). MS: 267 ($M^+ + 1$, 6), 266 (M^+, 6), 206(14), 167(10), 164(30), 139(69), 126(100), 111(62), 98(53), 97(25), 95(16), 84(58), 69(71), 57(14), 55(43), 43(39), 41(40). Analysis: Calc. for $C_{16}H_{26}O_3$: C, 72.14; H, 9.84. Found: C, 72.41; H, 9.68.

Preparation of cis*-4-Hydroxy-6-*n*-undecyltetrahydro-2-pyrone*, *XVb*. This was carried out as for the transformation XIVa → XVa, from XIVb (390 mg, 1.47 mmol) in ethanol (20 ml) and Ra-Ni (30 mg) for 24 hr. The product XVb (84% yield) had mp 53–56° (from chloroform–pentane). IR ($CHCl_3$): 3640, 3600–2600 (broad) 2960, 2900, 1720 cm^{-1}. [^{1}H]NMR

($CDCl_3$): δ 0.7–1.0 (broad t, 3 H), 1.0–1.8 (m, 20 H), 1.8–3.1 (m, 4 H), 3.9–4.6 (m, 2 H), 5.2–5.8 (broad s, deuterates with D_2O). MS: 271 ($M^+ + 1$, 3), 192(11), 166(21), 126(18), 115(61), 112(16), 111(17), 110(16), 97(79), 96(34), 89(44), 84(20), 83(29), 82(38), 81(31), 73(44), 71(43), 69(43), 67(29), 57(38), 55(57), 43(100), 41(74). Analysis: Calc. for $C_{16}H_{30}O_3$: C, 71.07; H, 11.18. Found: C: 70.85; H, 10.92.

Preparation of 6-n-Undecyl-5,6-dihydro-2-pyrone, XVIb. This was carried out as for the transformation XVa→XVIa, from XVb (260 mg, 0.96 mmol). The lactone XVIb (85% yield) had mp 32–34° and bp 143–147° (oven temp.)/0.08 mm Hg, lit. mp 27–29°, bp 140–142°/0.1 mm Hg (Wailes, 1959).

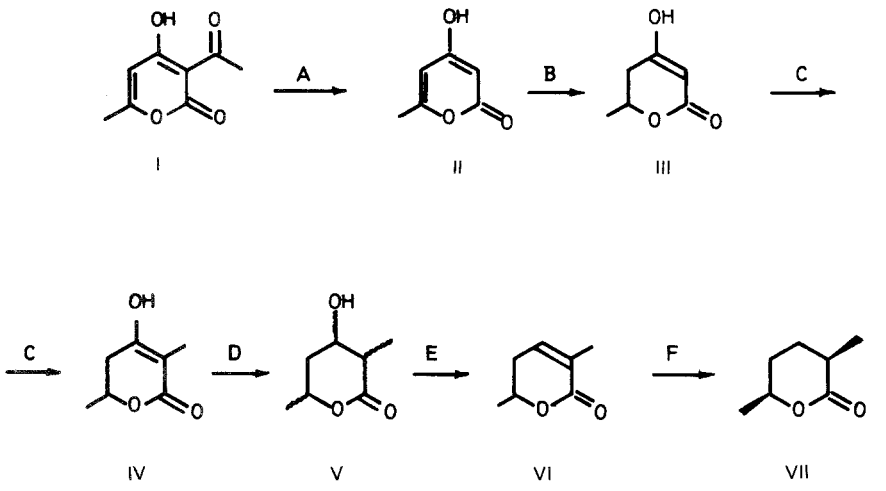
Preparation of 6-n-Undecyltetrahydro-2-pyrone, XVIIb. This was carried out as above for the transformation XVIa→XVIIa, from XVIb (142 mg, 0.56 mmol), in ethyl acetate (5 ml), with 10% Pd-C (20 mg). Product XVIIb had mp 29–30°, lit. mp 29.5–30° (Robinson, 1930). Its [^{1}H]NMR spectrum was coincident with that of an authentic specimen (Pirkle et al., 1979).

RESULTS AND DISCUSSION

The three target molecules required total or partial reduction of the original pyrone nucleus. Methylation at C-3 for the preparation of VII, and chain enlargement at the methyl group in C-6 for the synthesis of XVIa and XVIIb were also needed.

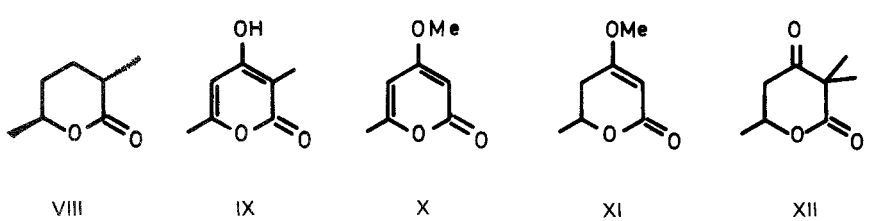
The lactone 4-hydroxy-3,6-dimethyl-2-pyrone, IX is a potentially useful precursor for the pheromone VII. However, all attempts to prepare it from II failed. In our best experiment (alkylation with i-PrOLi/i-PrOH and methyl iodide) 37% of the ether X and 15% of IX were formed. The ether X is always the predominant product (Suzuki et al., 1978). We turned our attention to III. Its reaction with methyl iodide and silver oxide has been reported to yield 4-hydroxy-3,6-dimethyl-5,6-dihydro-2-pyrone, IV, although in modest yield (Jones et al., 1949). However, treatment of III with one equivalent of lithium isopropoxide in isopropanol, followed by one equivalent of methyl iodide, gave 45% yield of isolated IV. Other products formed were XI and XII, some starting material being also recovered (Schemes 1 and 2). Other conditions tested for the preparation of IV which gave worse results were: (1) MeOLi/MeOH, and methyl iodide; (2) potassium carbonate/methyl iodide/acetone/reflux; (3) NaH/HMPT, and methyl iodide; (4) EtOLi/EtOH, and methyl iodide.

The methylated lactone IV was hydrogenated under platinum catalysis to a mixture of alcohols V, which without further purification was dehy-



SCHEME 1. A: H_2SO_4(90%), 130°, 15 min; B: H_2, 1 atm, room temp, EtOH, 10% Pd-C; C: (1) i-PrOLi/i-PrOH, (2) ICH_3 D: H_2, 1 atm, room temp, AcOEt, PtO_2; E: TsOH, C_6H_6, reflux; F: H_2, 1 atm, room temp, AcOEt, 10% Pd-C. Only one enantioisomer of constitution VII is shown.

drated (*p*-toluenesulfonic acid in benzene) to 3,6-dimethyl-5,6-dihydro-2-pyrone, VI. Hydrogenation under palladium catalysis of VI gave a mixture of the racemic pheromone VII and its *trans* isomer VIII (ratio ca. 9:1, correct elemental analysis), whose separation has already been described (Wheeler et al., 1976; Pyysalo et al., 1975; Pirkle et al., 1979). The [^{1}H]NMR spectrum of our mixture was identical to that described for pure VII in the mentioned references, and the elution order for both isomers in GC was also as described by Wheeler and by Pirkle. A [^{13}C]NMR spectrum of a ca. 1:1 mixture of VII and VIII was kindly provided to us by prof. Pyysalo (University of Helsinki). Our spectrum was identical, allowance being made for the ratio of isomers, thus providing further confirmation for the presence of VIII.



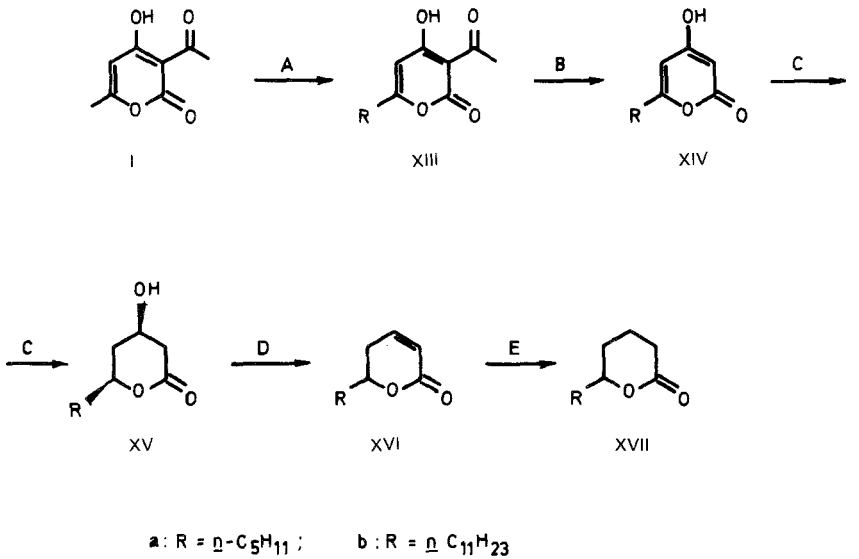
SCHEME 2. Only one enantioisomer of constitution VIII is shown.

It must be pointed out that the ratio of *cis* to *trans* isomers achieved in this synthesis is better than those previously reported, since an efficient control of the stereochemistry is possible in the hydrogenation step VI→VII, which, as expected, takes place with a *cis* addition of the incoming hydrogen preferentially by the least hindered side of the olefin VI.

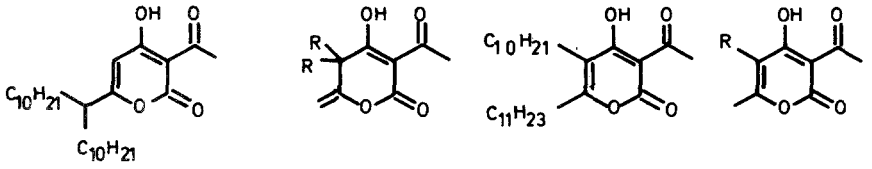
Our approach to the pheromone of *Vespa orientalis*, XVIIb, and to Massoia lactone, XVIa, takes advantage of the Harris method to lengthen the chain at C-6 of dehydroacetic acid, I, (Harris et al., 1968), based on the formation of the trianion of the lactone I (Schemes 3 and 4).

Thus, the anion of I was generated by treatment of dehydroacetic acid with three equivalents of sodium amide in liquid ammonia. Alkylation of the trianion with one equivalent of 1-bromodecane gave 3-acetyl-4-hydroxy-6-*n*-undecyl-2-pyrone, XIIIb, in good yield. Other alkylation products, namely XVIII, XIXb, XX, and XXIb were also isolated in pure form. All of them were inequivocally characterized by spectroscopic means as indicated in the experimental section.

Deacylation of XIIIb with sulfuric acid to yield 4-hydroxy-6-*n*-undecyl-2-pyrone, XIVb, paralleled the conversion of I into II (Collie, 1891, 1907). Hydrogenation of XIVb to afford *cis*-4-hydroxy-6-*n*-undecyl-2-pyrone, XVb, was achieved by Ra-Ni catalysis. The *cis* configuration was assigned



SCHEME 3. A: (1) $3NaNH_2$, liq. NH_3, (2) *n*-C_4H_9Br (to XIIIa) or *n*-$C_{10}H_{21}Br$ (to XIIIb); B: H_2SO_4 (90%), 130°, 15 min; C: H_2, 1 atm, room temp., EtOH/Ra-Ni; D: TsOH, C_6H_6, reflux; E: H_2, 1 atm, room temp., AcOEt, 10% Pd-C.



XVIII XIX XX XXI

a : R = n-C_5H_{11} b : R = n-$C_{11}H_{23}$

SCHEME. 4. By-products isolated in the preparation of XIIIa and XIIIb.

to XVb by analogy with the preparation of *cis*-4-hydroxy-6-methyl-2-pyrone (XV, R = CH_3) and consideration of the close similarity of the [^{1}H]NMR spectra of both alcohols in the region of C-3 protons. The stereochemistry of XV (R = CH_3) has been assigned (Bacardit et al., 1980).

Compound XVb was dehydrated by refluxing with *p*-toluenesulfonic acid in benzene to afford 6-*n*-undecyl-5,6-dihydro-2-pyrone, XVIb, which was catalytically hydrogenated under palladium on charcoal to give XVIIb in 42% overall yield from I. Product XVIIb was characterized by comparison of its [^{1}H]NMR and IR spectra with those of an authentic specimen (Pirkle et al., 1979) and in agreement with the melting point previously reported from the racemic XVIIb (Robinson, 1930).

Massoia lactone, XVIa, was prepared in 47% overall yield by a similar sequence of reactions, XIXa and XXIa being also formed in the alkylation step. Its IR spectrum was identical with that in the literature (Benoni et al., 1964). The conversion of XVIa into XVIIa has already been described (Cavill et al., 1968) and was repeated by us under palladium catalysis. The pyrone XVIIa is a component of the volatile fraction of apricots and peaches (Tang et al., 1968) and of black tea (Cazenave et al., 1974) and is also present in *Polianthes tuberosa L* (Kaiser et al., 1976).

Since the conversion of II and III into 6-methyltetrahydro-2-pyrone (XVII, R = CH_3) by hydrogenation under platinum catalysis has been described by us (Bacardit et al., 1980), we tried to perform similar reactions from XIVa to XVIIa and from XIVb to XVIIb. However, we have found erratic results in these experiments, depending very much on the batch of platinum catalyst. Although total hydrogenation to the saturated lactones XVII is always observed, several by-products with open-chain structures were also frequently formed. Thus, the best reduction of the 4-hydroxy-2-pyrone ring goes through intermediates such as the 4-hydroxytetrahydro-2-pyrone, XV.

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DOSE-RESPONSE RELATIONSHIP FOR TWO COMPONENTS OF THE SEX PHEROMONE OF LIGHTBROWN APPLE MOTH, *Epiphyas postvittana* (LEPIDOPTERA: TORTRICIDAE)

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Abstract—Bioassay studies with mixtures of the two components from the sex pheromone of *E. postvittana* are used to construct a dose-response surface. The surface clearly shows the existence of a response maximum and that an optimum ratio of the two components applies over a wide range of concentrations. Probit transformations of the same data are used to construct an isobologram. The isobole has a sharp minimum at a ratio for the two components close to the ratio produced by the females.

Key Words—*Epiphyas postvittana*, Lepidoptera, Tortricidae, sex pheromone, dose-response surface, isobole, male response.

INTRODUCTION

Epiphyas postvittana (Walker) is an omnivorous leaf roller common throughout southeastern Australia. The female produces a sex pheromone and two compounds, (*E*)-11-tetradecenyl acetate (I) and (*E,E*)-9,11-tetradecadienyl acetate (II), have been identified from extracts of female abdominal tips (Bellas et al., 1983). The two components were present in a ratio (I:II) of 20.4 ± 1.8:1. In laboratory bioassays based on sexual response (Bartell and Lawrence, 1977) neither compound was active alone. However, various combinations of the two compounds elicited male sexual behavior similar to that produced with extracts of virgin females under the same bioassay conditions. In field testing the two compounds did not have significant activity by themselves. A ratio of 20:1 of the two components (I:II) caught the most males, although captures were generally not significantly different between the ratios of 50:1 and 2:1.

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In a study of a group of tortricine moths which employ blends of (*E*)- and (*Z*)-11-tetradecenyl acetates, Cardé et al. (1977) concluded that the precision of the ratio is one means by which reproductive isolation is maintained by sympatric species. The same two compounds are found in extracts of the terminal segments of the female of *Argyrotaenia velutinana*. The ratio of the two compounds is confined within a narrow range (Miller and Roelofs, 1980) and males respond best to combinations near the midpoint of that range both in laboratory assays and in the field. Although responses are also obtained with ratios well outside this range, the amounts required to elicit the response are substantially higher than those required at ratios near those found in the female moths. In the species studied to date, the ratio of the pheromone components found in female moths and that needed for maximal male activity have usually been similar (Tamaki, 1977).

Although field trials are capable of giving an excellent indication of the optimal ratio (Miller et al., 1976), this is not usually the case and males may respond to yield large trap catches over a wide range of ratios, as with our field trials on *E. postvittana*. This may be due in part to a lack of specificity in the pheromone receptor system but is probably largely a result of the lack of control over the experimental variables.

Roelofs (1979) has shown that under the same environmental conditions the ratio of (*Z*)- to (*E*)-11-tetradecenyl acetate which is most effective in leading to the capture of males of the redbanded leafroller depends on the amount of the blend added to the lure and hence on the rate of release. In contrast to the lack of control of environmental variables in the field, laboratory bioassays do allow conditions to be more closely controlled, but such assays have been little used in attempts to define optimal ratios. This paper describes an analytical method based on laboratory bioassays for determining the optimal ratio of a two component system.

METHODS AND MATERIALS

Adult males of *E. postvittana* of the Bartell strain (Geier and Springett, 1976) were obtained from a laboratory culture which was maintained as described previously (Bartell and Shorey, 1969). Male pupae and adults were held segregated from females under a 24-hr photoperiod with 14.5-hr photophase. The first and last 3/4 hr of the photophase simulated a dawn and dusk period during which the fluorescent lights were brightened or dimmed.

(*E*)-11-Tetradecenyl acetate was purchased from the Chemical Samples Company, Columbus, Ohio. The proportion of *Z* isomer was determined by gas chromatography on 10% PEGA on 100/120 Diatomite C in a 1.5m × 2mm glass column at 170°. The column gave about 1800 plates. The retention times were 6.28 min for the *E* isomer and 6.69 min for the *Z* isomer.

There was 1.0% of the *Z* isomer present. The (*E,E*)-9,11-tetradecadienyl acetate was synthesized (Bellas et al., 1983) and contained 0.5% of the *Z,E* isomer.

The two components were dissolved in cyclohexane to prepare stock solutions. The ratio of concentrations of compounds I and II in the stock solutions was determined from a mixture of the two solutions using a Varian-Aerograph model 2100 gas chromatograph fitted with a 2 m × 3 mm glass column containing 5% Carbowax 20 M-TPA on 80/100 mesh Gas-chrom Z. The carrier gas was nitrogen at 30 ml/min, and the oven temperature was 140°. The retention time for I was 6.07 min and for II 11.58 min. The relative areas under the peaks for the two components were measured from four injections of the mixture. The molar responses of the two compounds were assumed to be equal.

The bioassay techniques developed for *E. postvittana* (Bartell and Shorey, 1969) and as subsequently modified (Bartell and Roelofs, 1973; Bartell and Lawrence, 1973) were used throughout. The pheromone dose is specified in terms of the quantity of compound I applied to the evaporating surface in the bioassay apparatus.

Treatments were arranged in randomized incomplete block designs to provide 12 replicates of each dose–ratio combination. Following the presentation of each treatment, the number of males out of five which performed the wing fanning and gyration behavior (= the sexual response) was counted to provide an estimate of the percentage sexual response (Bartell and Lawrence, 1973). The influence of a treatment was measured as the mean sexual response over all replicates for the treatment.

Data were collected in two series. The first series comprised the ratios 142:1, 43:1, 14.2:1, 4.3:1, and 1.4:1 over the concentration ranges 1×10^{-8}g to 1×10^{-11} g of I. A second series was designed to more closely define the optimum ratio and comprised the ratios 25.6:1, 17.1:1, 14.2:1, and 11.4:1 over the range 1×10^{-9} g to 1×10^{-12} g of I.

Contour diagrams were produced by a computer program which used the interpolation procedure of Akima (1973). A preliminary survey had established limits for the investigations and had, in addition, shown that wide departures from these values of dose and ratios resulted in no sexual response being exhibited. Accordingly, to control the extrapolation beyond the limits of the data, the dose–response matrix was imbedded in a field of zeroes. Zero responses were set at ratios of 3000:1 and 0.033:1 and at amounts of 1×10^{-13} g and 8×10^{-5} g of I.

RESULTS

The bioassay results for male sexual response expressed as the percentage of males responding (Table 1) indicate a maximal responsiveness in

TABLE 1. RESPONSES OF MALE *E. postvittana* AT VARIOUS DOSES AND RATIOS OF SEX PHEROMONE COMPOUNDS, I AND II[a]

Amount of I (g)	Ratio of compound I to compound II							
	142:1	43:1	25.6:1	17.1:1	14.2:1	11.4:1	4.3:1	1.4:1
10^{-8}	72.5 ± 5.8	75.0 ± 5.7			76.7 ± 5.3		72.5 ± 5.9	24.2 ± 5.1
10^{-9}	52.5 ± 7.3	85.0 ± 6.0 86.7 ± 4.0	95.8 ± 1.5	92.5 ± 4.6	86.7 ± 4.7	81.7 ± 4.2	70.0 ± 8.6	51.7 ± 9.3
10^{-10}	18.3 ± 6.0	53.3 ± 4.5 54.2 ± 7.3	70.0 ± 5.2	62.5 ± 6.9	58.3 ± 3.7	54.2 ± 5.8	17.5 ± 3.7	13.3 ± 2.8
10^{-11}	12.5 ± 3.7	16.7 ± 5.4 15.0 ± 4.7	31.7 ± 9.8	28.3 ± 6.8	24.2 ± 7.0	20.0 ± 6.3	12.5 ± 3.7	4.2 ± 2.9
10^{-12}		2.5 ± 2.5	6.7 ± 2.3	2.5 ± 1.8		0.0 ± 0.0		

[a]The responses in the upper line at each dose are data from the first series, in the lower line are data from the second series of experiments. The data are given as the mean percentage of males responding with the standard error.

the presence of 1×10^{-9} g of I and a ratio of I:II of 25.6:1. There was a decline in the percentage response above 1×10^{-9} g in all but the 142:1 ratio.

A probit analysis was carried out on the rising segment of the dose-response curves at each ratio for amounts of compound I of 10^{-9}–10^{-11} g or 10^{-9}–10^{-12} g, as appropriate in the lower ratios, and over the range 10^{-8}–10^{-11} g for the 142:1 ratio to yield the median response doses (RD_{50}) shown in Table 2. The slopes of the probit lines (Figure 1) do not differ significantly from parallel (Finney, 1971).

DISCUSSION

The dose–response matrix (Table 1) may be considered as a response surface represented as the computer-generated contour map in Figure 2. The main feature of the map is a peak, the response maximum, at a dose of around 8×10^{-10} g of I and at a ratio to II of about 24:1. The response falls away in all directions from this peak, but there is a pronounced ridge descending to lower doses of the compounds which represents the optimum ratio. Two other ridges at roughly right angles to the first are further features of the map, and they correspond to the response maxima at the various ratios. These features of the response surface may be even more clearly seen by viewing it as a stereo pair (Figure 3).

The contour map is a reasonable representation of the data since the median response doses calculated from the probit analyses lie close to the 50% contour (Figure 2). Certain assumptions have been made in order that

TABLE 2. MEDIAN RESPONSE DOSE (RD_{50}) FOR MALE E. *postvittana* AT EACH RATIO OF TWO COMPONENTS[a]

Ratio	RD_{50}	
	Quantity of compound I (g)	Quantity of compound II (g)
142:1	1.1×10^{-9}	7.9×10^{-12}
43:1	8.8×10^{-11}	2.1×10^{-12}
25.6:1	2.8×10^{-11}	1.1×10^{-12}
17.1:1	4.5×10^{-11}	2.6×10^{-12}
14.2:1	5.9×10^{-11}	4.2×10^{-12}
11.4:1	9.5×10^{-11}	8.3×10^{-12}
4.3:1	4.0×10^{-10}	9.4×10^{-11}
1.4:1	1.0×10^{-9}	7.2×10^{-10}

[a]The figures are calculated from probit transformation of the dose-response data (Table 1).

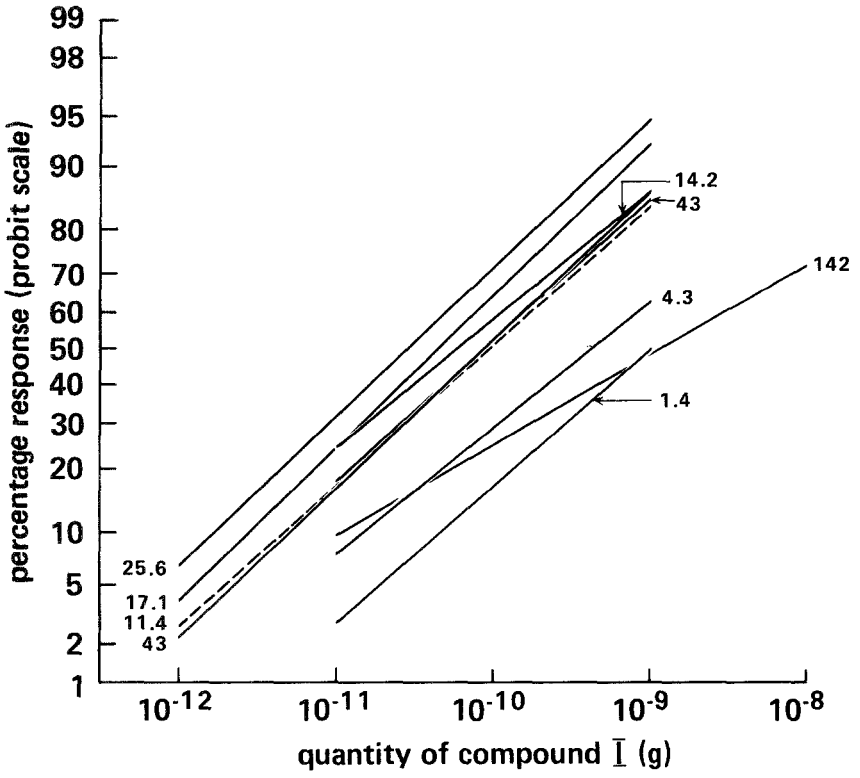

FIG. 1. Probit lines for the male *E. postvittana* sexual response data for the mixtures of (*E*)-11-tetradecenyl acetate and (*E,E*)-9,11-tetradecadienyl acetate.

the surface may be computed. For a given set of data, different methods of interpolation can result in different surfaces (Akima, 1973) and it is likely that another program would produce a different surface. However, these surfaces would still be qualitatively the same, even though the features may occur at slightly different positions, and our general observations would still be valid.

The dose–response surface may be explored in various ways. If the dose of one component is kept constant while the dose of the second is increased, then in the coordinate system used for this surface, this would correspond to traverses from left to right (component I constant), or along diagonals from bottom right to top left (component II constant). If the total dose (I and II) is kept constant while the ratio is varied, then this corresponds to traverses of the surface by a set of curved paths descending from the left towards the right-hand side. The dose–response surface readily allows one to see the results of such investigations. Baker et al., (1981) have used a sim-

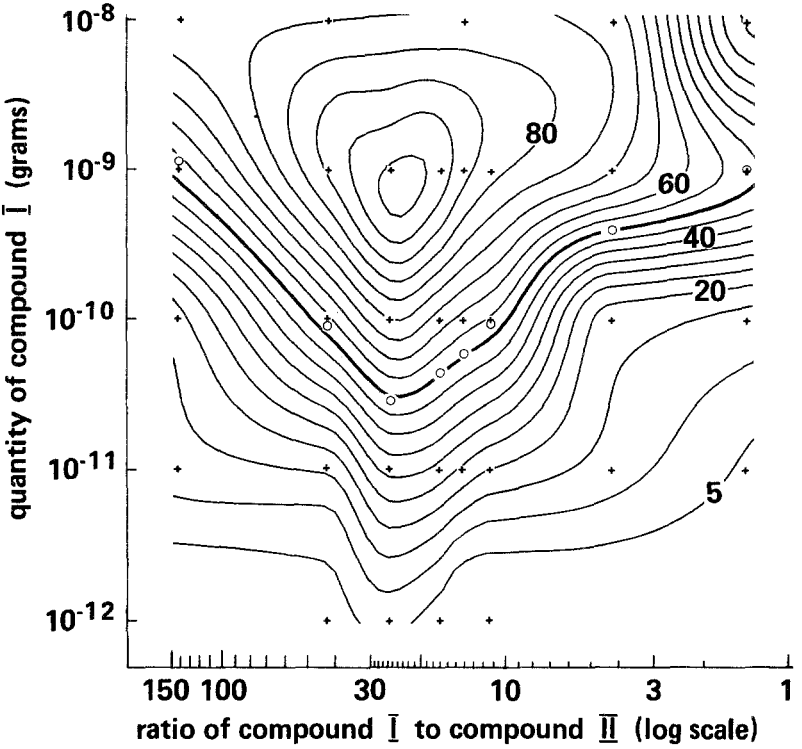

FIG. 2. Computed contour map of the dose–response surface for male *E. postvittana*. Contour levels of response are at intervals of 5%. The crosses indicate dose and ratio values where responses were measured. The open circle at each of the tested ratios is the median response dose (RD_{50}) calculated from the probit analyses. The heavy line is the 50% response contour.

ilar approach to explore the relationship between dosage and blend specificity of responses by Oriental fruit moth males.

A traverse of the surface at a constant dose of 1×10^{-8} g of I follows a path which is roughly parallel to the contour lines until 4.3:1 when it plunges steeply down the slope to 1.4:1. Except for the higher ratios, this dose is above the amount which produces the maximal responses. Bartell and Shorey (1969) showed that dose–response relationships, albeit to the natural blend, could be derived for several recognizable steps in the hierarchy of pheromone-mediated behaviors. It seems reasonable to assume, therefore, that response surfaces of a similar general form might apply to these behavioral parameters. If, for orientation behavior, such a dose-response relationship holds, then the traverse described above could offer an explanation of the lack of selectivity in field trials of males of *E. postvittana* and perhaps of

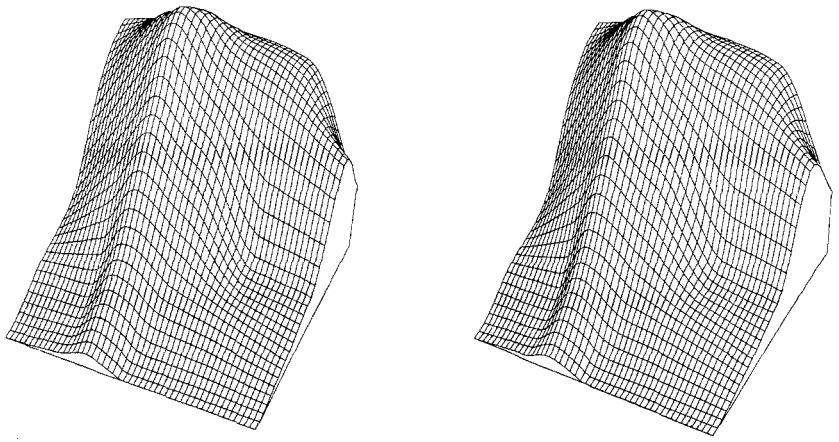

FIG. 3. Dose-response surface for male *E. postvittana* presented as a stereo pair. The lower right hand corner corresponds to a dose of 1×10^{-12} g of I at a ratio to II of 1.4:1.

other species. If the emission rate is above the amount required to produce maximal response, then the discrimination is low over a wide range of ratios. In order to increase the selectivity, the amount of attractant applied would have to be reduced so that as the males approach the trap they perceive doses which correspond to areas on the front part of the surface, that is, on Figure 2 equivalent to doses of less than 10^{-9} g of I. The capture rate will probably decline because the volume pervaded by the attractant at levels above the threshold is reduced, but the selectivity between ratios should increase. From the results of field surveys with traps baited with different amounts and ratios of components and consideration of the threshold for flight activation, Roelofs (1978) has reached a similar conclusion.

An alternative to the dose-response surface in representing the interaction of two components was suggested in the field of pharmacology by Loewe and Muischnek (1926). The representation was reduced to two dimensions by choosing a defined response, for example, the RD_{50}, and then plotting those doses of the two components which correspond to that response. The curve drawn through these points is a line of equal effect named an *isobole*. The derivation of this term is most apposite for pheromone work since it is comes from the Greek '*βόλοσ*' for which one of the meanings is the catch in a net (= trap?). The complete diagram is called an *isobologram*. The technique has also been applied to the fields of toxicology and pesticide studies (Tammes, 1964; Hewlett, 1969).

For a system in which both of the components are separately active, the isobole meets both axes. If the effect of the two components is additive, the isobole is a straight line joining these points. When the isobole lies outside

this line, the system exhibits antagonism. If the isobole lies inside the line, the system is said to show potentiation (Hewlett, 1969), and the greater the curvature the greater is the magnitude of the antagonism or the potentiation.

If only one of the components is separately active, the isobole meets one axis only. When the second component has no effect the isobole is a line parallel to the other axis; if the line curves away from that axis, the system shows antagonism; and if it curves towards that axis the system exhibits synergism. Hewlett (1969) has defined the joint action ratio to give a measure of the magnitude of the antagonism or potentiation or synergism.

There remains one other possibility: that neither of the components is separately active but a response is only obtained when they are present together. The isobole does not reach either axis and the system is said to show coalitive action since the components are working in coalition. The point on the isobole which is nearest the origin will be the optimal mixture. There are several examples of such a system involving pheromones, and it is the type of joint action exhibited by the two compounds from *E. postvittana*.

No universally accepted terminology exists to describe the results of the joint action of two drugs or pesticides (Morse, 1978). With two separately active components, both potentiation and synergism have been used as equivalent terms, and in pheromone studies synergism has usually been the term applied (e.g., Persoons et al., 1976). The isobologram gives a convenient graphical representation of the interactions, and the shape of the curve enables the type of joint action to be defined.

The amounts of the two compounds calculated at the RD_{50} for each ratio (Table 2) are plotted in Figure 4. Our expectations had been that one curve would be fitted to the points and that from the curve's closest approach to the origin an optimal ratio could be calculated. However, the shape of the isobole is such that no simple function could be fitted to the data. Simple functions can be fitted to the points which lie to either side of the ridge of the contour map. These curves are shown on the figure and provide a means of predicting the RD_{50} for ratios between those investigated. A ratio can be calculated for the point of intersection, but it is of doubtful statistical validity. The dashed line connecting the two lines is a rough indication of the form of this isobole, but the data are too few to define accurately the curve around the minimum. The optimal ratio lies between 17.1 : 1 and 25.6 : 1, but closer to the latter than to the former.

The shape of this isobole, where both arms of the curve move away from the axes with changes in ratio away from the optimum, shows that any deviation from the optimum requires more of both components to elicit the same level of response.

Miller and Roelofs (1980) have shown that there is a degree of precision in the blends of pheromone components produced by female moths. Our results show that males tend to reflect this precision by having an optimal response to mixtures close to the ratio produced by the females.

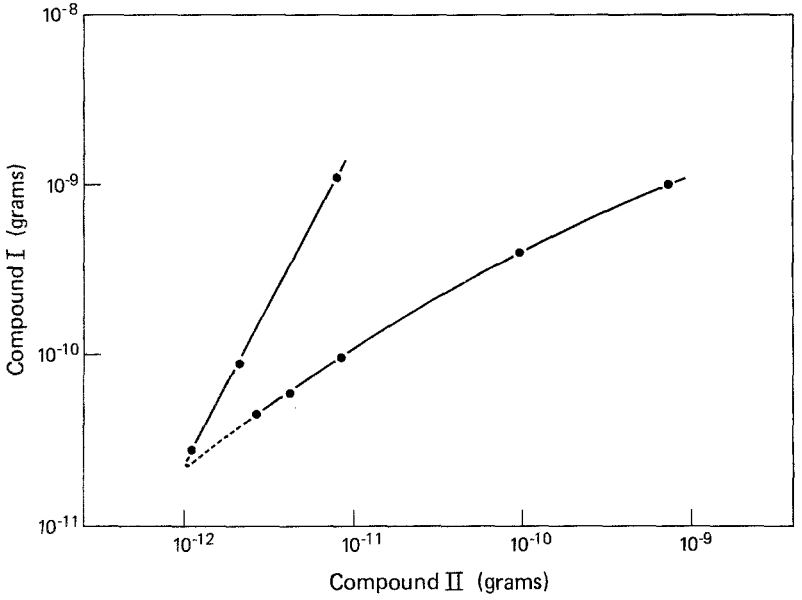

FIG. 4. Isobologram representing the 50% response in male *E. postvittana* at each ratio of (*E*)-11-tetradecenyl acetate and (*E,E*)-9,11-tetradecadienyl acetate. The functions corresponding to the lines have the formulas $y = 11.781 + 1.869x$ (for the three higher ratios) and $y = -10.443 - 0.7232x - 0.06181x^2$ for the remaining five.

The isobologram technique is probably applicable to field data. However, the variability of captures because of the vagaries of the weather and the lack of control over the release rates from lures would mean that much more effort would have to be expended to obtain statistically valid results and so it would always be easier to obtain the data in the laboratory provided a suitable assay exists. Since the isobole curves are derived from probit transformation on the original dose-ratio-response data, the technique provides a suitably predictive model for the optimal ratio, and a basis for interstrain or interspecific statistical comparisons.

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ANAL SAC SECRETION IN MUSTELIDS
A Comparison

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Abstract—The chemical compositions of anal sac secretions of seven mustelid species were examined by thin-layer chromatography, gas chromatography, and mass spectrometry. The analyses showed great similarities between species belonging to the genus *Mustela*, i.e., *M. erminea*, *M. nivalis*, *M. vison*, and *M. putorius*, whereas *Martes martes*, *Lutra lutra*, and *Meles meles* each showed a different pattern. Benzaldehyde was the predominant compound in the secretion of *M. martes*. Sulfur-containing compounds (thietanes and dithiacyclopentanes) were characteristic for the *Mustela* species. Some of the compounds were species-specific and the relative amounts of the compounds in common varied between the species. The chemical results are in agreement with systematics at the generic level. Species-specific chemical composition makes exchange of information possible between coexisting mustelids. The presence of the sulfur-containing compounds in the small mustelids may be an effect of the defense function of the anal sac secretion.

Key Words—Anal sac secretion, gland, thietanes, sulfur-containing compounds, mustelids, interspecific communication, mink, otter, stoat, weasel, badger.

INTRODUCTION

The mustelids are small to medium-sized carnivores which have diversified to exploit different habitats and food resources (Ewer, 1973). They are typically solitary, and the individuals in a population are usually distributed according to a territorial pattern (Powell, 1979). Often two or more species occur in the same area, which may result in interference (Erlinge, 1972; King and Moors, 1979; Simms, 1979).

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Scent communication is important in intra- and probably also in interspecific interactions. The scent material is deposited at certain sites according to specific behaviors (Ewer, 1968; Macdonald, 1980). Besides urination and defecation, body rubbing and anal drag are characteristic scent-marking behaviors in mustelids (Goethe, 1964; Erlinge et al., 1982).

The microscopic anatomy of the anal sacs in some mustelids was described by Stubbe (1970). Four types were distinguished, i.e., the *Meles*, *Lutra*, *Martes*, and *Mustela* type. The anal sacs of *Martes* and *Mustela* represented a more advanced type with a high concentration of tubular and alveolar glands.

The chemical compositions of the anal sac secretions have been examined in some mustelids, i.e., mink (*Mustela vison*, Schreber) by Sokolov et al. (1975, 1980), Schildknecht et al. (1976), and Brinck et al. (1978); otter (*Lutra lutra* L.) by Gorman et al. (1978); stoat (*Mustela erminea* L.) and ferret (*Mustela putorius furo* L., a domestic form of uncertain origin) by Crump (1978, 1980a,b); and badger (*Meles meles* L.) by Brundin et al. (unpublished).

On the basis of published information and our analyses, we compared the chemical composition of the anal sac secretions in eight mustelid species. The aim was to examine intergeneric and interspecific differences and similarities, and to make comparisons with phylogenetic relationships.

METHODS AND MATERIALS

Animals. Anal sac secretions were obtained from 12 anesthetized *Meles meles* (8 males and 4 females), 5 anesthetized *Lutra lutra* (1 male and 4 females), 14 killed *Martes martes* L. (9 males and 5 females), 47 killed and 3 anesthetized *Mustela erminea* (34 males and 16 females), 39 killed and 3 anesthetized *Mustela nivalis* L. (23 males and 19 females) and 15 anesthetized *Mustela putorius* L. (8 males and 7 females). Some complementary studies were performed on *Mustela vison* (3 males). All animals were caught in southern Sweden except one specimen of *Lutra lutra* which was captured in middle Norway.

Sampling Technique. From anesthetized *Meles meles* and *Mustela putorius* and killed *M. vison*, the secretion was collected by means of a catheter (Brinck et al., 1978). Before collecting the secretion of killed or anesthetized individuals of the other species (i.e., *Lutra lutra*, *Martes martes*, *Mustela erminea*, and *M. nivalis*), the area around the openings of the anal sacs was washed with diethyl ether. The contents were then pressed out of the sacs and absorbed on cotton plugs which had previously been washed with ethanol and diethyl ether. The organic material was extracted with 0.5 ml methylene chloride. The samples were stored at −20°C until examined. The secretion of each individual was analyzed separately.

Thin-Layer Chromatography (TLC). Analytical TLC was run on commercial silica gel plates (E. Merck, Darmstadt, West Germany, Kieselgel 60F, thickness 0.25 mm) with light petroleum bp 60–80°C–diethyl ether 90:10 (v/v) as solvent. The spots were visualized by exposure to iodine vapors and also by spraying with sulfuric acid (5% solution in ethanol) followed by charring at 180°C.

Gas Chromatography (GC). Analytical GC was performed on Perkin Elmer model 900 and 3920 instruments equipped with FID. A 25-m glass capillary column (ID 0.3 mm) dynamically coated with OV-101 in the laboratory was used for the secretions from *Mustela erminea*, *M. nivalis*, *M. putorius*, and *M. vison*. The carrier gas flow was 1.4 ml/min. Make-up gas corresponding to 25 ml/min was employed. For the low-molecular-weight components a split giving a ratio of 1:30 was used. For the secretions from *Martes martes*, *Lutra lutra*, and *Meles meles* a 25-m fused silica capillary column (ID 0.2 mm) dynamically coated with OV-101 was used. The split was closed for 1 min after injection, program 4°C/min from ambient to 230°C.

Mass Spectrometry (MS). Mass spectra were obtained with a Varian MAT 112 GC/MS instrument. In this GC/MS combination, the gas chromatograph was a Varian model 1400 equipped with a 25-m fused silica capillary column. The helium carrier gas flow was 2 ml/min. The split ratio

TABLE 1. REFERENCE SUBSTANCES USED FOR IDENTIFICATION OF COMPOUNDS IN ANAL SAC SECRETIONS IN EXAMINED MUSTELIDS

	Synthesized in laboratory[a]	Commercially available	Literature
2,2-Dimethylthietane	x		
2,4-Dimethylthietane (*cis*- and *trans*-)	x		
2,3-Dimethylthietane (*cis*- and *trans*-)	x		
3,3-Dimethylthietane	x		
2-Ethylthietane	x		
3-Ethylthietane	x		
Indole		x	
o-Aminoacetophenone		x	
Benzaldehyde		x	
2-Propylthietane			Crump, 1980a
3,3-Dimethyl-1,2-dithiacyclopentane			Sokolov et al., 1980
3-Ethyl-1,2-dithiacyclopentane			Crump, 1980a
2-Pentylthietane			Crump, 1980a
3-Propyl-1,2-dithiacyclopentane			Crump, 1980a

[a]The syntheses were performed by A. Brundin, Institute of Organic Chemistry, University of of Umeå, Sweden (unpublished).

at the injection port was 1:30. The spectra of *cis*- and *trans*-2,4-dimethylthietanes were obtained from a Ribermag R10-10 computerized GC-MS system. The mass spectra were compared with spectra from synthetic products prepared in the laboratory or with spectra available in literature (Table 1). The synthetic thietanes used were prepared by reacting different dioxan-2-ones with potassium thiocyanate.

Infrared Spectrometry (*IR*) was performed on a Perkin Elmer model 298.

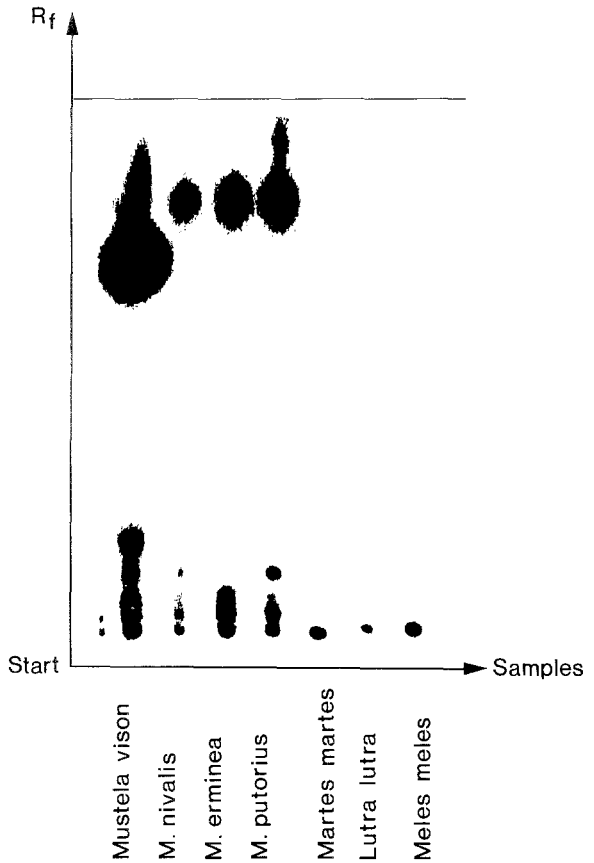


FIG. 1. TLC showing anal sac secretion of *Mustela vison*, *M. nivalis M. erminea*, *M. putorius*, *Martes martes*, *Lutra lutra*, and *Meles meles* with petroleum/ether 90:10 (v/v) as developer.

TABLE 2. DISTRIBUTION OF COMPOUNDS BETWEEN INVESTIGATED SPECIES OF MUSTELIDAE[a]

Peak number	Corresponding compound	MW	*Mustela erminea*	*Mustela nivalis*	*Mustela putorius*	*Mustela vison*	*Mustela putorius furo*[b]	*Martes martes*	*Lutra lutra*	*Meles meles*
1	2,2-Dimethylthietane	102	X	—	—	Ⓧ	X	—	—	—
2	2,4-Dimethylthietane (*cis-* or *trans-*)	102	—	X	—	—	—	—	—	—
3	2,3-Dimethylthietane (*trans-*)	102	X	X	X	—	X	—	—	—
4	2,3-Dimethylthietane (*cis-*)	102	—	—	—	—	X	—	—	—
5	2-Ethylthietane	102	X	—	—	X	—	—	—	—
6	2-Propylthietane, isomer of	116	X	—	Ⓧ	—	—	—	—	—
7	2-Propylthietane, isomer of	116	X	—	—	—	—	—	—	—
8	2-Propylthietane	116	Ⓧ	—	—	—	X	—	—	—
9	3,3-Dimethyl-1,2-dithiacyclopentane	134	—	Ⓧ	X	X	X	—	—	—
10	3-Ethyl-1,2-dithiacyclopentane	134	X	—	—	—	—	—	—	—
11	2-Pentylthietane	144	X	—	—	—	X	—	—	—
12	3-Propyl-1,2-dithiacyclopentane	148	X	—	—	—	X	—	—	—
13	Indole	117	X	X	X	X	X	—	—	—
14	*o*-Aminoacetophenone	135	X	—	—	—	—	—	—	—
15	Benzaldehyde	106	—	—	—	—	—	Ⓧ	—	—

[a]Predominant compounds are encircled. A compound not present in amounts over 10 ng/animal is denoted (—). MW = molecular weight.
[b]Data from Crump (1980b).

RESULTS AND DISCUSSION

Composition of Secretions. Anal sac secretions of the *Mustela* species analyzed with TLC showed great similarities, whereas *Meles meles*, *Lutra lutra*, and *Martes martes* each showed a quite different pattern. No components in the secretions of the latter three species moved on TLC when petroleum–ether was used as developer (Figure 1). GC analyses (Figure 2A–C) did not show any of the low-molecular-weight sulfur compounds typical for *Mustela* species. This was confirmed by MS analyses (SIM technique) (Table 2).

Meles meles *L., Badger.* The anal sac secretion, preliminary analyzed by means of TLC and GC (Brundin et al., unpublished), showed volatile esters, fatty acids, cholesterol, and cholesterol esters.

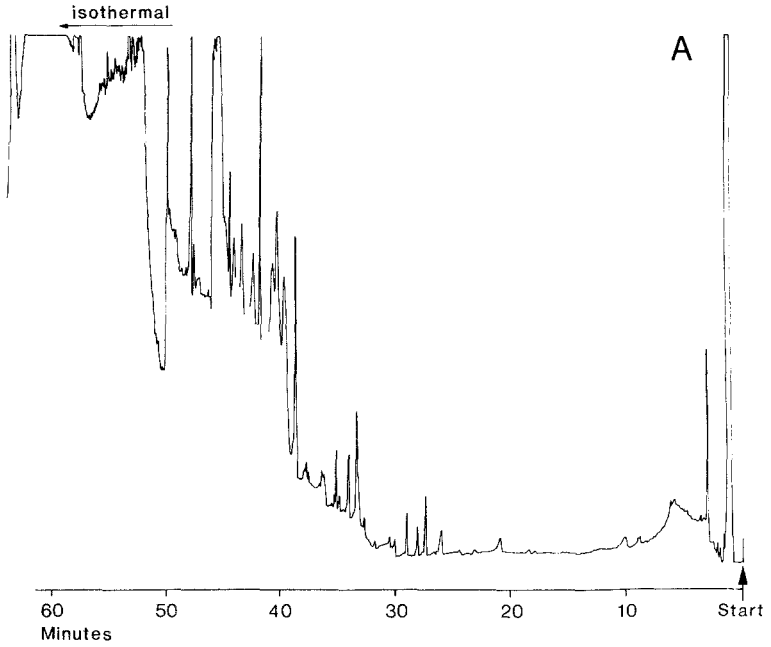
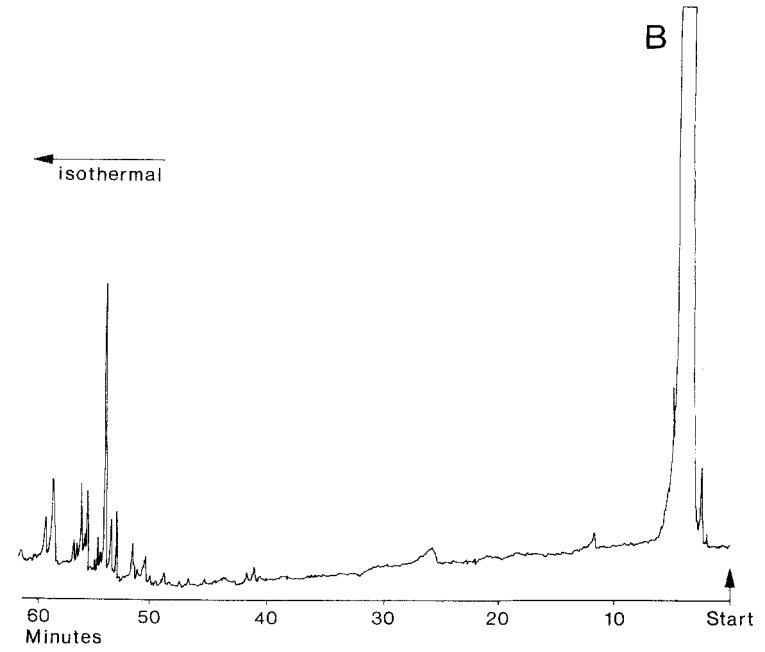


FIG. 2. Gas chromatograms of lower-molecular-weight components in the anal sac secretion from (A) *Meles meles*, (B) *Lutra lutra*, (C) *Martes martes*, (D) *Mustela erminea*, (E) *M. nivalis*, (F) *M. putorius*, (G) *M. vison.* Program 4°C/min, ambient–230°C. Split ratio 1:30.The split was closed 1 min after injection. For A, B, and C a 25-m fused silica capillary column (ID 0.2 mm) dynamically coated with OV-101 was used. For D, E, F, and G a 25-m glass capillary column (ID 0.3 mm) with OV-101 as stationary phase was used. Peaks not numbered in C–G are due to artifacts or belong to the solvent.



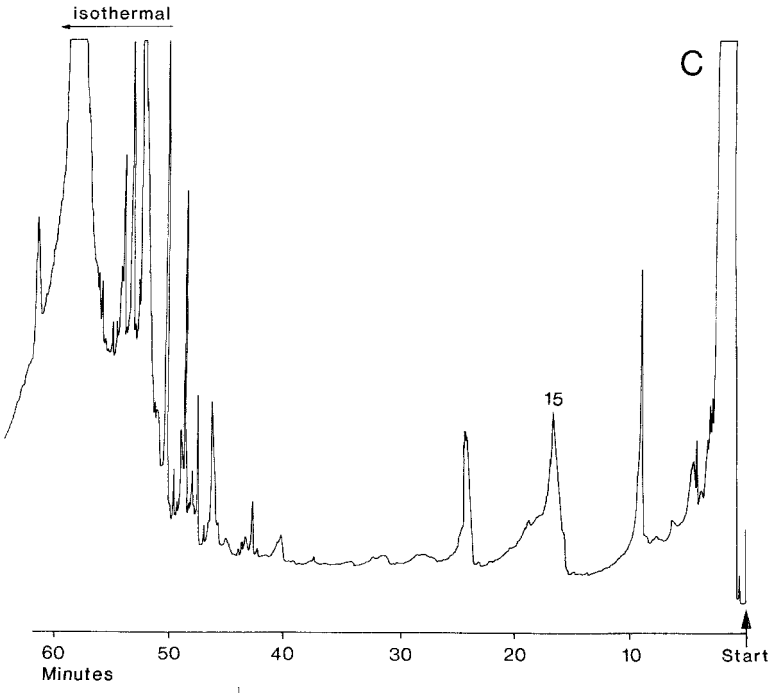
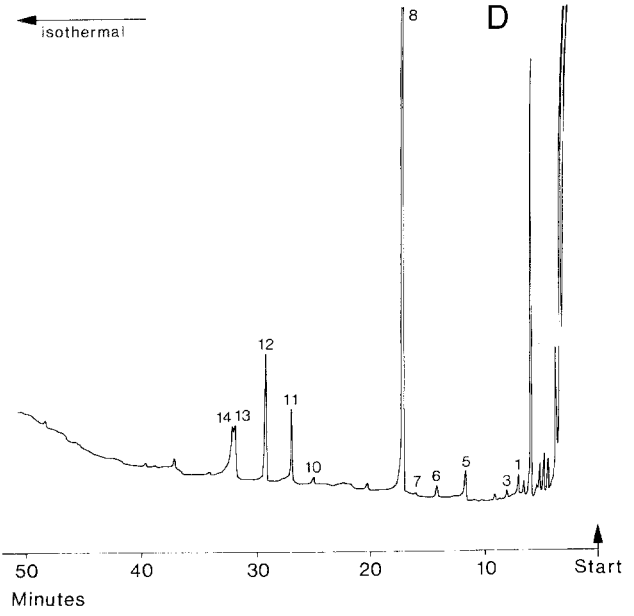


FIG. 2. Continued.



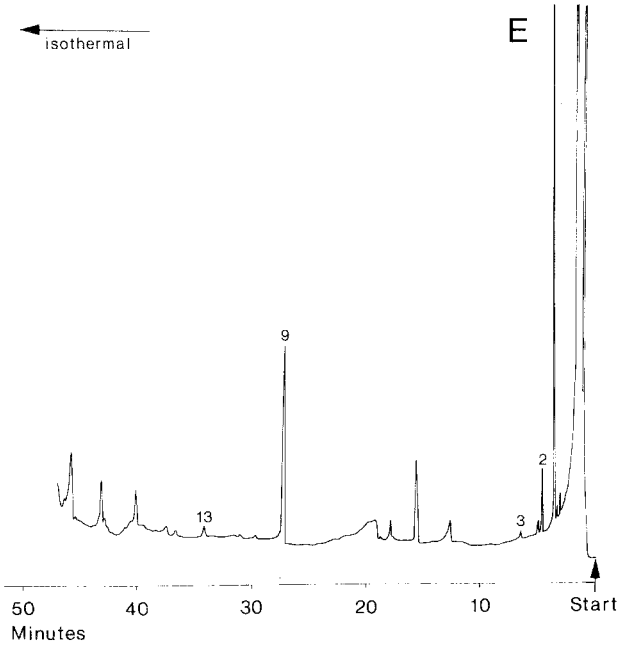


FIG. 2. Continued.

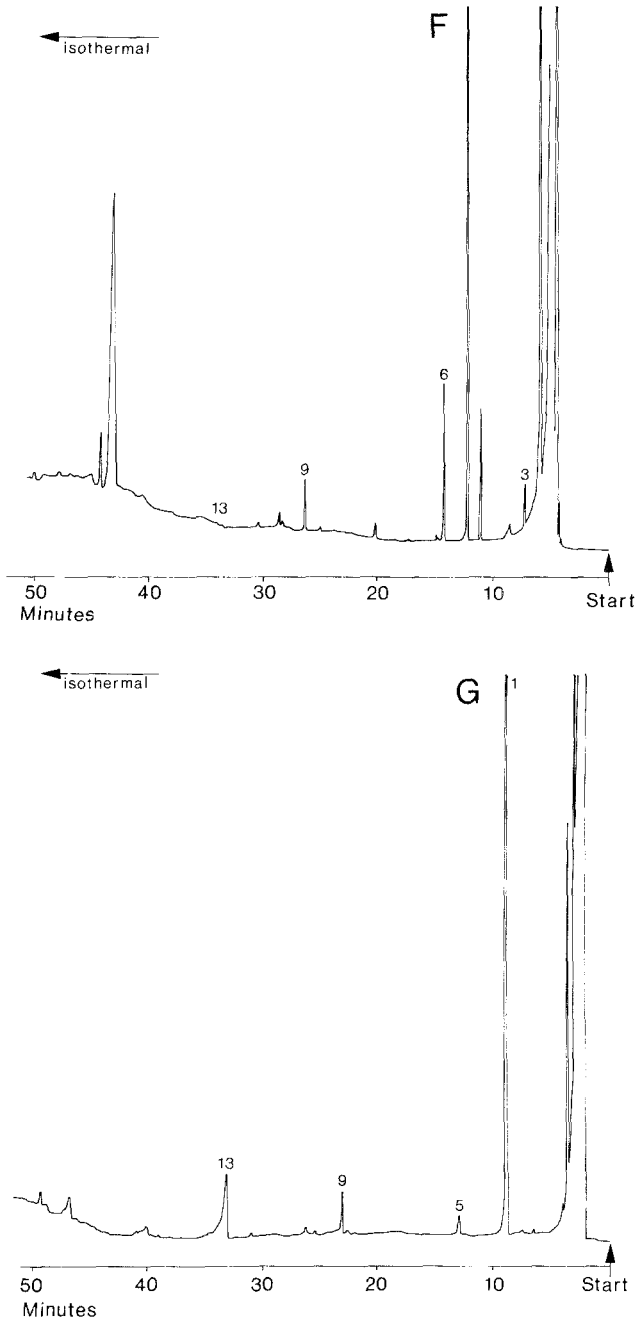


FIG. 2. Continued.

Lutra lutra *L.*, *Otter.* Gorman et al. (1978) analyzed the secretion by means of TLC, using petroleum, diethyl ether, and acetic acid (90:10:1 v/v) as developer, and found apocrine gland protein, polymucosaccharide, and sebaceous gland lipids.

Martes martes *L.*, *Pine Marten.* The GC showed some low-molecular-weight compounds, and several less volatile components were separated (Figure 2C). The predominant component, (15), molecular weight 106, had gas chromatographic and mass spectral data identical with those of benzaldehyde. The infrared spectra of the total secretion also indicated the presence of esters by carbonyl absorption at 1740 cm^{-1}.

Mustela *Species.* The secretion of the four *Mustela* species contained lipophilic components, which migrated on the TLC plates (Figure 1). Gas chromatograms of methylene chloride extracts of these fractions are shown in Figure 2D–G. Apart from some small individual variations (see Erlinge et al., 1982), the chromatograms of the low-molecular-weight region were consistent for each species. The chemical composition of the low-molecular-weight components in the secretions are presented below.

Mustela erminea *L.*, *Stoat.* The secretion of *erminea* showed the most diverse composition of the examined *Mustela* species. The GC (Figure 2D) revealed 11 peaks, some of them previously indentified by Crump (1980a), i.e., 2-ethylthietane (5), 2-propylthietane (8) (reported to be the major com-

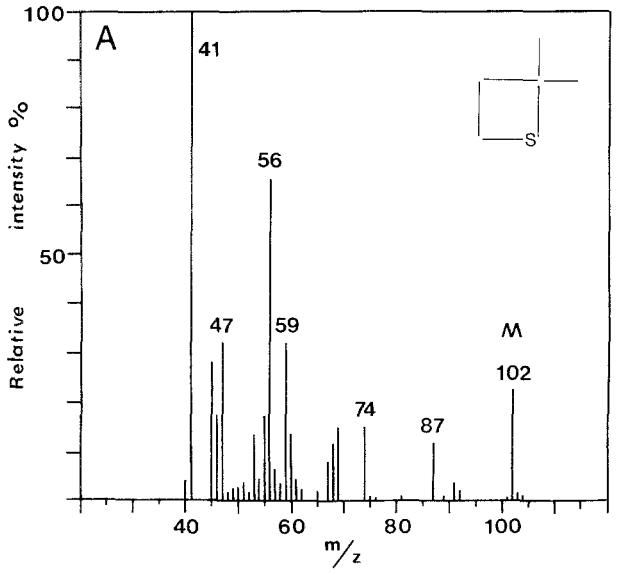
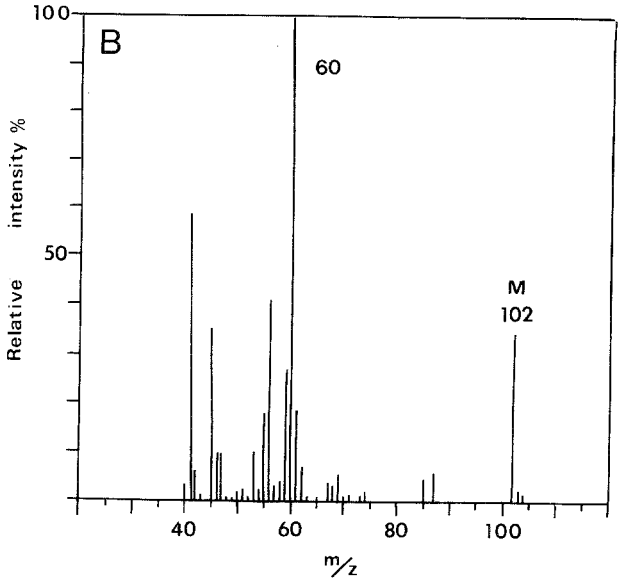


FIG. 3. Mass spectra of the peaks 1, 2, 3, and 5 with molecular weight 102, illustrated in A, B, C, D, respectively.



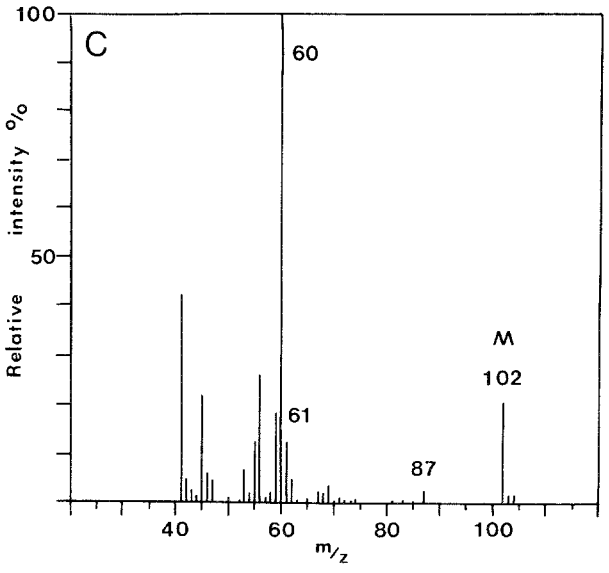


FIG. 3. Continued.

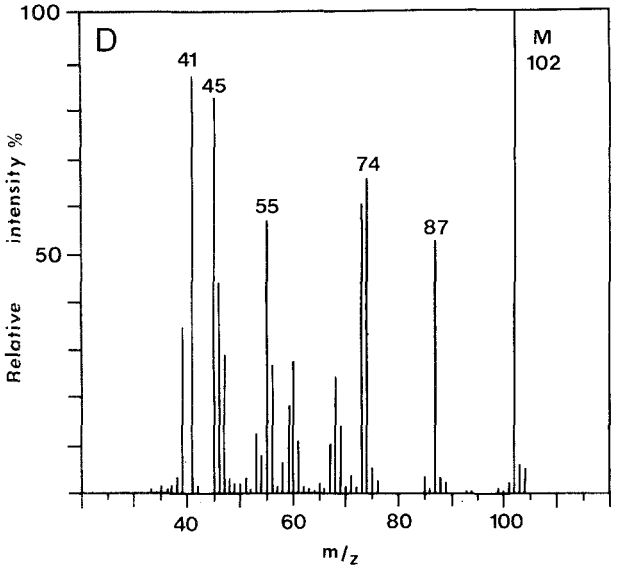


FIG. 3. Continued.

ponent), 3-ethyl-1,2-dithiacyclopentane (10), 2-pentylthietane (11), 3-propyl-1,2-dithiacyclopentane (12), and indole (13) (see Table 2).

In addition to these, we detected two isomers, peaks 1 and 3, of the composition $C_5H_{10}S$, (Figures 3A and C). For peak 1, gas chromatographic and mass spectral data were identical with those of 2,2-dimethylthietane. Peak 3 showed a mass spectrum superimposable on that of authentic *trans*-2,3-dimethylthietane.

Three components, peaks 6, 7, and 8 (Figure 4), had molecular weight 116, and were formally isomeric propylthietanes. The mass spectral data of peak 8 (*m/z* 73) corresponded to a 2-propylthietane (as shown by Crump, 1980b), while peaks 6 and 7 (*m/z* 74) represented thietanes with disubstitution.

Peak 14 (Figure 5), barely separable from indole on the column used, had a molecular weight of 135. The compound proved to be identical with *o*-aminoacetophenone as indicated by GC and MS data of an authentic sample (see also Crump, 1980a).

Mustela nivalis *L., weasel.* The volatile portion of the anal sac secretion of *M. nivalis* (Figure 2E) contained fewer low-molecular-weight compounds than were found for *M. erminea.* Peak 2 corresponded to a compound of molecular weight 102 (Figure 3B) and showed the same GC and MS data as authentic *cis-* or *trans*-2,4-dimethylthietane. The MS and GC data corresponding to peak 3 were identical with those of *trans*-2,3-dimethylthietane from *M. erminea* (Figure 3C). Peak 9 (Figure 6), corresponded to

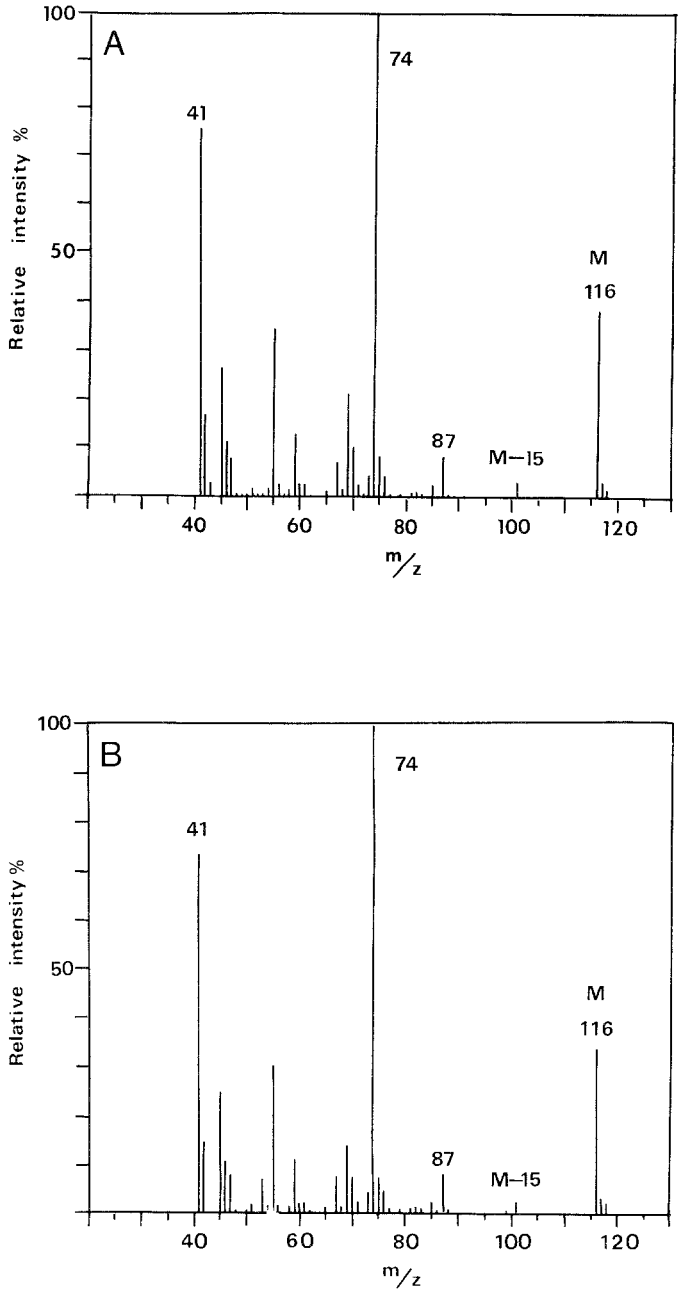


FIG. 4. Mass spectra of the peaks 6, 7, and 8 with the molecular weight 116, illustrated in A, B, C, respectively.

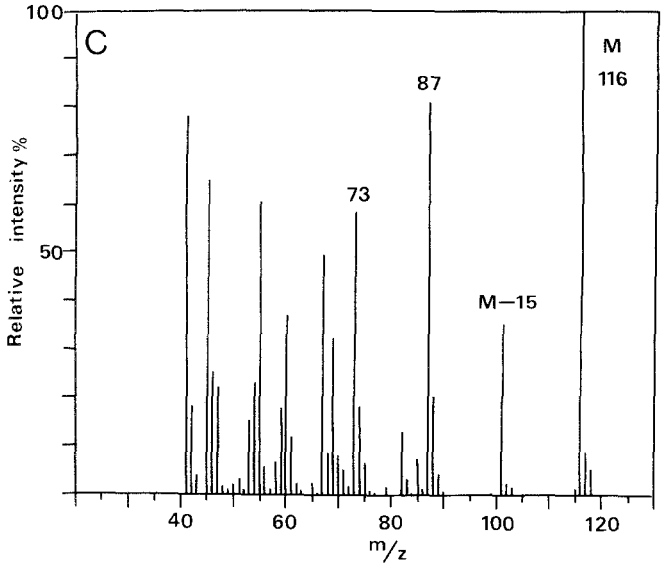


FIG. 4. Continued.

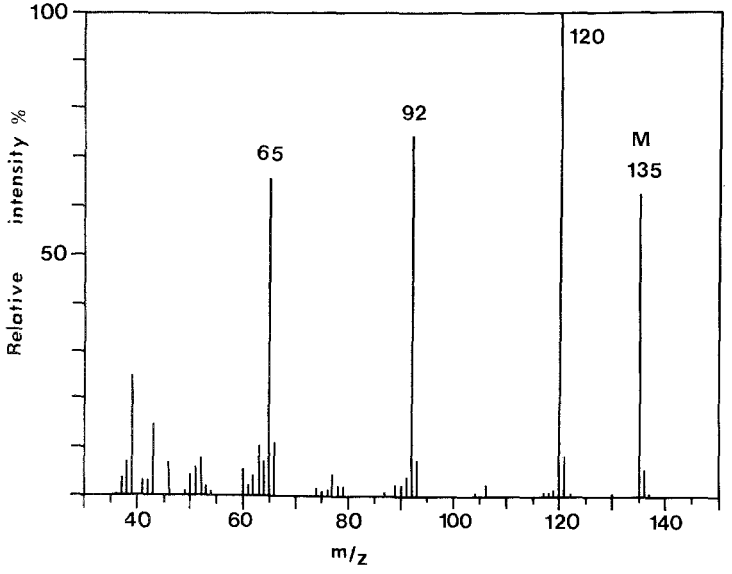


FIG. 5. Mass spectrum of the peak 14 with the molecular weight 135.

a compound of molecular weight 134, which contained two sulfur atoms as judged from the (M+2) peak. However, the absence of a significant peak at m/z M−29 indicated that it could not be 3-ethyl-1,2-dithiapentane. The most likely structure was 3,3-dimethyl-1,2-dithiacyclopentane (Sokolov et al., 1980). Peak 13 corresponded to indole.

Mustela putorius *L.*, *Polecat.* Four peaks appeared in the gas chromatogram (Figure 2F). The mass spectrum of the compound corresponding to peak 3 (Figure 3C) and comparison of its gas chromatographic data with synthetic compounds revealed it to be *trans*-2,3-dimethylthietane. Peak 6 proved to be an isomer identical with 2-propylthietane (Figure 4). The compound corresponding to peak 9 had the same MS data as the dithiacyclopentane of *M. nivalis* (Figure 6). Peak 13 was due to indole.

Mustela putorius furo, *Ferret.* Crump (1980b) reported 2,2-dimethylthietane (1), 2,3-dimethylthietane (4, 3; *cis*- and *trans*-), 2-propylthietane (8), 3,3-dimethyl-1,2-dithiacyclopentane (9), 2,3-dimethyl-1,2-dithiacyclopentane (*cis*- and *trans*-), 2-pentylthietane (11), 3-propyl-1,2-dithiacyclopentane (12), quinoline, and indole (13) (see Table 2).

Mustela vison *Schreber*, *American Mink.* The gas chromatogram for *M. vison* also showed four prominent peaks (Figure 2G) corresponding to 2,2-dimethylthietane, peak 1, reported to be the main component in the secretion (Schildknecht et al., 1976; Brinck et al., 1978), 2-ethylthietane, peak 5, and a cyclic disulfide, peak 9, suggested to have the structure 3,3-dimethyl-

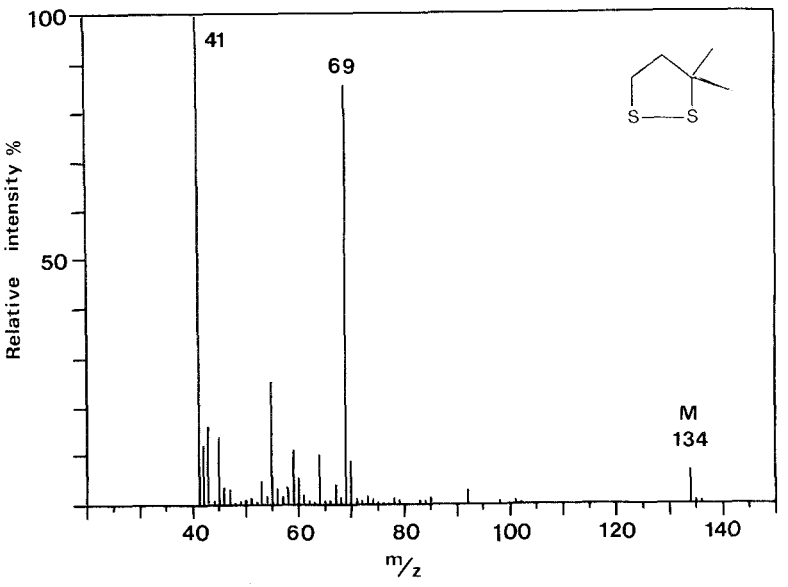


FIG. 6. Mass spectrum of the peak 9 with the molecular weight 134.

1,2-dithiacyclopentane (Schildknecht et al., 1976; Sokolov et al., 1980). Peak 13 corresponded to indole.

Comparison of Secretions in Examined Mustelids. Anal sac secretions of *Meles meles*, *Lutra lutra*, and *Martes martes* each had a characteristic composition of low-molecular-weight components, clearly distinguished from each other and the *Mustela* species. Several sulfur-containing compounds were found in the *Mustela* species (Table 2). Five different isomeric thietanes with the formula $C_5H_{10}S$ were found, and they were distributed between the species in various combinations and in different quantities. *trans*-2,3-Dimethylthietane (3) was prominent in the secretion of *M. putorius*, but occurred only to a minor extent in *M. erminea* and *M. nivalis*. 2,2-Dimethylthietane (1) and 2-ethylthietane (5) both occurred in *M. erminea* and *M. vison*, but (1) was the main component in *M. vison* (50% of the volatile fraction), whereas (5) was more pronounced in *M. erminea*. 2,4-Dimethylthietane (*cis*- or *trans*-) (2) occurred only in *M. nivalis*, where it was prominent. Three isomers of 2-propylthietane (6, 7, and 8) appeared in *M. erminea*, and one of them (8) was the predominant component. The isomer (6) was also found in *M. putorius*, where it was prodominant. 3-Ethyl-1,2-dithiacyclopentane (10) was only found in *M. erminea*, whereas 3,3-dimethyl-1,2-dithiacyclopentane (9) appeared in the other three species. In addition, two more unidentified isomers of 3,3-dimethyl-1,2-dithiacyclopentane were found in all four species.

Crump (1980b) reported several thietanes and dithiacyclopentanes in secretion of *M. putorius furo*. Of these only two were found in *M. putorius* examined by us.

Two nitrogen-containing compounds were found: indole in all species, but *o*-aminoacetophenone only in *M. erminea*.

Phylogeny of the Mustelids. The early evolutionary history of the mustelids is poorly known, but the recent subfamilies Mustelinae (weasels, polecats, martens, and others) and Lutrinae (otters) can be traced from the Oligocene, and the subfamily Melinae (badgers) from the Miocene (Romer, 1966). During the late Miocene and Pliocene the modern genera within the subfamily Mustelinae a.o. *Mustela* and *Martes* appeared (Kurtén, 1968). Some of the Pliocene forms were intermediate between *Martes* and *Mustela* (Andersson, 1970). Modern species within the genus *Mustela* are found from the middle Pleistocene (Hall, 1951). Phylogenetic relationships within the genus *Mustela* have been discussed from genetic data (Graphodatsky et al., 1976, 1977; Mandahl and Fredga, 1980). However, the pattern of karyotype evolution is still unclear (Fredga 1977; Imai and Crozier, 1980).

Based on available information on genetic, morphological, behavioral, and ecological characteristics, we have constructed a phylogenetic scheme (Figure 7). The subfamilies represent three different adaptive trends (Sokolov, 1968). Pocock (1921) considered the *Martes* group as a separate sub-

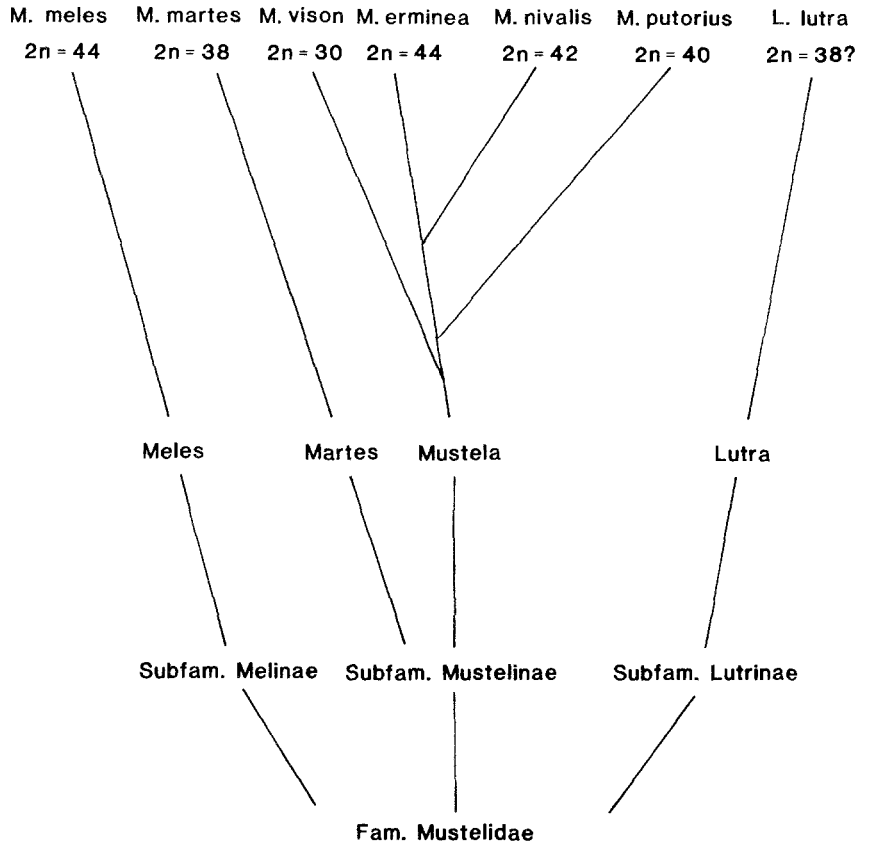


FIG. 7. Suggested phylogenetic relationships of examined mustelids. Chromosome numbers are from Fredga (1967) and Fredga (personal communication as concerns *Lutra lutra*).

family (Martinae), but today *Martes* is considered as a genus within the subfamily Mustelinae (Ewer, 1973). The close relationship between *Mustela erminea* and *M. nivalis* was questioned by Pohl (1910), but recent work by Mandahl and Fredga (1980) and also ecological and behavioral characteristics corroborate the close relationship between the two species. *Mustela putorius* and *M. vison* are both distinctly separated from *M. erminea* and *M. nivalis*, but *M. putorius* has more characteristics in common with them.

At the generic level the chemical results are in agreement with systematics. The anal sac secretions of all genera have a unique composition, with few, if any, components in common. Species within the genus *Mustela* on the other hand, showed a very concordant composition of the secretions, although each species had a distinct blend. The secretions contain a very

complex mixture of volatile components, and with present knowledge it is not possible to determine the degree of relatedness between the species, e.g., *M. erminea* and *M. nivalis* are considered to be closely related, but their secretions in the low-molecular-weight region have less compounds in common than have *M. erminea* and *M. vison* (Table 1). The systematic position of *M. putorius furo* in relation to *M. putorius* and *M. eversmanni* has been much discussed. Cranially *M. putorius furo* more closely resembles *M. eversmanni* but the karyotype is identical to that of *M. putorius* and differs from that of *M. eversmanni* (Volobuev et al., 1974; cited in Corbet, 1978). Of the examined low-molecular-weight compounds in *M. putorius furo* and *M. putorius* secretions, only a few were found in common (Table 1).

Intraspecific scent communication apparently is the main function of the anal sac secretion in mustelids (Goethe, 1964; Gorman, 1980; Erlinge et al., 1982).

Qualitative and quantitative differences in chemical composition between species provide a possibility for interspecific communication. Mustelids do react to scents from congeneric species, as was revealed in behavioral tests with *Mustela erminea* and *M. nivalis* (Sandell and Erlinge, unpublished). The anal sac secretions in small mustelids are also used for defense against predators. Frightened *Mustela vison*, *M. putorius*, *M. erminea*, and *M. nivalis* were observed to empty their anal sacs (our observations). This function may account for the presence of sulfur-containing compounds.

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BEHAVIORAL RESPONSES OF MALE *Heliothis virescens*[1] IN A SUSTAINED-FLIGHT TUNNEL TO COMBINATIONS OF SEVEN COMPOUNDS IDENTIFIED FROM FEMALE SEX PHEROMONE GLANDS

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Abstract—Each of the seven compounds that have been identified from female *Heliothis virescens* sex pheromone glands was examined for its ability to elicit sexual responses from male moths in a flight tunnel. The two compounds initially described as pheromone components, (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal, were necessary for behavioral activity to occur. Of the remaining five compounds, hexadecanal was most consistent in elevating behavioral activity of males when it was added to treatments. Live, calling females elicited greater sexual activity from males than did the 7-compound mixture on rubber septa.

Key Words—Tobacco budworm, *Heliothis virescens*, Lepidoptera, Noctuidae, flight tunnel, sex pheromone, moth behavior, rubber septa.

INTRODUCTION

The sex pheromone of the female tobacco budworm moth, *Heliothis virescens* (F.) was first identified as a 2-component mixture, (*Z*)-11-hexadecenal (*Z*11-16:ALD) and (*Z*)-9-tetradecenal (*Z*9-14:ALD) (Roelofs et al., 1974; Tumlinson et al., 1975). Klun et al., (1979, 1980) isolated and identified five additional compounds from washes of female sex pheromone glands: hexadecanal (16:ALD), tetradecanal (14:ALD), (*Z*)-7-hexadecenal (*Z*7-16:ALD), (*Z*)-9-hexadecenal (*Z*9-16:ALD), and (*Z*)-11-hexadecenol (*Z*11-16:OH). In tests with cotton wicks, the 7-compound mixture outperformed the 2-

[1]Lepidoptera:Noctuidae.

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component mixture in field-trapping experiments (Sparks et al., 1979; Hartstack et al., 1980). However, in other tests using rubber septa or plastic laminates, the two treatments were equally attractive or the 2-component mixture was superior (Hartstack et al., 1980). Due to the large number of possible permutations of compounds, only a limited number of combinations have been tested in the field. The purpose of this study was to systematically determine the role, if any, that each of the seven compounds plays in *H. virescens* sexual communication, using upwind flight and precopulatory behaviors of the male moths in a sustained-flight tunnel as the criteria for differentiation of the treatments.

METHODS AND MATERIALS

Rearing

Tobacco budworm larvae were raised on a modified pinto bean diet (Shorey and Hale, 1965). Pupae were segregated by sex, moths were aged, and the sexes kept in separate chambers after emergence. Larvae and adults were maintained at 25 ± 2°C on a 14:10 light–dark photoperiod. Adults always had access to a 8% sucrose solution.

Chemicals

All compounds were obtained from the Controlled Release Division of Albany International Corporation. Purity was determined by gas–liquid chromatography, using 10% XF-1150 (50% cyanoethyl, methyl silicone) on Chromosorb W, AW DMCS, 100/120 mesh (2.5 m × 2 mm). Purities for the compounds were as follows: 14:ALD (100%), Z11–16:OH (99%), Z9–14:ALD, Z7–16:ALD, and Z11–16:ALD (98%), Z9–16:ALD (96%), and 16:ALD (95%). The stock solutions were made prior to each series of tests and were stored at below −20°C. Rubber septa (A.H. Thomas Co. No 8753-D22, sleeve type, 5 × 9 mm) were loaded on the small end with 10 μl of solution in hexane such that the emission rates of the seven compounds, as measured with an airborne collection device (Baker et al., 1981), approximated those of the same compounds emitted from the forcibly extruded gland of a *H. virescens* female (Table 1). Z11–16:OH was not detectable in emissions from female glands and, therefore, it was loaded on the septum at 1 μg which was 1% of the total blend as found in extracts of gland tissues (Klun et al., 1979, 1980).

Behavioral Observations

Compound Mixtures on Septa. Male moths were tested during the 5th through 8th hr of their 4th or 5th scotophase in a clear plastic flight tunnel (1 m wide at the floor, 0.9 m high, 3.65 m long) modified after Miller and

TABLE 1. LOADING AND EMISSION RATES OF COMPOUNDS ON RUBBER SEPTA AND EMISSION RATES OF FORCIBLY EXTRUDED FEMALE SEX PHEROMONE GLANDS (DATA FROM POPE ET AL., 1982) AT 22°C

Compound	Loading on rubber septa (μg)	Emission rates ng/min (% of total)	
		Rubber septa	Female
Z11-16:ALD	100	1.14 (58)	2.19 (73)
Z9-14:ALD	2.5	0.18 (9)	0.16 (5)
16:ALD	50	0.36 (19)	0.39 (13)
14:ALD	5	0.25 (13)	0.22 (7)
Z7-16:ALD	1	0.02 (1)	0.02 (1)
Z9-16:ALD	1	0.03 (1.5)	
Z11-16:OH	1	ND (0)	ND (0)

Roelofs (1978). Further details of construction are found in Kuenen and Baker (1982). The males were transferred to the flight-tunnel room ca. 1.5 hr prior to the beginning of observations in order to acclimate the moths to wind-tunnel conditions (0.5 m/sec wind velocity, 24± 3°C, 0.3 lux light level). They were then transferred from the holding cage to individual release cages [6 cm long × 6 cm diam (3.15 wires/cm) galvanized mesh] at least 30 min prior to testing.

Septa were loaded daily with solutions ca. 30 min before testing. They were placed in an exhaust hood for 15 min, and then transferred to individual, capped, holding vials. Treatments within an experimental series were drawn in random order for each daily testing in a complete-block design. At the beginning of a trial, an impregnated septum was placed at the center of a piece of sheet metal (15 × 15 × 0.05 cm) situated 15 cm above the tunnel floor on a sheet-metal platform that was 50 cm from the upwind end of the tunnel. Each male was released ca. 3 m downwind of the source by placing the individual cage, open end up, on a metal platform, held by a ringstand positioned in the middle of the pheromone plume. The location of the plume was predetermined by a $TiCl_4$ smoke source. Cages and platforms were washed with acetone daily after use.

The attractiveness of a mixture was determined by the intensity of the males' responses upon leaving the release cage. Those males capable of sustained flight were scored for the following behaviors: *Up W*, moth flies upwind in the pheromone plume from the release point; *Pl*, moth flies within 10 cm of platform edge; *S*, moth lands on source; *Hp*, after landing on the source, moth everts his hairpencils; and *C*, after landing on the source, moth exhibits the copulatory response (i.e., full hairpencil eversion, curling of the abdomen). For each behavioral category, no male contributed more

than 1 data point and the number reported in the table is the percentage of males that exhibited at least that level of behavior (i.e., a male that exhibited a *Hp* response added one positive response to each of the first four categories but not the last). Observations of hairpencil extrusions near the source were aided with a flashlight that had several layers of red cellophane placed over the lens.

Males making multiple approaches were observed until they flew up and out of the plume toward the top of the flight tunnel. If there were uncertainty of the intensity of the male's response (e.g., septum blocking the observer's view), the most intense response observed was recorded. Males were used once and then discarded from further testing; no more than 8 males were flown to one treatment per day. Each trial (i.e., the amount of time the septum was in the flight tunnel airstream) lasted ca. 15 min. All rubber septa were used within 30 min to 4 hr of initial loading and discarded after use.

All statistical analysis was performed using a χ^2 2×2 test of independence with Yates' correction at the 0.05 level of significance.

Female vs. Septa. Rubber septa impregnated with the 7-compound mixture were tested against live, calling *H. virescens* females. Females were used during the 4th through 7th hr of their 4th scotophase. They were transferred to the flight tunnel room ca. 1 hr prior to the beginning of the bioassay and placed in individual cages in the rear of the flight tunnel, upwind of the exhaust hood. When a female was observed calling (i.e., pheromone gland exposed), she was gently transferred, in her cage, to the upwind end of the flight tunnel and placed in the center of the sheet-metal platform. When the rubber septum was tested, an identical cage was placed over it to mimic the conditions under which the female was contained. Data were recorded from males that flew upwind in the plume. If a male initiated flight but did not fly upwind in the plume, it was not known if he had not responded to the pheromone or if the female had stopped calling and no pheromone was present; observations on these males were discarded. Upon landing on the cage containing the female or rubber septum, the male was observed for 5 sec and then was removed. This action was necessary since the presence of the male occasionally disturbed the female, whereupon she would move about the cage and sometimes cease calling. [This should have caused no biasing of the data since in other series of experiments with synthetic mixtures to which there were 505 multiple approaches by males, the most intense response was exhibited on the first approach (91%); we should have retained discrimination regardless of the male removal procedure.] No more than eight males were flown to one female and most trials with a female lasted 15 min; none lasted more than 30 min.

Rubber Septa vs. Cotton Wicks. Two types of dispensers were compared: rubber septa and cotton wicks. Cotton dental wicks, 36 mm long $\times$ 15 mm diam., were cut in half (to approximate the height of a septum) and 10 μl

TABLE 2. RESPONSE OF MALE MOTHS TO 2-COMPOUND MIXTURES

Treatments	% Behavioral Response[a] 16 flights/treatment				
	UpW[b]	*Pl*	*S*	*Hp*	*C*
Z11-16:ALD + Z9-14:ALD	100a	81a	62a	38a	6a
All other possible permutations of two compounds (20 treatments)	0b	0b	0b	0b	0a

[a] *UpW*, moth flies upwind in plume from release point; *Pl*, moth flies <10 cm from platform edge; *S*, moth lands on source; *Hp*, after landing, moth everts hairpencils; *C*, after landing, moth exhibits copulatory response.

[b] Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).

of the 7-compound mixture in hexane was impregnated on the uncut end of the wick. The wick was placed in an exhaust hood 15 min prior to initiation of the bioassay. Rubber septa were prepared as before and males were flown to both dispensers.

RESULTS

Two-Compound Mixtures. All 21 possible combinations of binary mixtures from seven compounds were tested, but only one elicited upwind flight from the males (Table 2). This was the combination of Z11-16:ALD and Z9-14:ALD which was initially described as the pheromone for *H. virescens* (Roelofs et al., 1974; Tumlinson et al., 1975) and hereafter will be referred to as the 2-component mixture.

Three-Compound Mixtures. Using the information obtained from the first experiment, a series of 3-compound mixtures was run, admixing one of each of the additional five compounds to the 2-component mixture. These were then compared to the 2-component and 7-compound mixtures.

The 7-compound mixture evoked significantly more *S* and *Hp* behaviors than the 2-component mixture (Table 3). Of the five 3-compound treatments, only addition of 16:ALD elicited significantly more *Hp* responses than the 2-component mixture but not significantly greater *S* or *Hp* responses than the 7-compound mixture. The treatments containing 14:ALD, Z11-16:OH, Z7-16:ALD, and Z9-16:ALD did not elicit significantly greater responses than the 2-component mixture.

Six-Compound Mixtures. One of each of the seven compounds was deleted from the 7-compound mixture to determine which one(s), if any,

TABLE 3. RESPONSE OF MALE MOTHS TO 3-COMPOUND MIXTURES

Treatments	Additional compound	% Behavioral Response[a] 100 flights/treatment				
		UpW[b]	*Pl*	*S*	*Hp*	*C*
Z11-16:ALD + Z9-14:ALD	16:ALD	90a	83a	64ab	38ab	6a
	14:ALD	80ab	71ab	58ab	24c	1a
	Z7-16:ALD	85ab	74ab	58ab	32abc	3a
	Z9-16:ALD	86ab	75ab	57ab	34abc	5a
	Z11-16:OH	74b	63b	54b	27bc	2a
2-Component mixture		82ab	72ab	53b	24c	1a
7-Compound mixture		84ab	79a	69a	45a	4a

[a]Abbreviations for the behavioral responses are described in Table 2 and in Methods and Materials.
[b]Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).

would cause a significant reduction of behavioral response when compared to the blend of seven compounds. Since the first two components (Z11-16:ALD and Z9-14:ALD) together were crucial for eliciting upwind flight from males (Table 2), the first part of this experiment consisted of three treatments: the 2-component mixture and two 6-compound mixtures (one of the two initial components was deleted from the 7-compound mixture to form the appropriate 6-compound mixture). Again, it was obvious that both Z11-16:ALD and Z9-14:ALD were essential for upwind flight to occur (Table 4, Part A).

In the second set of experiments, each of the five additional compounds was deleted, one at a time, from a 7-compound mixture and compared to the 2-compound and 7-compound mixtures. The blend of seven compounds again elicited a significantly greater number of *S* and *Hp* behaviors than did the 2-component mixture (Table 4, Part B). Of the five 6-compound mixtures, the treatment lacking 16:ALD caused the greatest decrease at the *S* and *Hp* levels of behavioral activity and both levels were significantly lower than the 7-compound blend. Responses to this treatment were not significantly different from those of the 2-component mixture. The only other 6-compound mixture to which there was reduced response compared to the 7-compound blend was the one lacking 14:ALD; it was significantly weaker at the *Pl* and *S* levels.

Four-Compound Mixtures. Because the greatest change in activity from the last two series of experiments occurred with the addition of 16:ALD to the 2-component mixture or with the omission of 16:ALD from the 7-compound mixture, the basis for this series became a 3-compound blend comprised of the original 2-component mixture plus 16:ALD, henceforth referred to as the 3-component mixture. The 4-compound mixtures tested

TABLE 4. RESPONSE OF MALE MOTHS TO 6-COMPOUND MIXTURES

Treatments	Missing compound	% Behavioral response[a] 24 flights/treatment				
		UpW[b]	*Pl*	*S*	*Hp*	*C*
Part A						
6-Compound mixtures	Z11-16:ALD	0b	0b	0b	0b	0a
	Z9-14:ALD	0b	0b	0b	0b	0a
2-Component mixture		79a	79a	62a	46a	0a
		100 flights/treatment				
Part B						
6-Compound mixtures	16:ALD	85a	68ab	51c	37c	4a
	14:ALD	78a	62b	55bc	41bc	6a
	Z7-16:ALD	82a	73ab	68ab	57a	3a
	Z9-16:ALD	82a	74ab	67ab	54ab	9a
	Z11-16:OH	85a	77a	66ab	44abc	7a
2-Component mixture		83a	67ab	57bc	34c	6a
7-Compound mixture		85a	78a	72a	55ab	11a

[a]Abbreviations for the behavioral responses are found in Table 2 and in Methods and Materials.
[b]For each part, percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with a Yate's correction ($P < 0.05$).

in this series consisted of the 3-component mixture admixed with one of the remaining compounds (14:ALD, Z7-16:ALD, Z9-16:ALD, or Z11-16:OH (Table 5)). The reference treatments were the 2- and 3-component mixtures and the 7-compound mixture.

After 100 moth flights/treatment, no clear-cut differences were ap-

TABLE 5. RESPONSE OF MALE MOTHS TO 4-COMPOUND MIXTURES

Treatments	Additional compound	% Behavioral response[a] 100 flights/treatment				
		UpW[b]	*Pl*	*S*	*Hp*	*C*
Z11-16:ALD	14:ALD	86ab	73a	66a	37b	7a
+ Z9-14:ALD	Z7-16:ALD	92ab	78a	72a	36b	10a
+ 16:ALD	Z9-16:ALD	88ab	78a	72a	42ab	3a
	Z11-16:OH	86ab	83a	72a	55a	7a
2-Component mixture		93a	83a	71a	41ab	5a
3-Component mixture		91a	72a	61a	40a	5a
7-Compound mixture		82b	78a	73a	57a	4a

[a]Abbreviations for the behavioral responses are described in Table 2 and in Methods and Materials.
[b]Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yate's correction ($P < 0.05$).

parent due to the higher elicitation of activity by the two components, in contrast to the first sets of experiments. We could not discriminate among treatments containing compounds added to the 2 components, and here 16:ALD, when added to the 2-component mixture, did not elevate the males' responses above their already high levels. We include these results to show that the behavioral effects of adding 16:ALD could not always be observed in every experiment; this was the only experiment in which the effects of 16:ALD could not be seen.

Mixtures Containing Saturated Aldehydes and the Alcohol. A series of treatments was tested involving selected combinations of the saturated aldehydes and the alcohol with the 2-component blend which might provide further information on the effects of additional compounds. The treatments tested were two 3-compound blends containing the two components plus either 16:ALD or 14:ALD, two 4-compound mixes containing the 3-component mixture plus 14:ALD or Z11-16:OH, and a 5-compound mixture containing the 3-component blend plus 14:ALD and Z11-16:OH. The 2-component and 7-compound mixtures were included as the reference treatments.

The 7-compound mixture elicited significantly greater *S* and *Hp* behavioral responses than did the 2-component mixture, which was consistent with the first two experiments (Table 6). Of the remaining five treatments, those which contained 16:ALD caused behavioral activity similar to that of the 7-compound mixture. The only treatment lacking 16:ALD was the 3-compound blend which contained 14:ALD; this blend performed only as well as the 2-component mixture.

TABLE 6. RESPONSE OF MALE MOTHS TO TREATMENTS COMPRISED OF SELECTED COMBINATIONS OF 16:ALD, 14:ALD, AND Z11-16:OH[a]

Treatments	Additional compounds			% Behavioral response[b] 100 flights/treatment				
	16:ALD	14:ALD	Z11-16:OH	*UpW*[c]	*Pl*	*S*	*Hp*	*C*
Z11-16:ALD	+			89a	81a	68ab	48a	3a
plus		+		83a	73a	50c	28b	2a
Z9-14:ALD	+	+		84a	76a	67ab	51a	7a
	+		+	85a	75a	67ab	56a	5a
	+	+	+	89a	82a	72a	54a	7a
2-Component mixture				84a	80a	56bc	28b	1a
7-Compound mixture				88a	76a	71a	54a	3a

[a] A "+" indicates the presence of this compound in the treatment.
[b] Abbreviations for the behavioral responses are described in Table 2 and in Methods and Materials.
[c] Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).

TABLE 7. RESPONSE OF MALE MOTHS TO 7-COMPOUND MIXTURE EVAPORATED FROM RUBBER SEPTA AND TO LIVE, CALLING FEMALES

	% Behavioral response[a] 100 flights/treatment				
Treatments	*Up W*[b]	*Pl*	*S*	*Hp*	*C*
Calling female	99a	91a	91a	81a	15a
7-Compound mixture	96a	83a	70b	40b	0b

[a] Abbreviations for the behavioral responses are described in Table 2 and in Methods and Materials.
[b] Percentages in the same column with no letters in common are significantly different according to a χ^2 2×2 test of independence with a Yates' correction ($P < 0.05$).

Septum vs. Live Female. Live, calling females elicited a significantly greater number of *S*, *Hp*, and *C* responses than did the 7-compound mixture on rubber septa (Table 7). No treatment in any of the series was able to elicit such high levels of activity from the males in the *S* and *Hp* categories.

Septum vs. Cotton Wick. The dental wick loaded with the 7-compound mixture elicited significantly more *Up W* and *Pl* behaviors from the males than did the septum, but there was no difference between the dispensers in the percentage of males landing on the source. However, the 7-compound mixture on the rubber septum caused a significantly greater number of *Hp* behaviors in the males than did the cotton wick (Table 8). Cotton wicks emit the compounds at rates two to three times greater than do rubber septa at the loading used in this study.

DISCUSSION

Two components, *Z*11-16:ALD and *Z*9-14:ALD, are the major mediators of chemical communication in *H. virescens* (Roelofs et al., 1974; Tumlinson et al., 1975; Klun et al., 1980; this study). In our flight tunnel, behaviors associated with mate-finding and close-range sexual activity (i.e., upwind flight to the source and hairpencil eversion) were not exhibited by the male moths when either of these two components was missing from a pheromone test blend (Tables 2 and 4, Part A). The five additional compounds identified by Klun et al. (1980) were nonessential for the attraction of a male to the source since the omission of any of these compounds from the 7-compound mixture did not prevent upwind flight and source location (Table 4, Part B).

However, in three of four of our series where the 2-component and the 7-compound mixtures were compared, the blend of seven compounds elic-

TABLE 8. RESPONSE OF MALE MOTHS TO 7-COMPOUND MIXTURE FORMULATED AT A DOSAGE OF 100 μg Z11-16:ALD ON TWO DISPENSERS: COTTON WICK AND RUBBER SEPTA

Treatments	% Behavioral response[a] 100 flights/treatment				
	UpW[b]	*Pl*	*S*	*Hp*	*C*
Rubber septum	78b	67b	63a	48a	2a
Cotton wick	92a	82a	59a	34b	2a

[a] Abbreviations for the behavioral responses are described in Table 2 and in Methods and Materials.
[b] Percentages in the same column having no letters in common are significantly different according to a χ^2 2×2 test of independence with Yates' correction ($P < 0.05$).

ited significantly more responses in at least one of the close-range behavioral categories than did the two components (Tables 3, 4, Part B and 6). Therefore, the elevated behavioral response must have been due to one of the additional five compounds or to a combination of two or more compounds.

Of the five compounds tested in our flight tunnel, the saturated 16:ALD was the most important additional compound to the 2-component blend, accounting for all of the increased close-range behavior observed in the 7-compound mixture (Tables 3 and 6). Also, its omission from the 7-compound blend caused a significant decrease in close-range behaviors of males, making it only as effective as the 2-component mixture (Table 4, Part B). Of the five compounds, the presence or absence of 16:ALD was the most consistent in predictability of behavioral effects, although it was not absolute (Table 5). When the remaining compounds (14:ALD, Z7-16:ALD, Z9-16:ALD, and Z11-16:OH) were considered as singular additives, singular omissions, or in combination with other compounds, there was no consistent effect on attraction or close-range behaviors (Tables 4, Part B, 5, and 6).

We feel that there is now enough evidence that 16:ALD is a pheromone component of *H. virescens* since it usually increased close-range sexual behaviors of the male moths when added to blends containing Z11-16:ALD and Z9-14:ALD. None of the other four compounds identified by Klun et al. (1980) were consistent enough in our tests to warrant the classification of "pheromone component," although this does not preclude them from being such. Our test may not have been sensitive enough to distinguish any further function of these compounds. Nonetheless, we feel that 16:ALD is the most important of the five additional compounds, and its effects probably overshadowed any slight enhancement by the other four compounds.

Consistent with this, Pope et al. (1982) found that 16:ALD was not only present in the gland extracts but it was emitted by 98% of the females in their peak emission period. Other than Z11-16:ALD, which was detectable 100% of the time, no compound was as abundant or detected as often as 16:ALD in volatile collections.

In some previous studies (Sparks et al., 1979; Hartstack et al., 1980), Z11-16:OH significantly enhanced trap catch when added to treatments formulated on cotton wicks. However, in our experiments, the presence of Z11-16:OH with the 2-component mixture on rubber septa caused no increase in male response (Table 3). This is in agreement with Hartstack et al. (1980) who found the presence of Z11-16:OH as part of the 7-compound mixture in Hercon®-laminated flakes, Albany International® hollow fibers, or on rubber septa did not increase trap catch of males and sometimes significantly reduced captures. In the wind tunnel, our formulation of the seven compounds on a rubber septum attracted as many males to the source as did a cotton wick loaded with the same quantity of compounds (Table 8), and hence, there was nothing obviously inferior about septa as a release substrate that would account for the above-mentioned differences in field experiments. In a blend ratio series, males in the flight tunnel were attracted equally well to lower levels of the alcohol added to the two components, but exhibited significantly reduced levels of upwind flight to 3% or greater levels of the alcohol; 79%, 82%, 78%, 54%, 43%, and 30% of the males flew upwind to 0, 0.3, 1, 3, 10, and 30% of the alcohol relative to the Z11-16:ALD ($N = 29$, 22, 27, 28, 23, and 27, respectively). In airborne pheromone collection studies, no detectable amounts of Z11-16:OH were emitted by females, in contrast to the measurable quantities of the other six compounds that were collected (Pope et al., 1982). This information, plus our flight tunnel observations, indicate that Z11-16:OH may not contribute to sexual communication in this species.

The observations from the flight tunnel may be instructive in explaining the difficulty of discriminating subtle, close-range effects of purported pheromone compounds in field-trapping experiments using large traps. Most of the differences among treatments in the flight tunnel from which we made our conclusions occurred at the *S* and *Hp* levels of behavioral activity. If similar proportions of moths exhibiting *Pl* behaviors were trapped, then, from our results, it is easy to see why there might be no differences in trap catch among different treatments in the field; for example, between treatments containing and lacking 16:ALD. A *Pl* observation required that the male moth fly within 10 cm of the pheromone source. When no longer attracted to the source, these males would fly upward and most likely would be captured in the *Heliothis* cone trap which has an opening at the underside of 50 cm (Hartstack et al., 1979). On the other hand, flight tunnel studies may lack discrimination for compounds functioning mainly at longer range, and here trapping experiments would be superior. Flight tunnels normally cannot duplicate the plume dimensions and concentrations that occur at

great distances from a pheromone source and therefore cannot elicit from males the types of movements required to orient from those distances in a shifting wind field.

Possibly the most enlightening experiment was the comparison of the 7-compound mixture on a rubber septum to a live, calling female. When a female was tested against a rubber septum in the flight tunnel, there were no differences between *UpW* or *Pl* behavior elicitation. However, there was statistical significance at the remaining three levels. The greater number of *Hp* and *C* behaviors elicited by females could be due to better chemical as well as nonchemical (tactile, visual, and auditory) cues. However, the significantly greater number of *S* behaviors implies that the difference between septa and females may be explained best by chemical cues. Tactile cues are not possible since the male is still airborne just prior to landing on the source. Vision seems to play a minor role since males land on a pheromone-loaded cotton wick preferentially to a dead female pinned to an unloaded wick even when separated by only 2 cm (unpublished data) and females are relatively still when calling. Auditory cues cannot be ruled out and were not controlled in the experiment. Inasmuch as other cues may play a minor role in attracting males to a source, the factor more likely responsible for the elevated responses to live females in our study was chemical, due to components that are as yet unidentified or to blend quantity or quality differences that, despite our attempts to mimic female emissions, made the septa inferior at close range.

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ALLELOPATHIC EFFECTS OF *Polygonum aviculare* L. III. Isolation, Characterization, and Biological Activities of Phytotoxins Other Than Phenols

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Abstract—We previously reported that *Polygonum aviculare* has a strong allelopathic action against *Cynodon dactylon* (L.) Pers. and other test species. Moreover, we found that several phenolic compounds appeared to be important allelochemics in this activity. We have now isolated other potential inhibitors from *P. aviculare* residues and soil under *Polygonum* stands, and none of these occurred in soil under *C. dactylon* stands. GC-MS analysis revealed that these additional inhibitors are long-chain fatty acids with 14–22 carbons. Nine were identified in *P. aviculare* residues and seven in soil under *P. aviculare*. Sodium salts of all the identified fatty acids inhibited seedling growth of *C. dactylon* and at least some test strains of the nitrogen-fixing bacteria, *Azotobacter* and *Rhizobium*.

Key Words—Allelopathy, fatty acids, GC-MS, *Polygonum aviculare*, *Cynodon dactylon*, *Rhizobium*, *Azotobacter*.

INTRODUCTION

Polygonum aviculare (prostate knotweed) is a serious weed in crops and lawns in many parts of the world. We reported previously that this species exerts a strong allelopathic action against *Cynodon dactylon* (bermudagrass) and selected crop plants (AlSaadawi and Rice, 1982a). Moreover, four phenolic phytotoxins were isolated from *P.aviculare* residues and soil under *Polygonum*, and these allelochemics did not occur in soil under bermudagrass (AlSaadawi and Rice, 1982b).

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While using GC-MS in attempting to identify the phenolic compounds, we discovered relatively high concentrations of several other compounds which were not phenols. These compounds appeared in *Polygonum* residues and soil under *Polygonum*, but not in adjacent soil under bermudagrass. Preliminary experiments revealed that these compounds had certain characteristics of fatty acids. Experiments were designed, therefore, to isolate, identify, and determine the biological activities of the compounds.

METHODS AND MATERIALS

Collection and Extraction of Samples. Residues of *Polygonum aviculare* were collected from several plots and ground to pass a sieve with 1 mm openings. Several soil samples were collected from the top 10 cm in *P. aviculare* stands and similar collections were made in bermudagrass stands. All the samples were collected on March 20, 1981, four months after death of *Polygonum* in the field. The soil from each vegetation type was composited, mixed thoroughly, and air-dried.

Fifteen grams of *Polygonum* residues or 15 g of soil were extracted with 200 ml of petroleum ether for 24 hr in a Soxhlet extractor, after which the extracts were reduced to 10 ml in a flash evaporator.

Isolation and Identification of Inhibitors. The petroleum ether extract of each sample was concentrated to 2 ml by a jet of N_2 and methylated by addition of 5 ml of CH_2N_2. The solutions were kept at room temperature for 14 hr and then concentrated to 1 ml for GC-MS analysis.

The GC-MS analyses were performed on a Hewlett-Packard 5985 mass spectrometer. The GC was carried out using a 25-m, 0.2-mm internal diameter, methyl silicone Carbowax 20 M deactivated fused silica capillary column. The column was used under the following conditions: column pressure 20 psi; initial flow rate 0.6 ml/min; a split ratio of the petroleum ether extracts of 50:1; initial column temperature held at 150°C for 25 min after injection, programed from 150° to 180° at 4°/min, held at 180° for 10 min, then programed at 4°/min from 180° to 200°, held at 200° for 20 min, then programed from 200° to 216° at 4°/min.

Identification of the compounds separated by GC was accomplished by comparison of the unknown MS spectra with a collection of known MS spectra (the Hewlett-Packard Drug, Pollution, and Bio-Medical User Contributed Libraries) by the probability based searching technique. The reproduced comparative MS plots were obtained using the Hewlett-Packard SPDIF (spectral difference) program. A difference plot was generated by normalizing the base peaks of both spectra and plotting the relative differences. This difference, not shown in the compared MS plots, was less than 50% in every case, assuring a very high certainty of correct identification.

Bioassay of Identified Compounds. The sodium salts of all the identified fatty acids were tested against seed germination and seedling growth of bermudagrass in concentrations of 5, 10, 30, and 50 ppm. Salts were used because the pH of the soil in *Polygonum* field plots averaged 7.58, which suggests that the active form of the compounds under field conditions would be salts of the acids.

Twenty milliliters of a given test solution were placed in a 10-cm Petri dish containing washed quartz sand and 25 seeds of bermudagrass. Control dishes were made similarly, except distilled water was substituted for the test solutions. All test solutions and distilled water were buffered at a pH of 7.58. The Petri dishes were kept in a growth chamber on a 14-hr photoperiod (1000 ft-c) at 28°C and a 10-hr dark period at 20°. Germination, radicle, and epicotyl length were recorded 12 days after planting.

The same test solutions were bioassayed against the following nitrogen-fixing bacteria: *Rhizobium leguminosarum*, American Type Culture (ATC) strain 10314; *R. meliloti*, ATC strain 4400; *R. japonicum*, ATC strain 10324; *R. lupini*, ATC strain 10319; *Azotobacter vinelandii*, ATC strain 9104; and *A. chroococcum* ATC strain 9043. A yeast extract–mannitol medium (Society of American Bacteriologists, 1957, p. 113) was used for all strains of *Rhizobium* and a soil extract–mannitol medium (Society of American Bacteriologists, 1957, p. 109) was used for *Azotobacter.*

A sterilized sensitivity disk was saturated with a given test solution and placed on a Petri plate seeded with 0.2 ml of a 48-hr liquid inoculum of *Rhizobium* or *Azotobacter.* Distilled water was used again for controls and all test solutions and the distilled water were buffered at a pH of 7.58. All plates were incubated at 30°C and zones of inhibition were measured 3 days after inoculation.

RESULTS

Nine major unknown compounds were isolated by gas chromatography of the extract of *Polygonum* residue (Figure 1C). All the same unknown peaks, except peaks 1 and 8 appeared on the gas chromatogram of the extract of soil under *P. aviculare* (Figure 1B), but none of these appeared on the chromatogram of the extract of soil under bermudagrass (Figure 1A). All peaks labeled x or y were identified as impurities from the column.

Analysis of the peaks by mass spectroscopy revealed that the nine compounds in the *Polygonum* residue were all fatty acids (Figure 2). The peaks were identified as follows with the number of carbons and double bonds indicated in parentheses after each: (1) myristic acid (14:0), (2) palmitic acid (16:0), (3) linolelaidic acid (18:2), (4) oleic acid (18:1), (5) stearic acid (18:0), (6) arachidic acid (20:0), (7) 11,14-eicosadienoic acid (20:2), (8)

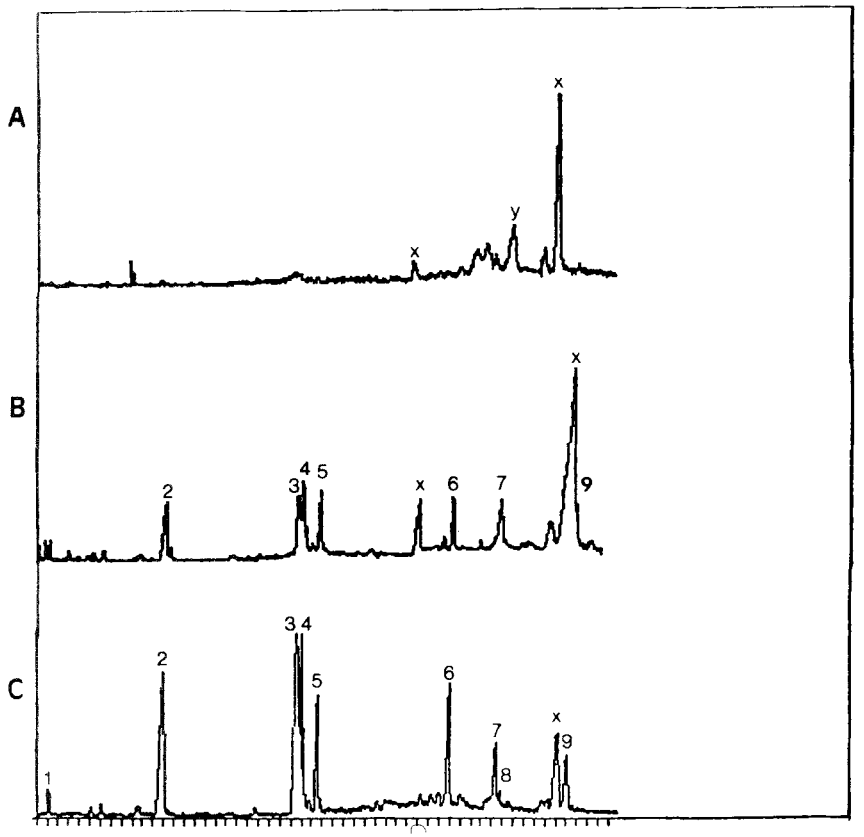

FIG. 1. Gas chromatograms of petroleum ether extracts of following samples after methylation: (A) extract of soil collected from bermudagrass stand, (B) extract of soil collected from *Polygonum aviculare* stand, and (C) extract of *P. aviculare* residue.

heneicosanoic acid (21:0), (9) behenic acid (22:0). As was indicated above, all of these fatty acids were present in the soil under *P. aviculare* also, except for myristic and heneicosanoic acids. None occurred in the soil under bermudagrass.

Bioassays of Identified Fatty Acids. Radicle growth of bermudagrass was significantly inhibited by all test concentrations of the sodium salts of all identified fatty acids, except the 10 ppm concentrations of 11,14-eicosadienoate and behenate (Table 1). Whole seedling growth (dry weight) was significantly inhibited by all test concentrations of all the compounds, except the same concentrations of the same two salts plus the 10 ppm concentration of linolelaidate. On the other hand, epicotyl growth of bermudagrass

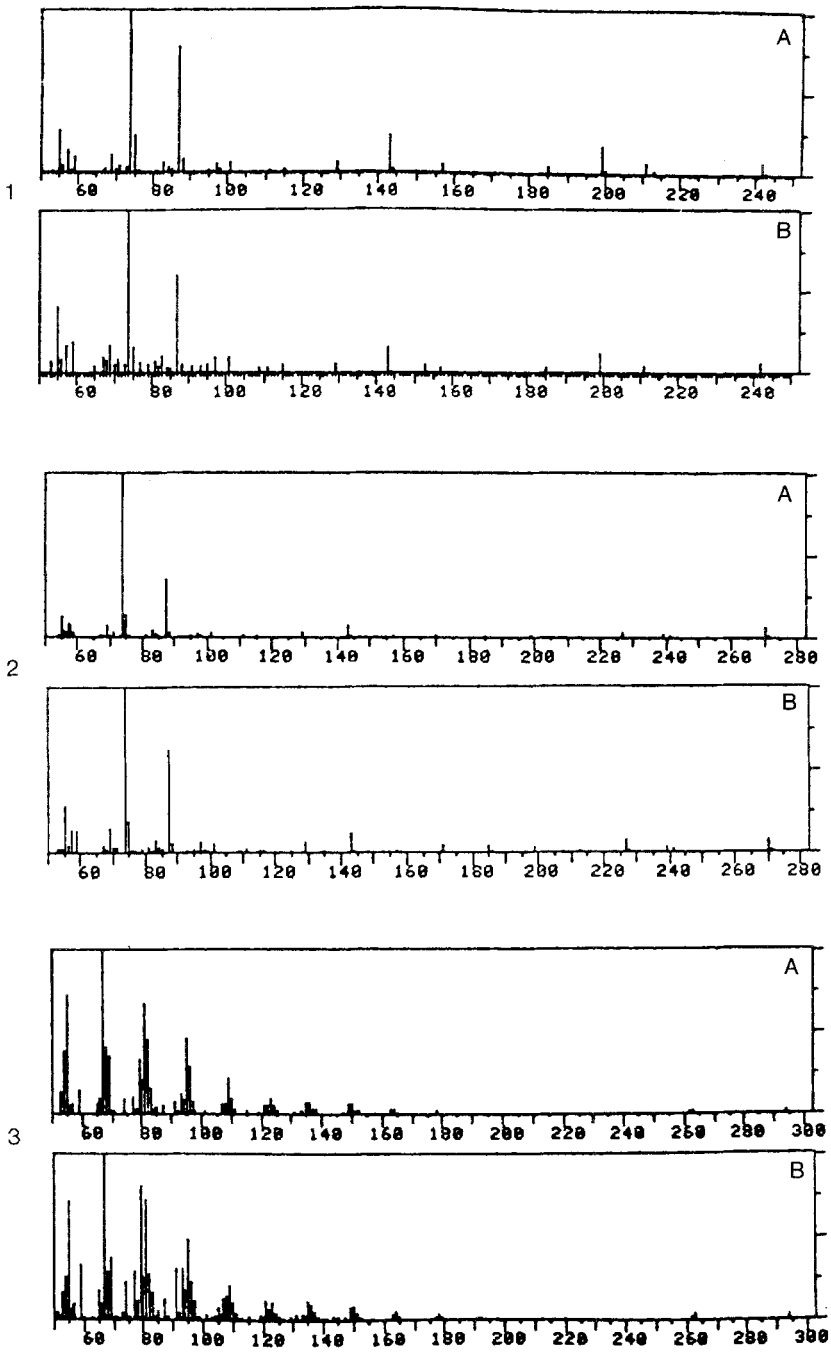

FIG. 2. Mass spectra of inhibitory compounds isolated from *P. aviculare* residue and soil under *P. aviculare* stands: (A) known compound, (B) suspected compound; 1, methyl myristate; 2, methyl palmitate; 3, methyl linolelaidate; 4, methyl oleate; 5, methyl stearate; 6, methyl arachidate; 7, methyl 11,14-eicosadienoate; 8, methyl heneicosanoate; 9, methyl behenate.

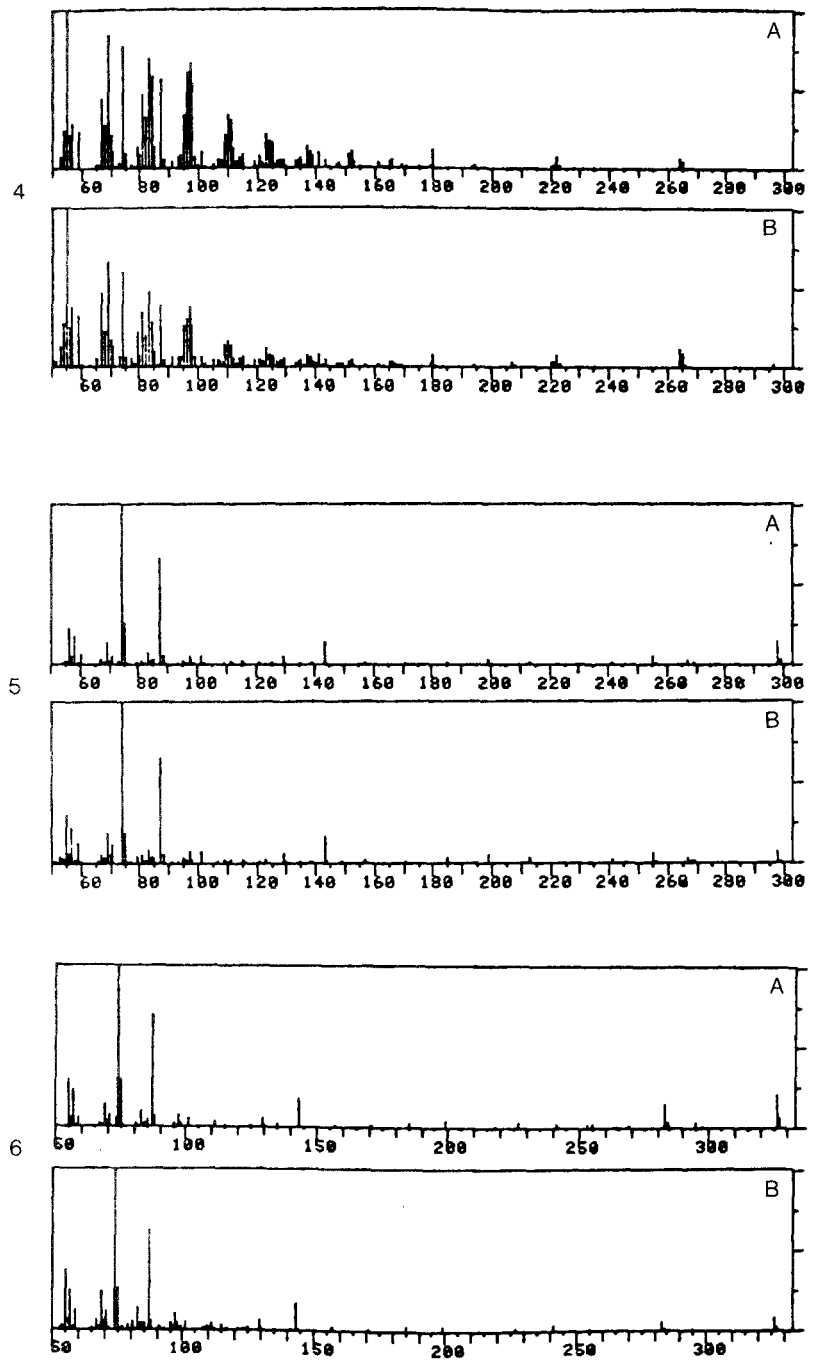

FIG. 2. Continued.

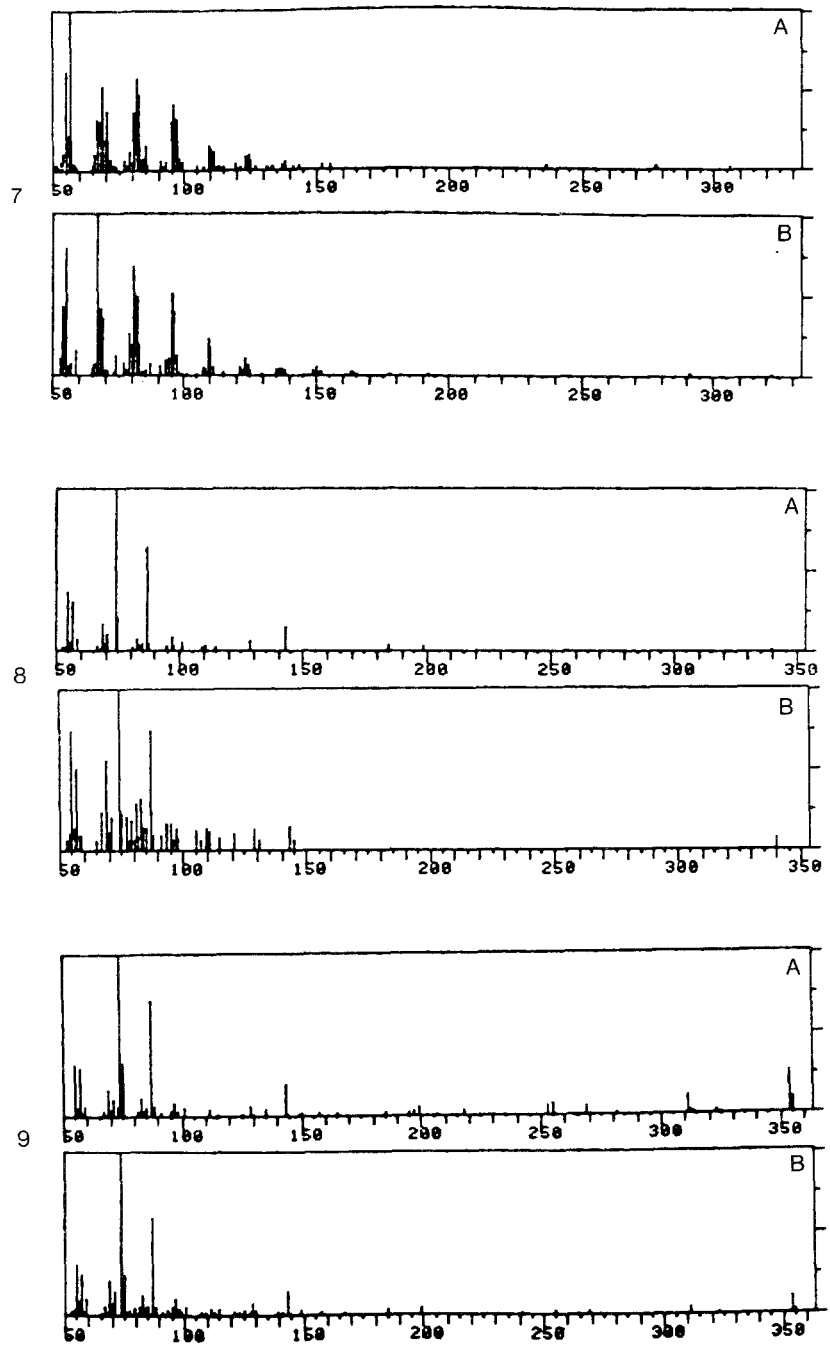

FIG. 2. Continued.

TABLE 1. EFFECTS OF SALTS OF FATTY ACIDS ISOLATED FROM *Polygonum* RESIDUES AND SOIL UNDER *Polygonum* STANDS ON SEED GERMINATION AND SEEDLING GROWTH OF *Cynodon dactylon*

Fatty acid salt	Concentration (ppm)	Mean length (mm)[a]			Germination % of control (0 ppm)
		Radicle	Epicotyl	Whole seedling	
Sodium myristate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	21.11 ± 1.53b	10.46 ± 0.28a	31.57 ± 1.61b	118.9
	10	20.04 ± 2.16b	10.77 ± 0.37a	30.81 ± 0.42b	121.2
	30	19.71 ± 1.43b	9.71 ± 0.36a	29.42 ± 1.54b	102.7
	50	20.61 ± 1.55b	9.96 ± 0.34a	30.57 ± 1.68b	91.9
Sodium palmitate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	9.00 ± 0.75c	8.81 ± 0.34b	17.81 ± 2.15c	83.3
	10	15.56 ± 1.85b	8.24 ± 0.40b	23.80 ± 2.19b	70.3
	30	15.68 ± 1.63b	8.16 ± 0.52b	23.84 ± 1.84b	67.7
	50	10.25 ± 0.78c	7.66 ± 0.44b	17.91 ± 1.32c	70.3
Sodium linolelaidate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	16.86 ± 1.69b	9.20 ± 0.34ab	26.06 ± 1.25b	108.1
	10	18.00 ± 1.91b	9.90 ± 0.33ab	27.90 ± 2.00a	102.7
	30	15.17 ± 1.02b	9.07 ± 0.44b	24.24 ± 1.22bc	102.7
	50	13.60 ± 1.72b	8.85 ± 0.34b	22.45 ± 1.83c	57.7
Sodium oleate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	20.55 ± 1.59b	9.77 ± 0.43a	30.32 ± 1.61b	100.0
	10	12.76 ± 1.35cd	9.70 ± 0.44a	22.46 ± 1.26c	108.0
	30	14.54 ± 1.65c	9.70 ± 0.35a	24.24 ± 2.00c	78.4
	50	8.87 ± 0.44d	8.41 ± 0.66a	17.28 ± 0.94d	75.7
Sodium stearate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	10.68 ± 1.02d	9.45 ± 0.53ab	20.13 ± 1.03d	97.3
	10	22.19 ± 1.64b	8.96 ± 0.28b	31.15 ± 1.99b	67.6
	30	14.76 ± 1.32cd	8.80 ± 0.32b	23.56 ± 1.35dc	83.8
	50	16.77 ± 1.58c	8.77 ± 1.69b	25.54 ± 1.70c	78.4

Sodium arachidate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	16.92 ± 1.28bc	10.96 ± 0.30a	27.88 ± 1.41b	113.5
	10	17.12 ± 1.58bc	10.12 ± 0.13a	27.24 ± 1.76b	129.7
	30	14.22 ± 2.13c	10.96 ± 0.50a	25.18 ± 2.30b	116.2
	50	18.68 ± 1.75b	10.37 ± 0.32a	29.05 ± 1.73b	97.3
Sodium 11,14-eicosadienoate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	20.31 ± 1.83b	9.96 ± 0.18ab	30.27 ± 2.14b	116.2
	10	25.32 ± 1.76a	10.92 ± 0.36a	36.24 ± 1.84a	113.5
	30	16.72 ± 1.32bc	9.75 ± 0.06b	26.47 ± 1.37bc	108.1
	50	13.96 ± 1.36c	9.89 ± 0.28ab	23.85 ± 1.62c	97.3
Sodium heneicosanoate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	17.26 ± 1.56b	9.53 ± 0.27a	26.79 ± 1.60b	110.8
	10	19.03 ± 1.82b	10.17 ± 0.28a	29.20 ± 1.98b	105.4
	30	14.80 ± 1.64b	9.57 ± 0.24a	24.37 ± 1.74b	102.7
	50	16.16 ± 1.31b	10.36 ± 0.49a	26.52 ± 1.28b	91.9
Sodium behenate	0	28.11 ± 1.42a	10.46 ± 0.73a	38.57 ± 1.67a	
	5	20.37 ± 1.69bc	11.18 ± 0.34a	31.45 ± 1.25bc	116.2
	10	25.17 ± 1.91ab	10.10 ± 0.33a	35.27 ± 2.00ab	108.1
	30	18.63 ± 1.02c	10.20 ± 0.44a	28.80 ± 1.22c	124.3
	50	21.02 ± 1.75bc	9.88 ± 0.34a	30.90 ± 1.83bc	108.1

[a]Average of at least 25 seedlings. Numbers with different letters within a column, associated with a given test compound, significantly different according to Duncan's multiple-range test.

TABLE 2. EFFECTS OF SALTS OF FATTY ACIDS ISOLATED FROM *Polygonum* RESIDUES AND SOIL UNDER *Polygonum* STANDS ON GROWTH OF NITROGEN-FIXING BACTERIA[a]

Fatty acid salt	Concentration (ppm)	Test organism[b]					
		R 10314	R 4400	R 10319	R 10324	A 9104	A 9043
Sodium myristate	5	0.00[c]	0.00	0.16	0.00	1.20	0.13
	10	0.00	0.00	0.00	0.00	0.66	0.26
	30	0.00	0.00	0.00	0.00	1.00	0.30
	50	0.00	0.00	0.16	0.00	1.33	0.30
Sodium palmitate	5	0.00	0.66	0.00	0.16	0.66	0.20
	10	0.00	0.66	0.00	0.16	0.66	0.30
	30	0.00	0.83	0.00	0.66	2.00	0.40
	50	0.00	0.83	0.00	0.66	2.00	0.40
Sodium linolelaidate	5	1.66	0.33	0.00	0.00	0.80	0.80
	10	1.66	0.33	0.00	0.00	1.23	1.23
	30	2.33	1.00	2.00	2.00	2.66	2.00
	50	3.33	3.00	2.66	3.33	5.30	4.30
Sodium oleate	5	0.00	0.00	0.00	0.00	0.80	0.60
	10	0.00	0.00	0.33	0.00	1.00	0.50
	30	0.00	0.00	0.33	0.30	1.80	2.00
	50	0.00	0.00	1.50	0.33	2.50	1.66
Sodium stearate	5	0.00	0.00	0.00	0.00	0.66	0.80
	10	0.00	0.00	1.66	0.06	0.66	0.80
	30	0.00	0.00	2.33	0.06	3.80	0.80
	50	0.00	0.00	3.00	0.06	3.00	1.00
Sodium arachidate	5	0.00	0.00	0.00	0.00	0.20	0.60
	10	0.00	0.20	0.00	0.00	0.20	0.60
	30	0.00	0.33	0.00	0.00	0.50	0.73
	50	0.00	0.33	0.00	0.00	0.50	1.06

Sodium 11,14-eicosadienoate	5	0.00	0.33	0.00	0.00	0.50	0.66
	10	0.00	0.33	0.00	0.00	0.66	0.66
	30	3.33	2.66	0.00	3.33	2.14	2.00
	50	6.33	3.66	0.00	6.50	2.14	2.00
Sodium heneicosanoate	5	0.00	0.33	0.00	0.00	0.00	0.00
	10	0.20	0.60	0.50	0.00	0.50	0.10
	30	0.20	2.00	0.50	0.00	0.60	0.20
	50	0.20	2.30	0.45	0.00	0.80	0.20
Sodium behenate	5	0.00	0.00	0.00	0.00	0.20	0.73
	10	0.20	0.00	0.00	0.00	0.20	0.73
	30	0.20	0.00	0.00	0.00	0.50	1.06
	50	0.20	0.00	0.00	0.00	0.00	1.06

[a]Control had no inhibition.
[b]Symbols: R, *Rhizobium;* A, *Azotobacter.* Numbers by these are ATC strain designations.
[c]Each figure is mean radius (mm) of inhibited zone of three trials.

seedlings was significantly inhibited only by some or all concentrations of four of the salts, palmitate, linolelaidate, stearate, and 11,14-eicosadienoate.

Seed germination of *Cynodon* was inhibited only by some or all concentrations of palmitate, linolelaidate, oleate, and stearate (Table 1). Germination was appreciably stimulated by the lower concentrations of the five remaining compounds.

Growth of test strains of both free-living nitrogen fixers, *Azotobacter vinelandii* and *A. chroococcum*, was inhibited by all test concentrations of all the salts except the 5 ppm concentration of heneicosanoate (Table 2). There was considerable variability, however, in the growth response of the test strains of the four species of *Rhizobium*, the symbiotic nitrogen-fixer on legumes (Table 2). Every test compound inhibited growth of at least one strain of *Rhizobium* and some or all concentrations of linolelaidate inhibited all test strains. Some or all concentrations of 11,14-eicosadienoate and heneicosanoate inhibited growth of three test strains of *Rhizobium* and some or all concentrations of palmitate, oleate, and stearate inhibited growth of two strains.

DISCUSSION

We reported previously that *Polygonum aviculare* invades heavy stands of bermudagrass rapidly, due at least in part to allelopathic effects of *Polygonum* (AlSaadawi and Rice, 1982a). *P. aviculare* was also found to be allelopathic to selected crop plants. Four phenolic phytotoxins were isolated from *P. aviculare* residues and soil under this species and found to inhibit growth of *Chenopodium album* and selected strains of the nitrogen-fixing bacteria, *Azotobacter* and *Rhizobium* (AlSaadawi and Rice, 1982b).

Our present results reveal that several fatty acids are also present in *P. aviculare* residues and soil under this species but are not present in soil under bermudagrass. Moreover, all nine isolated fatty acids significantly inhibited growth of bermudagrass seedlings even in the low concentration of 5 ppm. Moreover, all isolated fatty acids significantly inhibited growth of at least some test strains of the free-living nitrogen fixer, *Azotobacter*, and the symbiotic nitrogen fixer, *Rhizobium*. Thus, these allelochemics could have both a direct and indirect effect on growth of bermudagrass and crop species. The combinations of fatty acids and phenolic inhibitors undoubtedly have greater retarding effects on growth than either class of inhibitors alone.

It is significant that the fatty acids with two double bonds were most inhibitory to growth of *Rhizobium* and generally to *Azotobacter*. These results agree with those of Spoehr et al. (1949) and McCracken et al. (1980) described briefly below. No such relationship was found, however, in the inhibition of seed germination and seedling growth of bermudagrass. The

saturated fatty acids appeared to be just as inhibitory as the unsaturated ones in this case.

All of the fatty acids which occurred in the soil under a stand of *P. aviculare* occurred in the *Polygonum* residues also. This suggests that these fatty acids were produced by the *Polygonum* and not by microorganisms involved in the decomposition of the residues. If they were produced by microbial action, substrates from *P. aviculare* were evidently required for their production because no fatty acids were found in soil under the adjacent stand of bermudagrass.

It is noteworthy that nine fatty acids were still present in the *Polygonum* residues four months after death of the *P. aviculare* plants and seven of the fatty acids were still present in the soil. This indicates that these fatty acids are relatively resistant to decomposition and remain in the soil long enough to affect plant and microbial growth, at least early in the following growing season.

It has been known for many years that the allelochemics produced by an alga, *Chlorella*, are fatty acids (Spoehr et al., 1949). These researchers reported that the bacterial inhibitors produced by *Chlorella* are primarily unsaturated fatty acids of the C_{16} and C_{18} series, and these are active only after photooxidation resulting in fatty acids with 12 or fewer carbons. Proctor (1957) suggested that the inhibitor of other algae produced by *Chlamydomonas reinhardi* is a long-chain fatty acid or a mixture of such acids. This was confirmed by McCracken et al. (1980), who identified fifteen fatty acids ranging in length from 14 to 20 carbons. Seven of the identified compounds were tested against four algal genera, and it was concluded that unsaturated compounds were most inhibitory and that toxicity generally increased with an increase in double bonds.

A few short-chain fatty acids have previously been implicated in allelopathic effects of some of the higher plants. Patrick (1971) reported that acetic and butyric acids are among the toxins produced during decomposition of rye residues, and Chou and Patrick (1976) found that butyric acid is produced in decomposing corn residues. Tang and Waiss (1978) reported that salts of acetic, propionic, and butyric acids are the chief phytotoxins produced in decomposing wheat straw.

Our report is the first one implicating long-chain fatty acids in the allelopathic effects of higher plants. However, Tso (1964) and Cathey et al. (1966) demonstrated that growth of a variety of plants was inhibited by spraying the plants with alkyl or methyl esters of C_8–C_{14} fatty acids and C_8–C_{10} fatty alcohols.

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CHARACTERIZATION OF A SEX PHEROMONE IN THE JIRD (*Meriones tristrami*)[1]

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Abstract—The Israeli jird (*Meriones tristrami*) is a semidesert crecitid rodent found throughout the Mid-East. Jirds use a midventral sebaceous gland to scent-mark objects, the ground, and conspecifics. Volatile compounds from the male gland extract were found to contain *n*-aliphatic acetates by thin-layer chromatography, gas chromatography, and mass spectroscopy. In a digital olfactometer, female jirds demonstrated preference for male glandular extract and many of the *n*-aliphatic acetates. Other biologically important odors were also presented to the female jirds. Behavioral observations combined with olfactory bioassays implicate the involvement of the ventral sebaceous gland in jird sexual behavior.

Key Words—Ventral sebaceous gland, gland, sex attractant, pheromone, rodent behavior, gerbils, acetates, jirds, *Meriones tristrami*.

INTRODUCTION

The ventral sebaceous gland of the Mongolian gerbil (*Meriones unguiculatus*) has been studied extensively for over 15 years by numerous investigators, yet a definitive characterization of its role in the behavior of these rodents remains somewhat elusive (see Thiessen, 1977, for review). Evidence exists illustrating the influence of male sebum in a number of different behavioral contexts. It is becoming more and more apparent that multiple cues and responses are involved. Thiessen's studies suggest that male sebum is primarily involved in male–male chemocommunication, specifically in the context of territoriality and maintenance of dominance. However, studies by Daly (1977) and by Roper and Porioudakis (1977) contradict this theory, presenting evidence that

[1]Rodentia: Gerbillinae.

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females, not males, are the "primary targets" of male scent marks. This suggests a role in gerbil sexual behavior. In the only other previously reported study on an Israeli gerbil's (*Meriones tristrami*) scent marking, Thiessen (1973) found that only males were consistently interested in male sebum.

Chemical elucidation of mammalian scent gland sociochemicals has been carried out in relatively few species (Thiessen and Rice, 1976). A number of studies on male rodents have demonstrated the importance of aliphatic acetates from the preputial gland, as sex attractants (Spener et al., 1969; Stacewicz-Sapuntzakis and Gawienowski, 1977). Gerbils do not possess preputial glands; however, aliphatic acetates are present in the male ventral sebaceous glands of at least one *Meriones* species, *M. tristrami* (Ikan and Kagan, 1979).

Chemical analysis of Mongolian gerbil sebum by Jacob and Green (1977) did not reveal the presence of aliphatic acetates. The sebaceous lipids they found included hydrocarbons (especially cholesta-3,5-diene, a compound similar to the male boar sex attractant), cholesterol esters, free fatty acids, polar lipids, and a high percentage of triglycerides. Thiessen et al. identified phenylacetic acid as the biologically active component in male Mongolian gerbil sebum (1974).

Comparative information on ventral scent gland secretions and chemistry in other gerbil species is lacking. We present here findings which suggest that male ventral scent marking is influential in attracting the wild, female semidesert crecitid, *Meriones tristrami.*

Since our preliminary observations of jirds revealed an apparent female interest in the male's gland (Ikan et al., 1981) and since aliphatic acetates (known male rodent attractants) are present in male *M. tristrami* ventral sebaceous glands, we decided on two main objectives: (1) chemical analysis of the male glandular volatiles, with emphasis on the acetate fraction; and (2) bioassay of female olfactory preferences for various glands and compounds, again concentrating on aliphatic acetates.

METHODS AND MATERIALS

Extraction and Analysis of Ventral Sebaceous Volatiles. The ventral sebaceous glands of mature males and females were excised and homogenized in absolute ethanol. The homogenate was centrifuged and the supernatant dried over anhydrous magnesium sulfate. The alcohol extract was then reduced in vacuo to a volume of 1 ml and used for chemical and biological tests. Both the extract and supernatant portions (dry) were weighed.

The extract was subjected to thin-layer chromatography separation in silica gel G plates (0.5 mm thick) and developed with hexane–ethyl acetate, 9:1. The following markers were used: cholesterol, steryl ester, tri-

glyceride, alcohol (C_{16}), free fatty acid, hydrocarbon (C_{14}), and acetate (C_{16}). Identification of the bands was carried out in iodine baths.

The TLC band corresponding to aliphatic acetates ($R_f = 58$) was scraped off the plate and extracted with methylene chloride. Evaporation of the solvent left an oily residue which was used for gas chromatographic and mass spectral analysis.

Samples of the acetates were analyzed with a Packard model 7400 GC equipped with a 1.83-m × 3.2-mm stainless steel column containing gas-chrom Q, coated with 3% SE-30. The flow rate of nitrogen was 35 ml/min and temperature programing ran from 100°C to 260°C at 6°/min. Coinjection of synthetic acetate samples confirmed acetate identities.

GC data on acetates was reconfirmed using a Dupont 21-490 chemical ionization (CI) mode mass spectrometer. Isobutane served as the reagent gas.

Animals. Fourteen females (aged 6–12 months, weight 80–95 g) were removed from the breeding population. All animals were third-generation captive and presumably were genotypically wild. Female jirds were housed in pairs in an environmentally controlled room ($T_a = 22$–$25°$, light–dark cycle reversed 13:11) next to the bioassay room. A pair of mature males was also housed in this room so that natural odors and sounds would be available to the females. About half the females had previous sexual experience, but a systematic comparison of naive versus experienced females was not done. Recently a study on the effects of experience as well as estrus state on male–female interactions has been carried out in our laboratory.

Animals were amply supplied with standard rodent pellets as well as fresh fruits and vegetables. This jird does not require free drinking water.

Materials. Two groups of test substances, consisting of a total of thirty-one different materials, were individually tested on female jirds. The first group included 22 aliphatic acetates. Alcohols purchased from Fluka (Buchs, Switzerland), were treated with acetic anhydride yielding acetates. Both alkyl (saturated) and alkenyl (unsaturated) acetates were synthesized, none of them branched.

The substances of the second group included suspect pheromonal components from other gerbils (e.g., phenylacetic acid, protoporphyrin, cholesta-3,5-diene), precursors of aliphatic acetates (e.g., alcohols C_8 and C_{16}), and various glandular homogenates and/or extracts (e.g., *Microtus geuntheri* preputial gland, jird fat tissue, male and female jird ventral sebaceous extract).

Bioassay Apparatus and Procedure. A circular olfactometer and digital recorder were designed and constructed specifically for these tests. The basic design resembles the rat olfactometer of Gawienowski's group (Stacewicz-Sapuntzakis and Gawienowski, 1977). A galvanized metal structure 50 cm high and 50 cm in diameter was built. The floor was a removable

wire grid, beneath which wood shavings were placed to absorb urine and fecal matter. At the base of the olfactometer three openings (5 × 5 cm), spaced 120° apart, allowed access to tunnels. Floors of the tunnels also had grids to allow free circulation of air. The tunnel entrances permitted an animal to insert its head 3 cm at which point a screen prevented contact (touching, licking) with the sample odorant directly behind it. A photocell placed at the entrance to each tunnel was connected with a three-channel digital LED recorder. An autostop mechanism stopped the display of either cumulative time or events for each tunnel (substance) at the end of the 5-min trials. The recorder display, photocell, and experiment room lighting were all red light (gerbil darkness).

Test chemicals and glandular homogenates and/or extracts were dissolved in ethanol which also served as the control odor. Cotton wads, soaked with 0.5 ml of test substance or control, were placed in small glass containers behind the screen of each tunnel.

The ventral gland lipid extracts were tested at one concentration, 10 mg/ml. This test quantity approximates the amount of sebaceous lipid deposited by a gerbil during one or two scent marks (Thiessen and Yahr, 1977). Preputial gland and fat tissue were presented as whole homogenates at 100 mg/ml.

Acetates were tested at two concentration levels: low (0.01 mg/ml) and high (0.1 mg/ml). These levels were chosen since they probably simulate the individual acetate amount deposited during one or two scent marks.[2]

Each animal was tested for 5 min. After each pair was tested, the entire olfactometer (including floor and tunnels) was thoroughly washed with detergent and acetone, and wood chips were changed.

Gerbil olfactory preference for compounds was tested on at least two separate occasions at each of the two concentration levels. An experimental session was split in two. The lower concentration was tested first on 8–10 females (8–9:30 AM). Then the higher concentration was tested on the same females (10–11:30 AM). The morning testing period (jird evenings) corresponds with the major activity period of this crepuscular species. Of the three tunnels, at least one contained only ethanol, but the identity of each tunnel's contents was unknown to the experimenter. The positions of the test substances were changed between low and high concentration tests.

Statistics. Since our olfactometer could not register the cumulative investigation times as well as events for each animal, we chose to record the former. In any event, it has been reported that frequency of response (events)

[2]The TLC band corresponding to acetates accounted for up to 10% of total lipid content. Since an estimated 10 mg of sebum is deposited in two scent-marking bouts, 1 mg of acetates is an amount likely to be found in a natural setting. GC analyses showed the relative amounts of most individual acetates to be 1–10% of the total acetate fraction. Therefore, a male jird probably secretes 0.01–0.1 mg of each acetate during scent marking. This calculation led to the choice of the two concentrations used in behavioral testing.

is not a good measure of preference in rodents due to their spontaneous alternating behavior (Schultz, 1973).

The data was analyzed by the nonparametric Wilcoxon signed rank test. Control versus odorant were compared by a two-tailed test (attraction or repulsion versus indifference) (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

Chemical Analysis. Each male gland contains about 25 mg of extractable lipids. This is about 30% less than the amount of lipids recovered from Mongolian gerbil ventral pads. TLC of jird lipids demonstrated the presence of steryl esters ($R_f = 0.95$), hydrocarbons ($R_f = 0.91$), acetates ($R_f = 0.58$), triglycerides ($R_f = 0.47$), free fatty acids ($R_f = 0.29$), alcohols ($R_f = 0.25$), and cholesterol ($R_f = 0.19$). Acetates accounted for up to 10% of the total lipid content.

Figure 1 represents the typical GC pattern of male jird ventral sebaceous acetates. C_{16} and C_{24} compounds were the most abundant. Mass spectroscopy analysis confirmed the identity and presence of at least 21 saturated and unsaturated aliphatic acetates.

Acetates were not detected in Mongolian gerbil ventral pads by Jacob

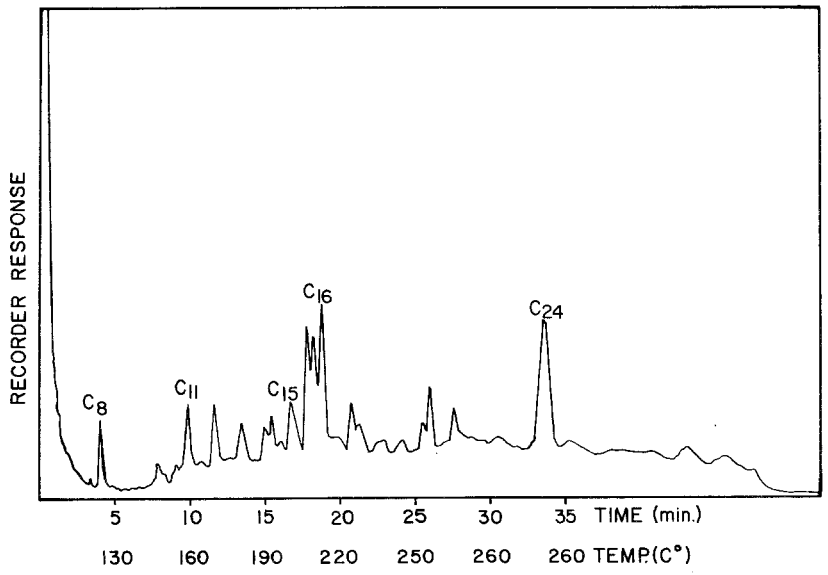

FIG 1. Gas chromatograph of jird ventral sebaceous aliphatic acetates. Column, 1.83 m × 3.2 mm of gas-chrom Q coated with 3% SE-30; N_2 flow rate, 35 ml/min. Temperature programing, 100–260°C at 6°/min.

et al. (1977). We have confirmed this absence (glands acquired from Thiessen) under identical chemical procedures to those used on *M. tristrami*. Recently we determined that Harderian gland secretions of the two *Meriones* species are chemically dissimilar as well. The chemical disparity found between these two species' glands provides further evidence for species specificity in mammalian pheromones, even within closely related members of a genus.

As noted earlier, aliphatic acetates have been identified as important components of other male rodent attractant pheromones. The lipid fraction from male mouse preputial glands (strain CD-1 and CD-57) is comprised of about 5% acetates (Spener et al., 1969). Acetate content of female glands was considerably lower. Interestingly, the major constituent of alkyl acetates was hexadecyl (C_{16}). As noted, we found this compound to be dominant in jird glandular acetates. Gawienowski and coworkers (Stacewicz-Sapuntzakis and Gawienowski, 1977) also found aliphatic acetates in rat preputial glands. Their subsequent behavioral testing showed that preputial gland extract from males attracted females and that acetates were primarily responsible for the attractance.

Behavioral Responses. Normally the jirds investigated one tunnel entrance after another, sniffing and sometimes trying to gain access to the test substance. Most of the investigation seemed to be within the first 3 min. Typical response times during a 5-min test were about 5–10 sec

TABLE 1. FEMALE JIRD OLFACTORY RESPONSES TO ALIPHATIC ACETATES[a]

Saturated Acetates			Unsaturated Acetates		
	Low	High		Low	High
C_6	+	+	$C_{11:1_{\Delta}}10$		
C_8	+++		$C_{14:1_{\Delta}}9$	+++	++++
C_9		+	$C_{14:2_{\Delta}}9,11$		++
C_{10}		++	$C_{16:1_{\Delta}}9$	++++	
C_{11}			$C_{16:2_{\Delta}}7,11$		
C_{12}			$C_{18:1_{\Delta}}9$	+++	
C_{13}		+	$C_{18:2_{\Delta}}9,12$	++++	
C_{14}			$C_{20:4_{\Delta}}5,8,11,14$		
C_{15}					
C_{16}	+	+			
C_{17}	−				
C_{18}					
C_{22}	+				
C_{24}	++	+			

[a]The signs (+) and (−) mark attraction and avoidance, respectively. Lack of a sign indicates indifference toward the compound. Probabilities were determined using T_s values obtained from the Wilcoxon signed rank test. +/−, $P < 0.1$; ++/−−, $P < 0.05$; +++/−−−, $P < 0.025$; ++++/−−−−, $P < 0.01$. Low concentration, 0.01 mg/ml; high concentration, 0.1 mg/ml.

TABLE 2. FEMALE JIRD OLFACTORY RESPONSES TO VARIOUS SUBSTANCES[a]

Ventral sebaceous extract (♂) (10 mg/ml)		++++
Ventral sebaceous extract (♀) (10 mg/ml)		+
Fat tissue (100 mg/ml)		
Preputial gland homogenate (♂ *Microtus*) (100 mg/ml)		
	Low	High
Alcohol (C_8)		
Alcohol (C_{16})		
Phenylacetic acid	++	
Protoporphyrin		
Cholesta-3,5-diene		

[a]See footnote to Table 1.

for control (i.e., no attractance or repulsion) and anywhere from 15 to 30 sec for substances that demonstrated attractance.

Tables 1 and 2 list the test compounds and indicate aversion or attraction in terms of statistical significance between control and sample.

Male gland extract was the only test compound that was consistently and clearly attractive to females. This response was evident in all preliminary testing and in all systematic olfactometer tests. Female gland extract barely met statistical criteria for attractance, and that in only one test session.

Approximately half the acetates tested were attractive to the females. However, rating of the acetates according to strength of attractance could not be achieved since so much individual variability, with respect to behavioral response, was observed. One acetate (C_{17}) seemed to be mildly aversive to females. This compound was conspicuously absent from the sebaceous acetates identified both by GC and MS.

Our results on female jird olfactory response to acetates are similar to those reported on female rats (Stacewicz-Sapuntzakis and Gawienowski, 1977). Most of the unsaturated acetates were biologically active in both studies. This suggests that bonding and not carbon number may be the determinant for attractance.

No chemical analysis of microtine preputial glands has been carried out to date. While it is likely that acetates are present in microtine preputial secretions, the type and quantity probably differ from jird ventral sebaceous acetates. Male microtine preputial gland homogenate showed no attractive qualities when offered to female jirds.

In the experiment reported here, neither cholesta-3,5-diene nor the two alcohols (precursors of acetates) elicited investigatory response in female *M. tristrami*. Phenylacetic acid did elicit female jird exploratory re-

sponse in one test session but not in others. These experiments therefore do not clarify the significance or species specificity of this substance.

Another possible gerbil sociochemical, protoporphyrin (present in Harderian gland secretions), was presented to the female jirds. However, it did not elicit interest or grooming, the response demonstrated when Mongolian gerbils and Israeli jirds are presented with the whole secretion (Kagan et al., 1982; Thiessen and Yahr, 1977).

Female jird olfactory responses to compounds varied somewhat at different concentrations and on different test days. In general, responses to acetates declined after initial exposure. It is quite likely that some animals began to habituate to the chemical cues since they failed to predict, or be associated with, a subsequent interaction with a male. Also it must be remembered that female mammals' olfactory physiology is altered by the estrous cycle. Technically, it is quite difficult to determine the estrous state of jirds, but in all likelihood the females were in estrous synchrony since they were housed in the same room. Despite this, individual differences in responses were evident. Recently we have found that ovariectomized jirds are even more interested in male glands (in vivo) when in artificially induced estrus (Kagan, 1982) but this contradicts Thiessen's findings in the Mongolian gerbil (Thiessen and Yahr, 1977). Finally, the significance of previous sexual experience on olfactory performance cannot be ignored. While this study was not designed to test this, it did seem that experienced females were more interested in the acetates and male glandular extract. In conclusion, we agree with Thiessen that gerbils demonstrate considerable plasticity to conspecific odors (Thiessen and Yahr, 1977).

Our results on *M. tristrami* seem to disagree with those of Thiessen et al (1973) with respect to a female attractant function for the male sebum. In that study, intact males but not females showed consistent interest in male sebum. Those findings were consistent with Thiessen's results for Mongolian gerbils. It is interesting to note that in comparing *M. tristrami* with *M. unguiculatus*, our findings are both similar and supportive to those of Roper and Porioudakis (1977) and Daly (1977). They suggest that male gerbil ventral marking is primarily, but not exclusively, involved in intersexual communication. However, the comparative picture is complicated by the fact that all studies use different bioassay techniques and, as previously stated, *Meriones* sebum is not chemically homogeneous between species. Furthermore, the flexibility of chemosignals in differing natural environments and social situations presents a serious limitation to any definitive conclusions.

In the research reported here, we did not investigate the almost certain involvement of male sebum in male-male chemocommunication. Our concern was restricted to revealing and identifying male attractants. We suggest that *n*-aliphatic acetates are major components of the male ventral gland pheromone and that they elicit sexual interest. Other sebum compounds may have similar and/or additional qualities.

Whether the investigatory interest shown by females should be termed sexual attractance is a question of semantics. The fundamental inference is that male glandular secretions, which contain aliphatic acetates, may function by bringing the sexes together (aggregation). Obviously, this is a crucial and necessary first step in successful reproduction. It is likely that learning (previous experience) and reproductive status play important roles in defining the significance of these chemical cues.

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IDENTIFICATION OF SEX PHEROMONE COMPONENTS OF THE FEMALE DRIEDFRUIT MOTH, *Vitula edmandsae serratilineella*,[1] AND A BLEND FOR ATTRACTION OF MALE MOTHS

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Abstract—Sixteen pheromone-like compounds were identified in abdomen tip washes and excised abdomen tip extracts of calling females of driedfruit moth, *Vitula edmandsae serratilineella* Ragonot. Identifications were by high-resolution gas chromatography–mass spectrometry, and gas chromatography with flame ionization and electroantennographic detectors. Male moths were attracted to a blend of (*Z*)-9,(*E*)-12-tetradecadienol and (*Z*)-9,(*E*)-12-tetradecadienal in a ratio of 100:1 at 500 μg/rubber septum dispenser, which is recommended for monitoring purposes. Low concentrations of (*Z*)-9-tetradecenol (0.5%) and (*Z*)-9-tetradecenal (0.1%) may be beneficial for the attraction of males, but 1–2% of (*E*)-9,(*E*)-12- or (*Z*)-9,(*Z*)-12-tetradecadienol, or (*Z*)-9,(*E*)-12-tetradecadienyl acetate inhibited their attraction. Gravid female moths were attracted to traps that captured large numbers of males. Females may be attracted to male hairpencil or forewing gland secretions emitted near the traps or that accumulate in the traps.

Key Words—Driedfruit moth, *Vitula edmandsae serratilineella*, Lepidoptera, Pyralidae, Phycitinae, sex pheromone, attractant inhibitor, (*Z*)-9,(*E*)-12-tetradecadienol, (*Z*)-9,(*E*)-12-tetradecadienal, alcohol, aldehyde.

INTRODUCTION

Larvae of the driedfruit moth, *Vitula edmandsae serratilineella* Ragonot, occur throughout North America as scavengers in bumble bee nests and they are known pests of honeybee combs, almonds, and dried fruits (Milum,

[1]Lepidoptera: Pyralidae, Phycitinae.

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1953; Okumura, 1966). Their threat to dried fruits may have been underestimated because damage was attributed erroneously to larvae of other closely related species such as the Indian meal moth, *Plodia interpunctella*, and the Mediterranean flour moth, *Anagasta kuehniella* (Okumura, 1966).

Driedfruit moth infestations were discovered recently in leafcutter bee hives and bee workshop–storage buildings (hereafter referred to as bee shops). Larvae invade the hive tunnels where they destroy the bee larvae and cocoons, and damage the polystyrene nesting material to the extent that it cannot be used again (Richards, 1983).

A sex pheromone monitoring or control system for the male moths of this pest would be advantageous. Weatherston and Percy (1968) reported evidence of a female sex pheromone, and Grant (1976) described the adult courtship behavior, but the chemical composition of the sex pheromone has not been described.

We report the identification of components isolated from the sex pheromone glands of female moths, a blend for attraction of male moths in bee shops, and attraction of female moths to traps that captured large numbers of males.

METHODS AND MATERIALS

Pheromone Recovery and Bioassay. Larvae were collected from leafcutter bee hives and fed on pollen and nectar from the hives until they pupated (Richards, 1983). Pupae were separated according to their sex. Unmated female moths, 1–4 days old, exhibited calling behavior (Weatherston and Percy, 1968) during a 2-hr simulated predawn (16L:8D, 20°–25°C, and 60–70% relative humidity) when the light intensity was increased from darkness to 4.3 lux.

Pheromone wash was recovered from calling females by washing their everted abdominal tips with *n*-hexane (BDH Omnisolv, glass distilled, and passed through silica gel before use). Pheromone extract was recovered from washed abdomen tips which were excised and allowed to stand in *n*-hexane for ca. 5 days before the solvent was decanted. Pheromone washes and extracts from 100–300 females (4000 females were used) were filtered and reduced to an appropriate volume under N_2 [0.5 female equivalents (FE)/μl] for analyses without further clean-up.

Bioassays were done with 1- to 6-day old male moths in a tube-type olfactometer (Struble et al., 1980a). Males were sexually responsive during a 2-hr simulated predawn period under the same conditions described for calling females. For bioassay purposes, abdomen tip washes and extracts at 1–2 FE, and synthetic compounds at 1 ng to 1 μg were placed on 1-cm strips of filter paper which were held in the air inlet of each olfactometer

tube for 10-sec exposures to the males. The air velocity in the olfactometer tubes was 12 cm/sec.

Instrumentation. Gas chromatography (GC) analyses were done with a Hewlett-Packard model 5830 using a flame ionization detector (FID), and with a modified Varian model 2700 with a FID and an electroantennographic detector (EAD) (Arn et al., 1975; Struble, 1983).

Mass spectra were recorded with a Hewlett-Packard model 5985B GC-mass spectrometer (GC-MS) using electron ionization (EI) and chemical ionization (CI) with isobutane as reactant gas.

Borosilicate glass capillary columns treated by the method of Grob et al. (1977), were coated by the dynamic method with Carbowax 20 M TPA (0.24 μm film thickness; 15, 28, and 61 m long) or SP-2340 (0.06 μm film thickness; 15, 28, and 75 m long). Columns were 0.25 mm ID for GC and 0.32 mm ID for GC-MS analyses. Helium carrier gas linear flow velocity was 30 cm/sec for all analyses, and various temperature programs were used with splitless injections.

Chemicals. Monounsaturated acetates and alcohols were either synthesized here (Struble and Swailes, 1975) or purchased from Farchan Research Laboratories and ChemSampCo., now a division of Albany International. A sample of *Z*9,*E*12–14:Ac was purchased from ChemSampCo. All compounds were purified to > 99% by argentation chromatography (Houx et al., 1974). Purified *Z*9,*E*12–14:Ac was hydrolyzed with methanolic KOH to *Z*9,*E*12–14:OH, a portion of which was oxidized with pyridinium chlorochromate (Corey and Suggs, 1975) to yield *Z*9,*E*12–14:ALD. The aldehyde was purified (> 99%) by chromatography on silica gel. Other aldehydes were prepared in a similar manner.

Purified (> 99%) samples of four isomers of 9,12–14:Ac and the corresponding alcohols were also purchased from Dr. S. Voerman, Laboratory for Research on Insecticides, Wageningen, The Netherlands.

Pheromone Entrainment. Female effluvium was collected on two glass bead filters (40–60 mesh, 15 g/filter) similar to those described by Weatherston et al. (1981). Females (20–200) were placed in a glass chamber (5 liter, modified desiccator) before the beginning of the dark period. Charcoal-filtered air was humidified and passed through the chamber at a rate of 1 liter/min for 3 hr during the predawn period. Volatiles adsorbed on the glass bead filters were recovered with *n*-hexane and concentrated for analyses.

Pheromone Acetylation. A pheromone wash from 443 females containing 1125 ng of the main pheromone component was evaporated (N_2) to dryness and reacted with acetic anhydride (50 μl) in pyridine (100 μl) at room temperature overnight. The reaction mixture was worked-up in the usual manner (Sower et al., 1974) and was analyzed without further clean-up.

Pheromone Ozonolysis and Epoxidation. A pheromone wash from

182 females was partially purified by liquid chromatography on silica gel (60 mg, Bio-Sil® HA, 325 mesh, Bio-Rad Laboratories); 2-ml eluates of *n*-hexane, *n*-hexane–10% CH_2Cl_2, and *n*-hexane–20% CH_2Cl_2 were discarded; and the main alcohol components were eluted with *n*-hexane–50% CH_2Cl_2. This fraction was acetylated as described above and chromatographed again on silica gel, and the acetates of interest were eluted with *n*-hexane–15% CH_2Cl_2. This fraction was evaporated (N_2), 70 FE was diluted with 30 μl of *n*-hexane for ozonolysis and 112 FE was diluted with 40 μl of CH_2Cl_2 for epoxidation.

The *n*-hexane solution was cooled (−78°C) and ozonized in two steps (Beroza and Bierl, 1967). The product, without any reducing agent, was analyzed immediately by GC using a 28-m Carbowax 20 M TPA column with splitless injection (0.5 min) at 50°C for 0.6 min, 20°/min to 120°C, and 2°/min to 175°C. The initial ozonolysis product contained 9-acetoxynonanal (19.56 min), and the acetylated pheromone components (*Z*)-9-tetradecenyl acetate (*Z*9–14:Ac) (19.83 min) and *Z*9,*E*12–14:Ac (22.72 min) were 100% and 80% reacted. The sample was ozonized again and GC analysis showed an increase in 9-acetoxynonanal and complete reaction of *Z*9,*E*12–14:Ac. The structure of 9-acetoxynonanal was confirmed by GC-MS, CI (isobutane); the base peak was the 201 ion (M+1).

The CH_2Cl_2 solution of acetylated pheromone was diluted with 40 μl of CH_2Cl_2 containing *m*-chloroperbenzoic acid (100 ng/μl). Progress of the reaction was followed by GC using a 15-m Carbowax 20 M TPA column with splitless injection (0.5 min) at 50°C for 0.6 min, 30°/min to 140°C, and 6°/min to 200°C. The retention times (min) of the reactants and products were: *Z*9–14:Ac, 7.4; *Z*9,*E*12–14:Ac, 8.3; *cis*-9,10-epoxy-14:Ac, 11.8; *trans*-12,13-epoxy-*Z*9–14:Ac, 12.2; *cis*-9,10-epoxy-*E*–14:Ac, 12.8. The reaction was stopped after 48 hr at room temperature, excess *m*-chloroperbenzoic acid was removed by filtering through 20 mg of activity three neutral alumina (Bierl-Leonhardt et al., 1980; Tumlinson et al., 1974). Authentic samples of the epoxides were prepared in a similar manner from the corresponding acetates.

Moth Trapping. Male moths (ca. 10,000 during 4 months) were released in a controlled environment flight room (2.3 × 2.3 × 3 m, 20–24°C, 50–60% relative humidity, 16 hr light–8 hr dark). Four traps, plastic funnels (17-cm diam.) with plastic bags (46 × 80 cm) attached, were placed 1.5 m from the floor and 1 m from each corner of the room. Traps were baited with chemical blends on red rubber septa (A.H. Thomas Co., catalog No. 8753-D22), unmated female moths (1♀/trap), or excised female abdomen tips (6/trap). One unbaited trap was used throughout each test, moth catches were recorded, and the relative trap positions were changed daily.

Traps made from 4-liter plastic pails, containing 1 liter of water and 10 ml of mineral oil, were used in bee shops near Brooks, Alberta (see Tables 1 and 3). The shops were irregular shapes and 10 baited traps were evenly

spaced around the interior of each shop with one unbaited trap in the center of the area. Treatments on red rubber septa were randomized within each shop, their relative positions were changed at least once/week, and captured moths were recorded and removed weekly. Each shop was considered a replication.

Analyses of variance were done on the data and treatment means were

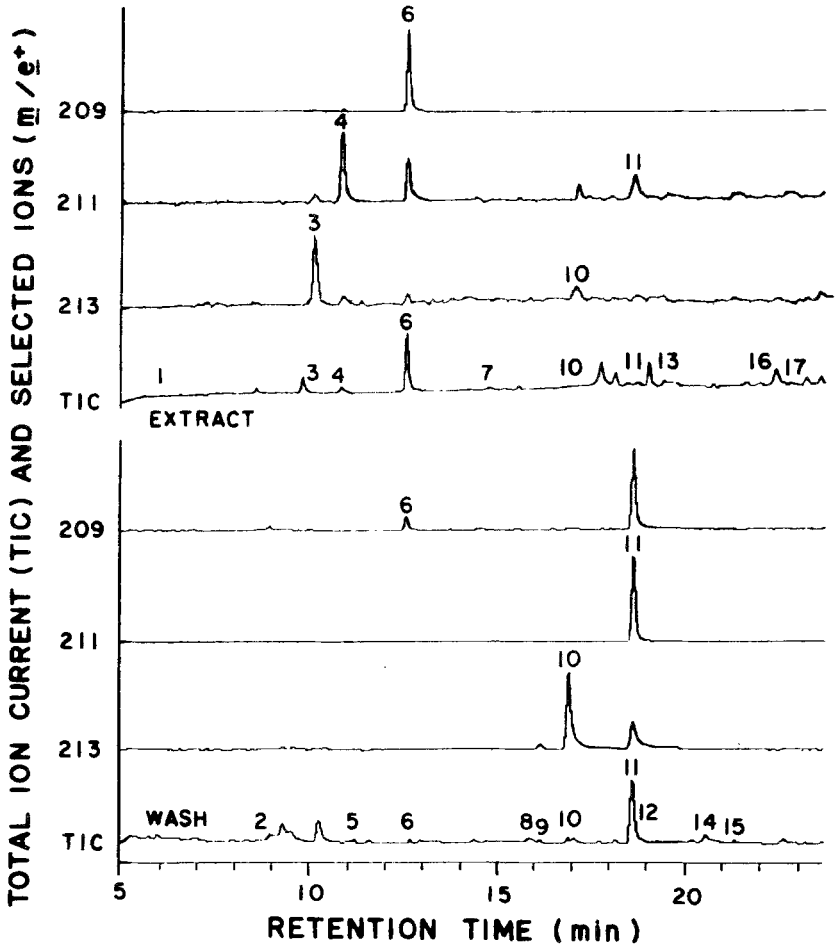

FIG. 1. Total ion chromatograms, CI isobutane, and selected ion mass chromatograms of the main pheromone components of a female abdomen tip extract and wash. Ten FE was injected on a 28-m Carbowax 20 M TPA capillary GC-MS column. The components were: (1) 12:ALD; (2) 12:Ac; (3) 14:ALD; (4) Z9-14:ALD; (5) 12:OH; (6) Z9,E12-14:ALD; (7) 16:ALD; (8) Z9-16:ALD; (9) 14:OH; (10) Z9-14:OH; (11) Z9,E12-14:OH; (12) 16:Ac; (13) 18:ALD; (14) 16:OH; (15) Z9-16:OH; (16) Unknown; (17) 18:Ac.

compared by Tukey's or Duncan's new multiple-range tests (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

GC and GC-MS Analyses. Typical reconstructed total ion chromatograms of an abdomen tip wash and extract are illustrated in Figure 1. The corresponding mass chromatograms, based on M+1 ions, for the main pheromone components 3, 4, 6, 10, and 11 are also illustrated. Total ion chromatogram data were scanned in a similar manner for C_{10}–C_{18} saturated, mono-, di-, and triunsaturated aldehydes, alcohols, and acetates, and the detectable compounds of this series are numbered in Figure 1. Complete CI differential mass spectra were obtained for all of the components, except 16, and they were comparable to those of authentic samples. These components also cochromotographed with authentic samples on GC columns of Carbowax 20 M TPA (15 m and 61 m) and SP-2340 (28 m and 75 m) under various temperature program conditions. Abdomen tip washes contained an average of 2.5 ng/FE of component 11, based on GC and GC-MS analyses. The structure of components 1–17 and their quantities relative to component 11 in typical abdomen tip washes and extracts were: (1) 12:ALD, ND, <1; (2) 12:Ac, <1, ND; (3) 14:ALD, ND, <1; (4) *Z*9–14:ALD, ND, 8; (5) 12:OH, <1, ND; (6) *Z*9,*E*12–14:ALD, 5, 87; (7) 16:ALD, <1, 3; (8), *Z*9–16:ALD, <1, ND; (9) 14:OH, 5, <1; (10) *Z*9–14:OH, 8, 1; (11) *Z*9, *E*12–14:OH, 100, 10; (12) 16:Ac, 4, <1; (13) 18:ALD, ND, <1; (14) 16:OH, 10, <1; (15) *Z*9–16:OH, 3, <1; (16) unknown, <1 < 1; (17) 18:Ac, ND, <1.

The selected ion mass chromatograms and corresponding data obtained by GC-MS analyses on a 75-m SP-2340 column showed that *Z*9,*E*12–14:OH and *Z*9,*E*12–14:ALD were the only isomers of these components detectable in the pheromone washes and extracts. The four geometrical isomers of 9,12–14:OH and 9,12–14:Ac were completely resolved on the SP-2340 column, but all of the corresponding aldehydes were not available. There was no evidence of conjugated dienes or any triene components in the pheromone. Component 16 may be a nonconjugated hexadecadienol, but there was insufficient quantity to obtain a complete mass spectrum, and its structure is unknown.

The alcohol components of an abdomen tip wash were acetylated, and GC and GC-MS analyses confirmed the presence of the expected corresponding acetates. *Z*9,*E*12–14:Ac was the only C_{14} diene acetate detectable.

Partially purified acetylated pheromone wash was ozonized in two steps. GC analysis of the initial ozonolysis products showed that *Z*9–14:Ac was completely reacted along with 80% of *Z*9,*E*12–14:Ac, and the ex-

pected ozonolysis product, 9-acetoxynonanal, was readily detectable. The initial reaction mixture was ozonized again, and GC analyses showed that $Z9,E12$–14:Ac was completely reacted and the quantity of 9-acetoxynonanal increased. This supported the positional isomeric assignments of a double bond at C_9 in components 10 and 11. The retention times of component 11 and its corresponding acetate indicated that it was a nonconju-

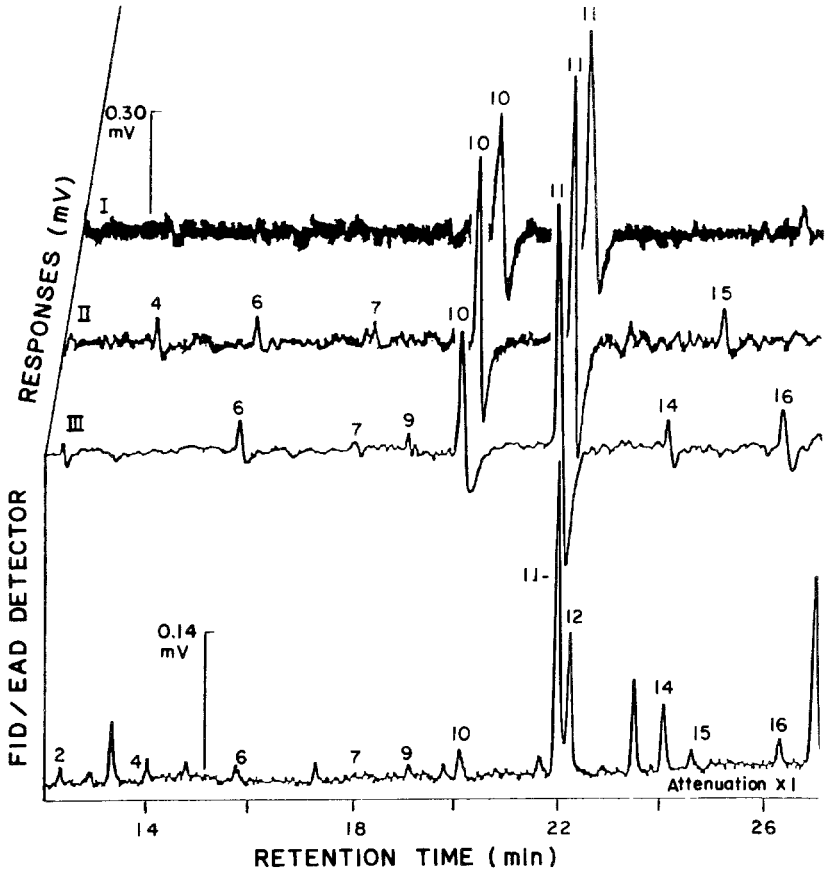

FIG. 2. EAD-FID chromatograms with detector antennae from males of: I. sunflower moth; II. F_1 hybrid of *Euxoa tessellata* (male) and *E. plagigera* (female); and III. driedfruit moth. EAD and FID responses were recorded simultaneously with one detector antenna, and the EAD responses were combined and offset. Two FE of driedfruit moth abdomen tip wash was injected on a 28-m Carbowax 20 M TPA capillary column, and the components are numbered as in Figure 1. GC conditions were: splitless injection at 50°C for 0.3 min, 25°/min to 110°C, 4°/min to 205°C; EAD-FID split ratio 1:3.

gated diene. Since the ozonolysis data showed that one double bond was at C_9, the only possible positions for the second double bond would be at C_{12}, or in the terminal position, C_{13}. Therefore, the positional isomeric assignment of 9,12 must be correct, as a 9,13-isomer would be expected to have a longer retention time.

Partially purified acetylated pheromone wash was epoxidized, and GC and GC-MS analyses showed the expected *cis*-9,10-epoxy-14:Ac, *trans*-12,13-epoxy-*Z*9-14:Ac, and *cis*-9,10-epoxy-*E*12-14:Ac were in a range of 60–80% yields. EI and CI mass spectra of the three compounds were consistent with those of authentic samples and with the fragmentations described by Bierl-Leonhardt et al. (1980) and Tumlinson et al. (1974). These data were consistent with the positional isomeric assignments for components 10 and 11. The epoxidation of component 6, *Z*9,*E*12-14:ALD, was not attempted.

EAD-FID Analyses. EAD-FID chromatograms of a typical abdomen tip wash are illustrated in Figure 2. Driedfruit moth male antennae (III) gave consistent, major responses to pheromone components 6, 10, and 11 with minor responses to components 2, 7, 9, 14, and 16. EAD-FID chromatograms of abdomen tip extracts had major responses to components 4, 6, 10, and 11. Components 4 and 6, *Z*9-14:ALD and *Z*9,*E*12-14:ALD, were at a greater concentration in the abdomen tip extracts than in the washes (Figure 1). EAD responses of similar intensities were recorded to the corresponding synthetic chemicals, which helped confirm the structural assignments (Figure 3).

Similar EAD-FID data were obtained for pheromone washes and extracts and synthetic chemicals on a 28-m column of SP-2340, where the relative retention times of the various components were different.

Structural assignments of components 4, 6, 7, 10, 11, and 15 were further confirmed by a special EAD technique in which the detector antennae were from males of other species that were known to have specific responses to some of these components (Struble et al., 1980b). EAD data with detector antennae from the sunflower moth, *Homoeosoma electellum* (I), and the F_1 hybrid of *Euxoa tessellata* (male) and *E. plagigera* (female) (II) are included in Figure 2.

Sunflower moth antennae were known to respond specifically to *Z*9-14:OH and *Z*9,*E*12-14:OH (Underhill et al., 1979). They gave strong responses to components 10 and 11 in the driedfruit moth washes and extracts (Figure 2, antenna I). F_1 hybrid antennae were known to respond specifically to *Z*9-14:ALD, *Z*9-14:OH, *Z*9,*E*12-14:ALD, *Z*9,*E*12-14:OH and *Z*9-16:OH (Byers and Struble, unpublished results) and responded to components 4, 6, 10, 11, and 15 (Figure 2, antenna II).

These data were all consistent with the structural assignments of components 1–15 and 17. The main pheromone components in the abdomen

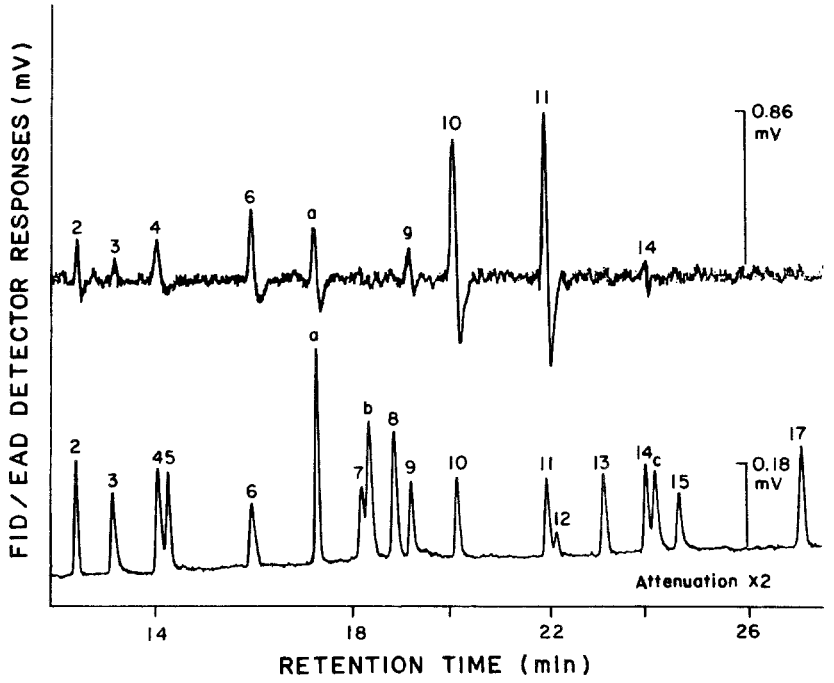

FIG. 3. EAD-FID chromatograms with driedfruit moth male antennal responses to 1–2 ng of synthetic compounds, numbered as in Figure 1 and injected under the same conditions as in Figure 2. Additional compounds were: (a) 14:Ac; (b) *Z*9,*E*11–14:ALD; and (c) *Z*9,*E*11–14:OH.

tip washes were *Z*9,*E*12–14:OH and *Z*9–14:OH, with the corresponding aldehydes in the extracts.

Female Effluvium. Female effluvium was collected on glass bead filters and EAD-FID analyses with driedfruit moth males as detector antennae showed *Z*9,*E*12–14:OH as the only detectable component. It was concluded that the females released this as their main pheromone component.

Olfactometer Behavior Tests. Male moths exhibited strong sexual behavior, which included flight to the source, extended claspers and attempted copulations when exposed to calling females, excised abdomen tips, pheromone washes or extracts, or selected synthetic chemicals. *Z*9,*E*12–14:OH at 10 ng/test elicited strong sexual responses from the males. However, it was not possible to differentiate conclusively among male responses elicited by two- to four-component blends involving *Z*9,*E*12–14:OH with *Z*9–14:OH and the corresponding aldehydes. There were essentially no responses to the other isomers of 9,12–14:OH, *Z*9,*E*11–14:OH, *E*12–14:OH and the corresponding acetates.

These tests helped establish that *Z*9,*E*12-14:OH was the primary (Roelofs and Cardé, 1977) pheromone component. The olfactometer was useful in determining when the females were emitting pheromone.

Attractancy Tests in a Flight Room and in Bee Shops. Initial attractancy of males to various combinations of the main pheromone components, *Z*9,*E*12-14:OH, *Z*9-14:OH and the corresponding aldehydes, was determined in a flight room where males could be effectively trapped and their behavior observed. During a 2-hr simulated sunrise they approached traps baited with 10, 25, or 100 μg of *Z*9,*E*12-14:OH, but they hovered about 20 cm from the dispensers and few moths were captured. Addition of *Z*9-14:OH and the corresponding aldehydes, at ratios similar to those in the female washes, resulted in the capture of more moths. Many males approached the dispensers directly, where they exhibited prolonged sexual responses. Blends that elicited the strongest behavioral responses in these flight room tests were included in the initial attractancy tests in bee shops during April to September, 1981.

In the initial tests in bee shops, the greatest numbers of males ($P < 0.05$) were captured with four-component blends of *Z*9,*E*12-14:OH, *Z*9-14:OH, *Z*9,*E*12-14:ALD, and *Z*9-14:ALD, and the most promising ratio was

TABLE 1. MEAN MALE DRIEDFRUIT MOTHS ATTRACTED TO VARIOUS COMBINATIONS OF *Z*9,*E*12-14:OH (100 μg) WITH *Z*9-14:OH, *Z*9,*E*12-14:ALD, AND *Z*9-14:ALD IN BEE SHOPS

Components added (μg)			Mean[a] males/period	
*Z*9-14:OH	*Z*9,*E*12-14:ALD	*Z*9-14:ALD	I	II
			10c	
8			16bc	10bc
8	4	0.4	35ab	19b
8		0.4		7c
8	4			9bc
8	1	0.1	39a	16bc
8		0.1		10bc
8	1			15bc
2	1	0.1		34a
One unbaited trap/shop			1, (1♀)	2, (3♀)
Total males (in baited traps)			1009	595
Total females (in baited traps)			104	297
Mean females/treatment/rep.			3	8

[a]Means within test periods followed by the same letters do not differ ($P > 0.05$) by Duncan's new multiple-range test and unbaited trap catches were not included in the analyses of variance. Periods I and II were 10 and 5 replications (shops) during May 20 to July 7 and Sept. 3 to Oct. 7, 1981.

TABLE 2. MEAN MALE DRIEDFRUIT MOTHS ATTRACTED TO VARIOUS COMBINATIONS OF *Z*9,*E*12-14:OH (100) WITH *Z*9-14:OH, *Z*9,*E*12-14:ALD, AND *Z*9-14:ALD IN A FLIGHT ROOM

Components added (%)[a]			Mean[b] males/test		
*Z*9-14:OH	*Z*9,*E*12-14:ALD	*Z*9-14:ALD	I	II	III
0.5	1	0.1	53a	13a	15a
0.5	10	0.1	9b		
2	1	0.1	18b		
0.5	1			9ab	
0.5		0.1		5b	
0.5				5b	
	1	0.1		10ab	
	1			8ab	
		0.1		3b	
0.5	1	0.5			18a
Eleven females					4b
Six female abdomen tips					2b
Unbaited trap			1	1	1

[a]Blends were based on 25 μg of *Z*9,*E*12-14:OH/septum.
[b]Tests I and III each involved 5 days. Test II was in 2 parts, each involved four treatments for 10 days. Trap catches were recorded daily and treatments were randomized daily. Means within a test period followed by the same letters do not differ ($P > 0.05$) by Tukey's test.

100:2:1:0.1 (Table 1). Omission of *Z*9,*E*12-14:ALD resulted in the capture of fewer ($P < 0.05$) moths, which indicated that this diene aldehyde was an essential secondary pheromone component. This was confirmed in later tests.

Numerous ratios of these 4 components were further tested in a flight room during the winter of 1981-1982 and blends in ratios of 100:0.5:1:0.1 or 100:0.5:1:0.5 captured the greatest number ($P < 0.05$) of males (Table 2). Both of these blends captured more ($P < 0.05$) males than traps baited with females or excised abdomen tips from calling females. Females used to bait traps exhibited calling behavior before and during the attractancy tests. Observations of the male behavior showed that omission of the monounsaturated aldehyde and alcohol resulted in reduced sexual activity of the males near the pheromone dispensers. Male moths were not captured in traps baited with a single component, *Z*9,*E*12-14:ALD, *Z*9-14:ALD, or *Z*9-14:OH. The most promising blends from these tests were repeated in bee shops during April to June, 1982 (Table 3).

Bee shops varied in size and shape from rectangular quonsets to converted houses, which increased positional effects within tests and limited the number of treatments to 11/building. The data in Table 3 were in agree-

TABLE 3. MEAN MALE DRIEDFRUIT MOTHS ATTRACTED TO VARIOUS COMBINATIONS OF *Z*9,*E*12-14:OH (100 μg) WITH *Z*9-14:OH, *Z*9,*E*12-14:ALD, AND *Z*9-14:ALD IN BEE SHOPS

Components added (μg)			
*Z*9-14:OH	*Z*9,*E*12-14:ALD	*Z*9-14:ALD	Mean[a] males
0.5	1	0.1	66
0.5	1	0.5	50
2	1	0.1	49
			41
	1		64
	10		50
0.5			32
0.5		0.5	30
0.5	1		62
	1	0.5	62
One unbaited trap/shop			3, (3 ♀)
Total males (in baited traps)			2526
Total females (in baited traps)			619
Mean females/treatment/rep.			12

[a]Means from five replications were not different ($P > 0.05$) and unbaited trap catches were not included in the analyses of variance. Tests were from May 10 to June 25, 1982.

ment with those from the flight room as the greatest ($P > 0.05$) numbers of males were attracted to the four-component blend in a ratio of 100:0.5:1:0.1. However, a blend of *Z*9,*E*12-14:OH and the corresponding aldehyde in a ratio of 100:1 captured similar numbers ($P > 0.05$) of males as the four-component blend. *Z*9,*E*12-14:ALD was essential as its omission resulted in the capture of fewer ($P > 0.05$) males.

It was important to establish the quantity/dispenser that functioned the best throughout a flight period of 8 weeks. Two blends of the four components were tested at 100 and 500 μg/septum (Table 4). Blend A at 500 μg/septum captured the most ($P > 0.05$) males over a 7-week period. To determine the effectiveness of the original treatments after 7 weeks, blend B at 100 μg/dispenser was replaced with a freshly treated blend A at 100 μg/dispenser. This captured more ($P < 0.05$) males than the remaining original treatments, which indicated that dispensers should not be used more than 7 weeks in bee shops.

Since this manuscript was submitted, additional attractancy tests were done in bee shops and in bee shelters in the field. In both tests, similar numbers ($P > 0.05$) of males were attracted to a two-component blend of *Z*9,*E*12-14:OH and *Z*9,*E*12-14:ALD in a ratio of 100:1 at 500 μg/septum and to a four-component blend (blend A, Table 4) at 500 μg/septum. It was

TABLE 4. MEAN MALE AND FEMALE DRIEDFRUIT MOTHS IN TRAPS BAITED WITH *Z*9,*E*12-14:OH, *Z*9-14:OH, *Z*9,*E*12-14:ALD, AND *Z*9-14:ALD, BLEND A, 100:0.5:1:0.1, AND B, 100:0.5:1:0.5, AT TWO QUANTITIES (μg) PER DISPENSER

Quantity (μg)/septum	Mean[a] males and females/trap/day					
	April 23–May 31		June 1–9		June 10–18	
	Males	Females	Males	Females	Males	Females
Blend A						
100	6.5	3.3	4.3	0.9	3.4b	2.6
500	9.8	2.2	11.7	5.3	6.6b	1.4
Blend B						
100[b]	5.7	2.7	5.6	3.2	14.6a	3.8
500	9.0	4.1	9.6	2.3	3.9b	3.3
Unbaited	0.9	1.1	0.6	1.6	0.3	1.1

[a]Treatments were tested in 1 leafcutter bee shop, and moths were removed and traps randomized three times/week. Each randomization was considered a replication and means followed by the same letter do not differ ($P > 0.05$) by Duncan's new multiple-range test. All traps were replaced May 31 and June 9, and the original dispensers were used throughout the test.
[b]This treatment was replaced June 9 with a freshly prepared treatment of blend A at 100 μg/septum.

concluded that the two-component blend is satisfactory for monitoring purposes and that further tests, preferably under field conditions, are required to establish the function of *Z*9-14:OH and *Z*9-14:ALD in the four-component blend.

Capture of Female Moths. Female moths were captured in all unbaited and synthetic female pheromone-baited traps in bee shops (Tables 1, 3, and 4). This was most dramatic in the test summarized in Table 4, and the mean males and females captured in all pheromone-baited traps during 2- and 3-day periods throughout the test are illustrated in Figure 4. There were similar numbers ($P > 0.05$) of females in the baited and unbaited traps from April 23 to May 5; however, from May 7 to 31 there were greater numbers ($P < 0.01$) of females in the baited traps (means/treatment/replication for baited and unbaited traps was 10 and 3). The traps were all replaced on May 31 and June 9, and baited with the original dispensers. This resulted in an initial decrease in the capture of females (June 2 and 11), which then increased, especially by June 9.

These data suggest that the females were attracted by the males or by male secretions that accumulated in the traps. Close-range attraction of females by male hairpencil and forewing gland secretions have been reported in other Pyralidae (Kuwahara, 1980; Baker et al., 1981; McLaughlin,

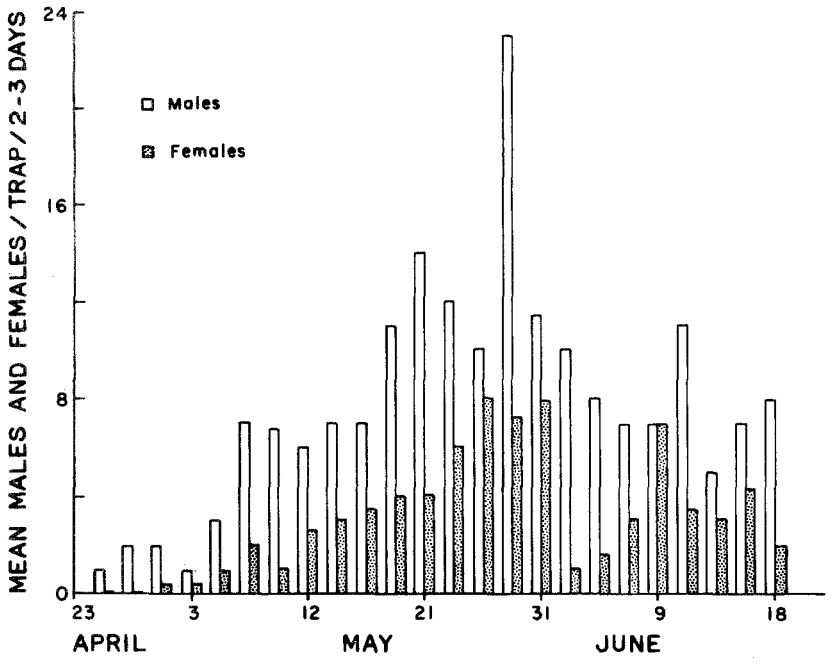

FIG. 4. Mean catches of male and female driedfruit moths in traps baited with synthetic four-component blends A and B (Table 4). Traps were replaced May 31 and June 9. There were greater numbers ($P < 0.01$) of females in the baited than in the unbaited traps from May 7 to 31.

1982, and references therein). Females had no detectable (EAD-FID) olfactory responses to their own pheromone or to the synthetic pheromone blends, and their responses to male gland secretions were not determined.

This interesting phenomenon may have considerable practical importance for control of this pest, at least within bee shops.Examination of the captured females showed that they were mated at least once, but they still contained a majority (100–200) of their eggs. A considerable number of gravid females may be trapped by using the synthetic female pheromone to capture males which in turn appear to attract the females.

Attractant Inhibitors. Attractancy tests in the flight room showed that the captures of males to the four-component blend *Z*9,*E*12–14:OH, *Z*9–14:OH, *Z*9,*E*12–14:ALD and *Z*9–14:ALD in a ratio of 100:0.5:1:0.1 were reduced ($P < 0.05$) by the addition of *Z*9,*Z*12–14:OH (1%) or *E*9,*E*12–14:OH (2%). The addition of *E*9,*Z*12–14:OH (2%) or *E*12–14:OH (2%) to the four-component blend had no obvious ($P > 0.05$) effect on the attraction of males. Similarly, tests in bee shops showed that the addition of

*Z*9,*E*12–14:Ac (0.5 μg) or *Z*9–14:Ac (0.5 μg) to the four-component blend in a microgram ratio of 100:8:4:0.4 resulted in the mean capture of 3*c* and 16*bc* males (comparable data Table 1, test I). *Z*9,*E*12–14:Ac was a potent attractant inhibitor and *Z*9–14:Ac had little effect.

These data established that *Z*9,*E*12–14:OH used for the preparation of trap baits must be essentially free of *E*9,*E*12–14:OH, *Z*9,*Z*12–14:OH, and *Z*9,*E*12–14:Ac.

The addition of the following compounds (μg, in parentheses) to the four-component blend in a microgram ratio of 100:8:4:0.4 had no obvious effect on the attraction of males: 12:OH(3), 12:ALD(2), 14:OH(3), 14:ALD(2), 16:OH(10), 16:ALD(2), 16:Ac(30), *Z*9–16:OH(3), 18:OH(10), and 18:ALD(2). These components were not tested individually with the four-component blend. Two micrograms of *Z*9–16:ALD, *Z*9,*E*11–14:ALD, and *Z*9,*E*11–14:OH were added separately and together to the four-component blend, but these had no obvious effects on the attraction of males. The pheromone-like compounds identified in the female pheromone that had no effect on trap catches may be involved with some other aspect of their mating behavior. This was not investigated.

Reproductive Isolation. Driedfruit moth is classified in the subfamily Phycitinae and to our knowledge this is the first species of this group that produces *Z*9,*E*12–14:OH and the corresponding aldehyde as pheromone components. *Z*9,*E*12–14:Ac, a potent attractant inhibitor for the driedfruit moth, has been identified as the sex pheromone of some other species of Phycitinae, such as: Indian meal moth (Kuwahara et al., 1971a; Brady et al., 1971); almond moth (Kuwahara et al., 1971a); Mediterranean flour moth (Kuwahara et al., 1971); raisin moth (Brady and Daley, 1972), and tobacco moth (Brady and Nordlund, 1971).

*Z*9,*E*12–14:OH and *Z*9–14:OH were identified in the sunflower moth (Underhill et al., 1979). *Z*9,*E*12–14:OH was also reported to be emitted by Indian meal moth which inhibited the response of almond moth to *Z*9,*E*12–14:Ac (Sower et al., 1974). Since several of these species may occur in the same habitat (Okumura, 1966) it is likely that they maintain reproductive isolation, at least in part, by their pheromones.

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BIOCHEMICAL ADAPTATIONS OF THE BRUCHID BEETLE, *Caryedes brasiliensis* TO L-CANAVANINE, A HIGHER PLANT ALLELOCHEMICAL

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Abstract—This communication reviews recent biochemical studies of L-canavanine that have provided significant understanding of the interaction between the seed of *Dioclea megacarpa* (Leguminosae) and the bruchid beetle *Caryedes brasiliensis* (Bruchidae). The principal biochemical bases are proposed for: canavanine toxicity, the ability of the beetle larvae to adapt to its presence, the metabolic sequestration and detoxification of ammonia, and the potential amplification by microbial symbionts of the beetle's abilities to adapt to toxic components of the host.

Key Words—*Caryedes brasiliensis*, Coleoptera, Bruchidae, L-canavanine, L-canaline, *Dioclea megacarpa* (Leguminosae), allelochemicals, detoxification, adaptation.

INTRODUCTION

Certain toxic compounds synthesized by higher plants function in plant chemical defense (Harborne, 1982). These allelochemicals are involved in interspecific interactions between the plant and phytophagous insects and other herbivores, pathogens, and even other higher plants (Rosenthal and Janzen, 1979). The nonprotein amino acids are an important but lesser known component of plant allelochemicals (Rosenthal, 1982); L-canavanine is illustrative of this group of toxic plant constituents. It elicits a broad range of detrimental biological effects in virtually all living systems to which it is a foreign compound (Rosenthal, 1977a).

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BRUCHID BEETLE, *CARYEDES BRASILIENSIS*

The neotropical leguminous vine, *Dioclea megacarpa* Rolfe is a veritable canavanine storehouse; canavanine may comprise 13% of the seed dry weight, constitute 55% of all seed nitrogen, and more than 95% of the nitrogen allocated for stored free amino acids (Rosenthal, 1977b). Accumulation of so much toxic canavanine creates a highly effective chemical barrier to predation since the only known insect seed consumer is the larva of the bruchid beetle, *Caryedes brasiliensis* Thunberg (Bruchidae). The bruchid beetle oviposits her egg clusters on the outside of the ovary wall, along the suture of the fruit. These eggs are produced about a month before the pod matures; pods suitable for egg deposition are available for only 2–3 months of the year (Janzen, 1981). The newly emerged larvae bore into the testa and feed upon the canavanine-laden cotyledonous tissues of the seed; more than 50 larvae can develop within an infected seed. The young adult carves an exit portal in the testa and ultimately escapes via openings such as that between the semi-dehiscent values of the mature pod. The adult beetles, released from April to July, are solely pollen feeders. These adults do not oviposit on detached pods and must await the rainy season for the advent of the new seed crop before completing their life cycle (Janzen, 1981). The ability of this bruchid beetle to pursue successfully its specialized feeding activity in this highly poisonous seed results from a series of fundamental biochemical adaptations which are the subject of this communication.

ABERRANT, CANAVANYL PROTEIN FORMATION

A principal biochemical basis for canavanine's toxicity is its structural similarity to L-arginine which results in its activation and aminoacylation by arginyl-tRNA synthetase. Arginyl-tRNA is charged with canavanine, and this arginine analog is incorporated into the elongating polypeptide chains, thereby producing structurally anamolous proteins. Replacement of arginine by the much less basic canavanine molecule can significantly alter the basic physicochemical properties of the proteins (Pines et al., 1981). Recent experimental studies have demonstrated the deleterious effect of canavanine incorporation into protein. However, it is not possible presently to resolve the question of how such replacement specifically affects the various functional parameters of the altered proteins (Rosenthal, 1982).

Injection of [^{14}C]canavanine into the tobacco hornworm, *Manduca sexta* [Sphingidae], an insect highly susceptible to the adverse effects of canavanine, revealed that at least 3.5% of the administered canavanine was incorporated into de novo synthesized protein (Rosenthal et al., 1976). An identical analysis with *Caryedes brasiliensis* failed to detect appreciable

canavanine-laden protein. Analysis of the arginyl-tRNA synthetase of this bruchid beetle established that arginine was a substrate, but it failed to charge canavanine (Rosenthal et al., 1976).

In a direct determination of the protein-synthesizing system of these insects, a series of five arginine analogs including canavanine were provided to *M. sexta* and *C. brasiliensis*. The tobacco hornworm fixed all of the tested compounds into de novo-synthesized proteins. Comparable determination with the bruchid beetle revealed remarkably little assimilation of these analogs into protein (Rosenthal and Janzen, in review). This finding indicates a highly discriminatory protein-producing system for insects such as *C. brasiliensis*. Indeed, the marked ability of this seed predator to distinguish molecules structurally akin to arginine confers general resistance to the incorporation of such nonprotein amino acids. These studies reveal a fascinating and important biochemical adaptation of these larvae that prevents aberrant protein formation.

CANAVANINE AS A DIETARY NITROGEN SOURCE

Among the other biochemical adaptations discovered for this feeding specialist are those associated with the use of canavanine as a dietary nitrogen source for the developing larvae. On first inspection, canavanine represents an obvious dietary source of metabolizable nitrogen, but an example of significant diversion of a toxic nonprotein amino acid's nitrogen into fundamental reactions of insectan nitrogen metabolism had not been demonstrated previously. Reliance upon canavanine's nitrogen is encouraged by the limited proteolytic activity of the gut of the bruchid beetle larva. This deficiency in protein-degrading ability is related to the synthesis of inhibitors of these enzymes that occur in seeds normally consumed by bruchids (Applebaum, 1964).

Bruchid beetles share with other insects the ability to produce arginase which mediates the cleavage of arginine to urea and ornithine; ornithine is vital to insects as a precursor of glutamic acid. Arginase also provides a means for the efficient conversion of canavanine to canaline and urea:

$$\underset{\text{L-canavanine}}{H_2N{-}C(=NH){-}NH{-}O{-}CH_2{-}CH_2{-}CH(NH_2)COOH} \rightarrow \underset{\text{L-canaline}}{H_2N{-}O{-}CH_2{-}CH_2{-}CH(NH_2)COOH} + \underset{\text{urea}}{H_2N{-}C(=O){-}NH_2} \quad (1)$$

Urea is not a dietary nitrogenous resource since its direct assimilation into amino acids or other useful nitrogenous components does not occur. Urease, which transforms urea to usable ammonia, is rarely reported in insects and accounts of its presence are usually discounted (Cochran, 1975). This stands in

striking contrast to its abundance in bruchid beetle larvae. The biochemical acquisition of the ability to rapidly degrade urea to CO_2 and ammonia makes available nitrogen that can be fixed into amino acids (Rosenthal et al., 1977).

$$H_2N—C(=O)NH_2 \rightarrow CO_2 + 2NH_3 \quad (2)$$

By reactions (1) and (2), two thirds of the nitrogen of the terminal guanidinooxy moiety of canavanine is mobilized to meet the nitrogen requirements of the developing larva; thus, this arginine analog is detoxified and in such a manner as to provide dietary nitrogen. Canavanine is an excellent example of a potentially toxic allelochemical which, by virtue of its effective utilization, assumes a decisively beneficial or kairomonic role in this insect.

CANALINE FORMATION

The stoichiometric formation of canaline, resulting from arginase-mediated cleavage of canavanine, creates a serious biological hazard for the larva since canaline is also highly toxic. For unadapted insects, this ornithine analog causes underdeveloped larvae, reduces successful larval–pupal ecdysis, increases the severity of pupal and adult malformation, decreases survival of all developmental stages, and elicits neurotoxic actions in the adult tobacco hornworm moth (Rosenthal and Dahlman, 1975; Kammer et al., 1978). Bruchid beetle larvae circumvent these adverse biological effects by the detoxification of canaline to homoserine and ammonia:

$$\underset{\text{L-canaline}}{H_2N—O—CH_2—CH_2—CH(NH_2)COOH} + NADH + H^+ \rightarrow \underset{\text{L-homoserine}}{HO—CH_2—CH_2—CH(NH_2)COOH} + NAD^+ + NH_3 \quad (3)$$

It has not been determined if this catalytic process is directed by a novel enzyme specifically able to conduct reaction (3) or rather by an existing protein, e.g., a dehydrogenase with a broad specificity for amino acids.

NITROGEN EXCRETION

As the larvae of *C. brasiliensis* feed on the seed of *D. megacarpa*, they defecate into the seed cavity. Examination of this fecal material reveals that uric acid nitrogen constitutes only 11% of the excreted nitrogen; ammonia and urea account for 42% and 47%, respectively, of the fecal nitrogen (Rosenthal and Janzen, 1981).

This finding is in accord with the research of Mullins and Cochran (1972, 1976) asserting to the potential importance of ammonia as a nitrogen excretory product of terrestrial insects. Thus, the excretion of ammoniacal

nitrogen by this seed-eating insect may not be unusual. By contrast, nitrogen elimination as urea, i.e., ureotelism, is of marginal importance in the overwhelming majority of insects (Schoeffeniels and Gilles, 1970). Urea is only a minor component of the hemolymph (Buck, 1953) and the excreta of numerous insects (Razet, 1966; Powning, 1953). The question of whether the Krebs-Henseleit ornithine cycle functions in insects has still not been adequately addressed or resolved. These cyclic reaction constituents are insectan natural products (Garcia et al., 1956), but in the hawk moth, *Celerio euphorbiae*, this cyclic reaction sequence is incomplete even though the various cycle intermediates are present (Porembska and Mochnacka, 1964). This seed predator represents an atypical terrestrial insect, for it is strongly ureotelic and ammoniotelic with uricotelism being of relatively limited significance. The ability to excrete ammonia and urea, derived from the metabolism and detoxification of canavanine and canaline, has adaptive significance in providing the insect with a means for eliminating nitrogen without the metabolic cost of converting it to uric acid or some other nitrogen excretory product.

METABOLIC AMMONIA ASSIMILATION

While excretion in the frass is an effective means for extraneous ammonia elimination, appreciable ammonia is also sequestered metabolically; for example, by a glutamine synthetase-catalyzed reaction in which glutamic acid forms glutamine. This enzyme is very active in *C. brasiliensis*, and glutamine is a major free amino acid of the larvae. Aspartic acid is a very minor component of larval nitrogen storage and the amide group of asparagine is of little consequence in nitrogen containment (Figure 1).

The commercial availability of [^{15}N] urea and the bruchid beetle larval ability to release the guanidinooxy group nitrogen of canavanine as ammonia via urea permitted a direct assessment of canavanine's capacity to support the amino acid metabolism of this seed predator. This was achieved experimentally by administrating [^{15}N]urea and direct evaluation of the de novo synthesis of [^{15}N]containing amino acids by combined gas chromatography–mass spectroscopy techniques (Rosenthal et al., 1982).

The abundance of certain key ion fragments was used to quantify the $^{15}NH_3$ incorporation occurring for 13 amino acids. More than 25% of the nitrogen content was contributed by the heavier isotope for glutamic acid–glutamine, aspartic acid–asparagine, proline, 3-alanine, 2-aminobutyric acid, and alanine. A lesser but very appreciable ^{15}N level occurred in glycine, serine, valine, homoserine, and methionine. Urea nitrogen did not transfer significantly to threonine, leucine, isoleucine, lysine, histidine, or hydroxyproline (Figure 2).

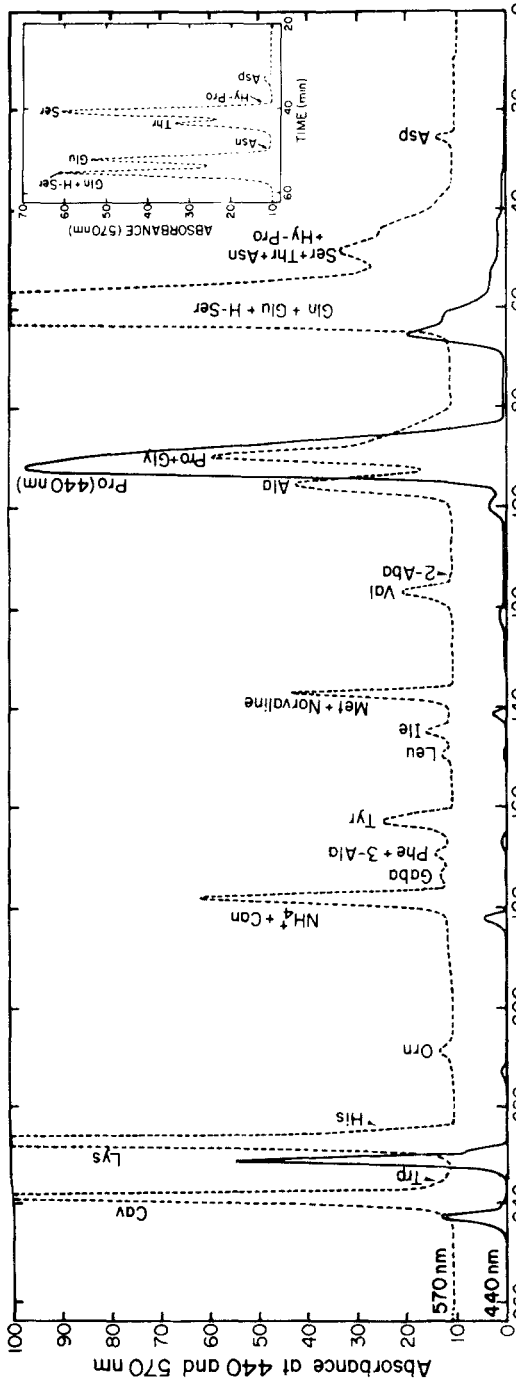

FIG. 1. The free amino acids of the larvae of the bruchid beetle, *Caryedes brasiliensis*. Automated amino acid analysis was conducted with the highly purified free amino acid extract as previously described (Rosenthal et al., 1982). Highly purified free amino acid extract diluted to permit resolution of the indicated amino acids. Norvaline served as an internal control. The amino acid designations are: Ala, alanine; 2-Aba, 2-aminobutyric acid; Thr, threonine; Ser, serine; 3-Ala, 3-alanine; Val, valine; Leu, leucine; Ile, isoleucine; H-Ser, homoserine; Pro, proline; Hy-Pro, hydroxyproline; Met, methionine; Asn, asparagine; Asp, aspartic acid; Orn, ornithine; Phe, phenylalanine; Cav, canavanine; Can, canaline; Lys, lysine; Tyr, tyrosine; Gln, glutamine; Glu, glutamic acid; His, histidine; Trp, tryptophan; Gaba, 4-aminobutyric acid.

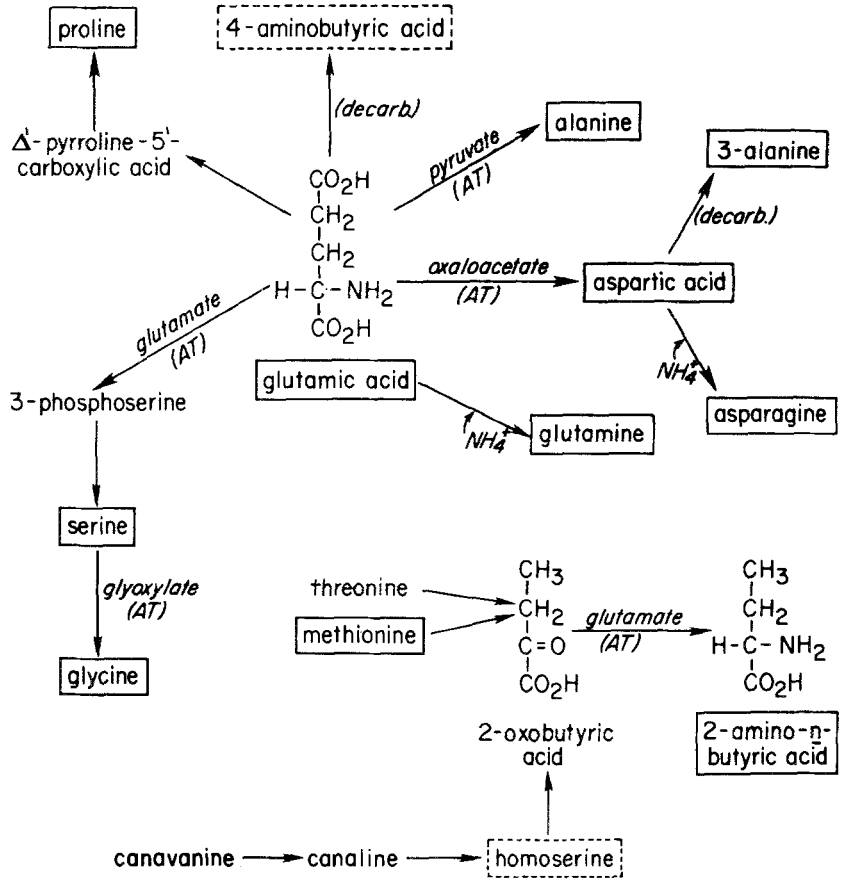

FIG. 2. Putative reactions for the transfer of $^{15}NH_3$ into the amino acid metabolism of the bruchid beetle, *Caryedes brasiliensis*. The amino acids enclosed with solid lines contained appreciable nitrogen-15. 4-Aminobutyric acid was unique in being detected by amino acid analysis while failing to appear in the gas chromatographic runs. AT refers to various aminotransferases that catalyze the indicated reactions.

It is evident that the nitrogen of canavanine, released as urea and converted to ammonia, supports insect amino acid biosynthesis. Canavanine functions so effectively as a dietary nitrogen resource kairomone for this highly adapted seed predator as to make one wonder if a legume comparable to *D. megacarpa* but containing much less canavanine, would be a suitable host plant for this seed predator. Would these larvae languish on a food resource deficient in canavanine? Other questions of *C. brasiliensis* biochemistry remain to be answered. Are the aminotransferases of the bruchid beetle larvae resistant to canaline? This ornithine analog is a potent inhibitor

of ornithine-keto acid transaminase of *Manduca sexta* (D.L. Dahlman, personal communication) and other animals (Kito et al., 1978). It would be of interest to compare the inhibition kinetics of this enzyme from *C. brasiliensis* with that of canavanine- and canaline-sensitive insects. Similarly, insect arginase is inhibited by canavanine (Harry et al., 1976), but this enzyme sensitivity must be circumvented in *C. brasiliensis*, whose arginase must function in the presence of abundant canavanine.

ACQUISITION OF APPROPRIATE BIOSYNTHETIC CAPACITIES

Acquisition of the distinctive biochemical adaptations described above has permitted *C. brasiliensis* to: (1) avoid producing canavanyl proteins, (2) detoxify canavanine and canaline, (3) utilize the nitrogen in canavanine and canaline, and (4) efficiently assimilate ammonia into amino acids. It is germane to ask how these metabolic abilities and those remaining to be detailed were acquired.

It is proposed that among the earliest adaptations achieved by *C. brasiliensis* was the development of a marked discriminatory capacity in protein synthesis so that aberrant or dysfunctional protein molecules were not produced. It is difficult to imagine significant reliance upon a food resource rich in this highly toxic nonprotein amino acid without a mechanism for avoiding or minimizing synthesis of aberrant proteins. Generation of an adequate pool of functional enzymes is essential to the existence of all living systems. Once avoidance of erroneous protein production was achieved, several different possibilities might have occurred. Firstly, canavanine and canaline could have been excreted or otherwise avoided while other nitrogen-rich metabolites such as storage proteins were marshaled to meet the fundamental nitrogen needs of the insect. The overall Darwinian fitness of these pioneering predators of *D. megacarpa* undoubtedly plummeted in the face of canavanine and canaline. Individuals emerged from the remnant population able to survive the formidable chemical barrier represented by these toxic natural products.

In time, perhaps by a process of sequential adaptations, this seed consumer became progressively better able to cope with and ultimately to utilize the nitrogen resource formerly locked in the canavanine and canaline molecules. With the assumption of each new biochemical ability, *D. megacarpa* became a more suitable and beneficial food and ovipositional resource; this may have instigated a closer linkage with this canavanine-containing legume. With time, this bruchid beetle came to utilize *D. megacarpa* to the exclusion of other seeds in its habitat (Janzen, 1981). (*Caryedes brasiliensis* attacks two other Costa Rican legumes: *D. wilsonii* and *D. reflexa* but neither occurs in this habitat.) This utilization was

made possible by its detoxification ability and was stimulated by virtue of at least three additional benefits: First, there is unencumbered access to its food resource. It does not necessarily follow that in giving up all other food sources, even with an abundance of competitors, this beetle accrued significant benefit in terms of available food. Nevertheless, it is reasonable to contend that *Caryedes brasiliensis* probably benefits from the absence of competition. Second, there is the need to maintain only minimum detoxification abilities. Since the spectrum of toxic allelochemicals of its food is limited, so too is the need to maintain the capacity to detoxify a conglomerate of secondary metabolites. Third, the larvae are assured a safer haven for development since canavanine's intrinsic toxicity minimizes exposure to various predators. Bruchid beetle larvae enjoy marked freedom from predation (Janzen, 1971) and the sequestration of canavanine may be a contributing factor.

A recent analysis of various insects for urease revealed an extremely low but detectable level of this enzyme in certain tested species (Rosenthal et al., 1977). Many of the enzymes required by this bruchid beetle may have been present initially but at a low concentration. The sudden consumption of canavanine and canaline may have stimulated production of enzymes required for their metabolism. Thus, a high seed canavanine and canaline concentration, which would more effectively select for individuals able to cope with these toxic metabolites, may have characterized this legume at the time of the initial contact between these organisms.

It is also worthwhile to view this from a different perspective. When a progenitor of *C. brasiliensis* first used *D. megacarpa*, the canavanine content of the seed may have been far less. If so, the sequential biochemical acquisitions necessary for eventual larval dependency on *D. megacarpa* had much more time to occur. As the canavanine level rose, the associated ability of *C. brasiliensis* to utilize the augmented canavanine of the seed also developed and magnified. While there are no data bearing on this point, their many close biological and biochemical links speak for considerable co-evolutionary interaction between these species.

SYMBIONTS AND CANAVANINE UTILIZATION

Insect ability to adapt rapidly to a multitude of toxins needs no further elaboration. It is reasonable to propose that their intrinsic adaptive capacity would be amplified greatly by appropriate microbial gut symbionts. The rapid assumption of the ability of this seed predator to accommodate *D. megacarpa* may be nothing more than fortuitous acquisition of the proper prokaryotes typically found associated with decaying *D. megacarpa* seeds. Such a symbiotic relationship would possess the potential to accelerate acquisition of many biochemical adaptations. The symbiotic gut and fat body flora of the

bruchid beetle larvae are being evaluated at this time, and many bacteria capable of utilizing canavanine and/or canaline as the sole nitrogen source and in some cases carbon as well have been isolated. These efforts will permit a direct assessment of the biochemical competency of their microbial flora for conducting the above reactions. The existence of such a competent flora would provide an outstanding potential means for rapid and effective insectan acquisition of novel biochemical capacities.

In this vein, it would be highly interesting to compare the amino acid sequence of the urease molecule synthesized by *C. brasiliensis* and by other urease-containing beetles to that in *D. megacarpa* and other canavanine-containing legumes (e.g. *Canavalia brasiliensis*) of their common habitat not consumed by this seed predator. Discovery of significant urease homology unique to *C. brasiliensis* and *D. megacarpa* would provide further evidence for the close genetic association between these biological systems and indicate strong coevolutionary interaction between these species. While a close coevolutionary linkage between certain insects and higher plants is often assumed, Janzen and Martin (1981) have argued cogently that the underlying biological properties of fruit and seeds may have been shaped by evolutionary interactions with their Pleistocene megafauna.

RISK OF EXTINCTION

Another relevant consideration is the risk of extinction due to the monophagous feeding habit of these larvae in the face of a precipitous loss in its present host plant. While the advantages of the host-specific nature of *C. brasiliensis* are significant, this species is placed in a precarious situation by its dependence on *Dioclea*. The virtual elimination of the American chestnut, *Castanea dentata* by the fungus *Endothia parasitica* may have caused a parallel extinction of certain insect predators of this forest tree (Opler, 1979) On the other hand, selection of the normal ovipositional substratum is an imperfect process; some gravid females fail to integrate the appropriate physical and chemical clues as adroitly as others. Some individuals highly motivated to oviposit will deposit their egg clusters on alternate hosts and thereby serve as the vanguard for adapting to the use of new food resources. It seems reasonable to propose that canavanine-storing members of the present habitat that are not presently attacked by this bruchid beetle, e.g., certain members of *Canavalia*, would possess a high probability of becoming novel hosts. The detoxification and utilization pathways necessary for efficient insect utilization of these hew hosts would already be present to some extent, having been acquired in the accommodation to *D. megacarpa*. The olfactory, visual, and chemical signals specific to *Dioclea* that guide *C. brasiliensis* in its food and oviposition selection behavior may not be common to other canavanine-containing legumes of the habitat. The presence of the pre-

requisite cues for host location may still not prevent the eventual demise of this seed predator, if the competition for the new host resources is too keen. If this insect were to survive, it would be most interesting to note if canavanine-containing legumes not presently attacked by *C. brasiliensis* were to become its preferred host and, if so, whether the beetle would remain a specialist or feed upon a broader range of legumes.

PLANT SEQUESTRATION OF CANAVANINE

The abundance of seed canavanine is not unique to *D. megacarpa* but rather has been recorded for a number of tested leguminous species (Rosenthal, 1977b). This disproportionate placement of seed nitrogen into canavanine becomes more explicable when it is realized that arginase and urease are abundant in canavanine-containing seeds. Indeed, the seed urease activity correlates well with the level of stored canavanine (Rosenthal, 1974). These enzymes rapidly mobilize the nitrogen of canavanine and facilitate its movement into primary metabolic pathways. Thus, no intrinsic disadvantage accrues to the plant in storing canavanine over arginine; for example, since canavanine's nitrogen readily enters into primary metaboilc pathways. Thus, a seed protected chemically by canavanine may sequester a greater amount of its available nitrogen into this allelochemical, even at the possible expense of arginine or other nitrogen-storing metabolites. In time, as more and more canavanine came to be stored, it must have assumed an ever increasing role in the defense of the seed. This unusual and disproportional reliance of certain legumes on canavanine or some toxic nonprotein amino acid (Rehr et al., 1973; Bell and Janzen, 1971) arouses speculation as to whether this process of nitrogen resource allocation may have run away with a resulting cascading dependency on a single nitrogen-storing metabolite for chemical defense. While many questions remain to be resolved, it is evident that the study of *D. megacarpa* and its seed predator, *C. brasiliensis*, has provided significant insight into the biochemical adaptations underlying the basic biological interactions between these two species.

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ANTIFOULING AGENTS AGAINST THE BENTHIC MARINE DIATOM, *Navicula salinicola* Homarine from the Gorgonians *Leptogorgia virgulata* and *L. setacea* and Analogs

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Abstract—At concentrations found in the gorgonian corals, *Leptogorgia virgulata* (L.) and *L. setacea* (L.), homarine (*N*-methyl-2-carboxypyridine) and water-soluble extracts from the gorgonians that contained homarine inhibited the growth of the potential fouling diatom, *Navicula salinicola* Hust., by 50–60%. Homarine comprised 0.3 and 0.25% of the fresh weight of *L. virgulata* and *L. setacea*, respectively, and the water-soluble extracts comprised 4.0 and 3.0% of the fresh weight of the gorgonians, respectively. Three compounds structurally related to homarine including, in order of most to least active, nicotinic acid, picolinic acid, and pyridine, also reduced growth of *N. salinicola*. The activity of these compounds in the diatom assay suggests that the carboxyl group at the 2 position of the pyridine ring is important for activity and that *N*-methylation is not important. We conclude that chemical defense against fouling is operative in *Leptogorgia* species. Evidence from the literature for combined chemical and mechanical defenses by *Leptogorgia* and other organisms against fouling is presented.

Key Words—Chemical defense, fouling inhibitors, homarine, *Leptogorgia virgulata*, *Leptogorgia setacea*, *Navicula salinicola*.

INTRODUCTION

Horny corals or gorgonians (sea fans and whips; Phylum Cnidaria, Order Gorgonaceae) are large, showy, perennial, and sessile animals that remain remarkably free from predation and fouling or encrustation (Bakus, 1981;

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Burkholder, 1973; Ciereszko and Karns, 1973; Fenical et al., 1981; Sieburth, 1968; Tursch, 1976; Tursch et al., 1978). The lack of fouling is characteristic of living gorgonian colonies only; dead gorgonian colonies become heavily fouled by algae, hydroids, tunicates, and other sessile organisms within several weeks (Burkholder, 1973). Because gorgonians are sessile and perennial, they cannot escape in space and time from predators and fouling organisms as some algae can (Ogden and Lobel, 1978; Vadas, 1977). Therefore, gorgonian colonies might be expected to maintain chemical and/or mechanical mechanisms of defense to remain free from epiphytic and epizooic organisms.

Diatoms, bacteria, and protozoa are the primary colonizers of most marine surfaces; they generally precede settlement of invertebrate larvae (Conover and Sieburth, 1964; Fuller, 1946; Graham and Gray, 1945; Marszalek et al., 1979; O'Neill and Wilcox, 1971; Sieburth, 1975; Wood, 1950). The presence of extracts, fatty acid-derived butenolides, prostaglandins, and nitrogen heterocycles (Baker and Murphy, 1976; Tursch et al., 1978) with antibacterial, antialgal, antiprotozoal, ichthyotoxic, and several other types of biological activity strongly suggests that chemical defense is operative in gorgonians, and more specifically, that primary film formation is inhibited in many of these animals (Baker and Murphy, 1976; Burkholder, 1973; Burkholder and Burkholder, 1958; Ciereszko, 1962; Ciereszko and Karns, 1973; Ciereszko et al., 1960; Perkins and Ciereszko, 1973; Tursch, 1976; Tursch et al., 1978; Weinheimer and Matson, 1975; Weinheimer et al., 1977). The ability of some gorgonians to slough or shed noncellular organic membranes or slime that are faily well covered with diatoms and other microorganisms suggests that in at least some species, mechanical defense may be operative (Kinzie, 1973; Patton, 1972; Ciereszko et al., 1973). Chemical and mechanical defenses against fouling in gorgonians may not be mutually exclusive.

The branched sea whip, *Leptogorgia virgulata* (Lamarck), and the unbranched sea whip, *L. setacea* (Lamarck), are commonly found in shallow waters along the Atlantic coastline of the United States south of Chesapeake Bay, and in the Gulf of Mexico from Florida to Texas (Bayer, 1961). During the course of chemical studies of marine organisms collected along the coast of Georgia, we determined that *L. virgulata* and *L. setacea* contain high concentrations of homarine (*N*-methyl-2-carboxypyridine), a compound common to numerous marine organisms (Baker and Murphy, 1976; Halstead, 1965; Weinheimer et al., 1973; Welsh and Prock, 1958). Here we report the results of experiments to determine whether a chemical defense against fouling is operative in *L. virgulata* and *L. setacea*. We employed a bioassay whereby we measured growth of the benthic pennate diatom, *Navicula salinicola* Hust., when exposed to various concentrations of homarine,

analogs of homarine, and extracts from *L. virgulata* and *L. setacea*. *Navicula salinicola* was chosen as the asssay organism because it is a common fouling organism in areas where *Leptogorgia* thrives (Darley et al., 1979; Sullivan, 1978; Williams, 1962; Williams, 1964), and can be grown in the laboratory on a solid agar medium (Chan et al., 1980; Harrison and Chan, 1980).

METHODS AND MATERIALS

Isolation and Characterization of Homarine from Leptogorgia virgulata (*Lamarck*) *and* L. setacea (*Lamarck*). *Leptogorgia setacea* (Lamarck)[2] was collected by SCUBA at −17 m from Gray's Reef, 20 miles offshore of Sapelo Island, Georgia, in the summer of 1980. Several collections of *L. virgulata* (Lamarck) were made by trawling and dredging in the Skidaway and Wilmington Rivers, which are adjacent to Skidaway Island, Georgia, at depths of 6–14 m in the fall of 1979 and 1980. Both animals were immediately cut into 5 to 10-cm lengths and frozen.

Leptogorgia setacea and *L. virgulata* were extracted separately with 100% MeOH and the solvent ws removed in vacuo. The residues were partitioned between deionized and distilled H_2O and EtOAc. The EtOAc layers were discarded, and the aqueous layers were lyophilized. The lyophilized aqueous layers comprised 3.0% and 4.0% of the fresh weights of *L. setacea* and *L. virgulata*, respectively.

Homarine was purified from the aqueous extract by Si gel preparative thick-layer chromatography (Analtech Si gel GF, 2.0 mm, $CHCl_3$–MeOH 1:2, R_f = 0.25–0.35) and by reverse-phase HPLC (Whatman Partisil ODS-2, 50 cm, MeOH–H_2O, 3:1 to 2:1). Homarine exhibited [^{1}H]NMR (400 MHz, DMSO, and D_2O), UV, TLC, and HPLC data identical with data from a synthetic sample prepared by treating commercially available homarine hydrochloride (Aldrich) with NaOH. Homarine comprised 0.25% of the fresh weight of *L. setacea* and 0.3% of the fresh weight of *L. virgulata*. Variation in concentrations of homarine from different collections of *L. virgulata* was insignificant.

Isolation and Culture of Navicula salinicola. The common benthic pennate diatom *Navicula salinicola* Hust.[3] was isolated from the Skidaway River in the fall of 1980. Microscope slides (3.5 × 7.6 cm) were suspended at −7 m from the Skidaway Institute of Oceanography dock. After 27 days, areas colonized by diatoms were transferred to the surface of 9-cm agar plates

[2] Taxonomic identification of *L. virgulata* and *L. setacea* were made by F. Bayer at the National Museum of Natural History, Smithsonian Institution, Washington, D.C.

[3] Taxonomic identification of *N. salinicola* was made by M. Sullivan at Mississippi State University.

prepared from nutrients, equivalent to f/2 media (Guillard and Ryther, 1962) in filtered water from the Skidaway River ($S = 20 \times 10^{-3}$), which were solidified with Difco-Bacto agar (1.5% w/v). The plates were incubated at 19.5° C at an irradiance of 80–100 $\mu\epsilon$/m/sec provided by "cool white" fluorescent lamps on a 12-hr light–dark cycle. Chan et al. (1980) and Harrison and Chan (1980) employed similar diatom culture methods.

Navicula salinicola was among the fastest growing organisms on the plates. A unialgal culture was obtained by several transfers of agar "plugs" containing the alga to new agar plates. Growth was enhanced by doubling the nitrate concentrations of the agar media. Stock cultures of *N. salinicola* were maintained under incubation conditions described above.

Bioassay. Compounds or extracts were dissolved in 1.0 ml 2× distilled H_2O and stirred into 15 ml of agar medium in a 15 × 100-mm Petri dish before the agar solidified. After solidification of the agar, 1 ml from a *N. salinicola* stock culture was evenly spread over each Petri plate. Six test plates at each concentration were then incubated for 8 days, the duration of the exponential growth phase in the *Navicula* control plates. Growth was determined for five test plates at each concentration by measuring in vitro chlorophyll *a* fluorescence after the diatoms and growth media had been homogenized and extracted with 90% aqueous acetone (method modified from Yentsch and Menzel, 1963). Fluorescence was measured with a Turner model 110 fluorometer.

Because organic acids and bases were being added to the media for test purposes, the pH had to be monitored. The buffer capacity of the Trizma buffer already present in the f/2 media was quickly exceeded as the test concentrations increased. The addition of 0.02 mol $NaHCO_3$/liter seawater media effectively stabilized the pH in the 7–8 range for all test concentrations. A sixth plate at each test concentration was used to monitor pH throughout the experiment. Blanks containing only growth media and test sample were also run for each sample at each concentration.

A mean value was calculated at each concentration and normalized to obtain the percentage growth of test sample relative to control. Propagated error (PE) was calculated according to the following:

$$x = \frac{\text{mean of test}}{\text{mean of control}}$$

$$s = \text{standard deviation}$$

$$PE = \pm\left[\left(\frac{x}{\text{mean of test}}\right)^2 (s \text{ of test})^2 + \left(\frac{x}{\text{mean of control}}\right)^2 (s \text{ of control})^2\right]^{1/2}$$

The Mann Whitney U test, a nonparametric statistic, was used to determine if the data obtained at various concentrations were significantly different from the control at the 95% confidence level (Sokal and Rohlf, 1969; Zar, 1974).

RESULTS

Water-soluble material containing homarine extracted from both *Leptogorgia* species inhibited diatom growth, with fluorescence falling to approximately 40% of the controls at 2000 μg/ml (Figure 1). Significant differences occurred between algal growth in control and test plates at concentrations $\geqslant$100 μg/ml for *L. setacea* and 500 μg/ml for *L. virgulata*.

Homarine (I) and three commercially available analogs (Aldrich), picolinic acid (2-carboxypyridine) (II), nicotinic acid (3-carboxypyridine, niacin) (III), and pyridine (IV) were also tested for their effects on the growth of *N. salinicola* (Figure 2).

Homarine test plates showed significant decreases in growth relative to control plates at concentrations $\geqslant$250 μg/ml. At 2000 μg/ml, a concentration approaching that which we estimate occurs naturally in *L. setacea* and *L. virgulata*, growth was inhibited 41% (Figure 1).

Picolinic acid test plates showed significant decreases relative to control at concentrations $\geqslant$500 μg/ml (Figure 3). The major structural difference

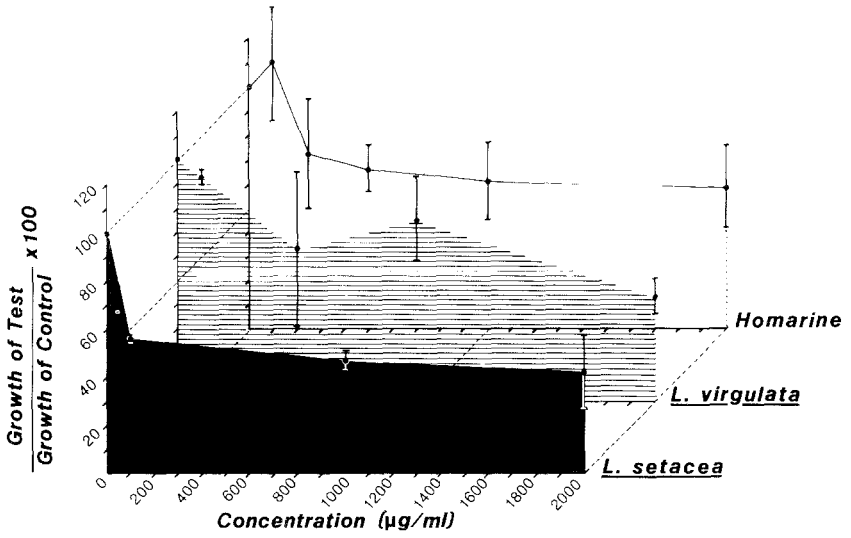

FIG. 1. Percentage growth of *Navicula* on test plates relative to control for *L. setacea*, *L. virgulata* and homarine.

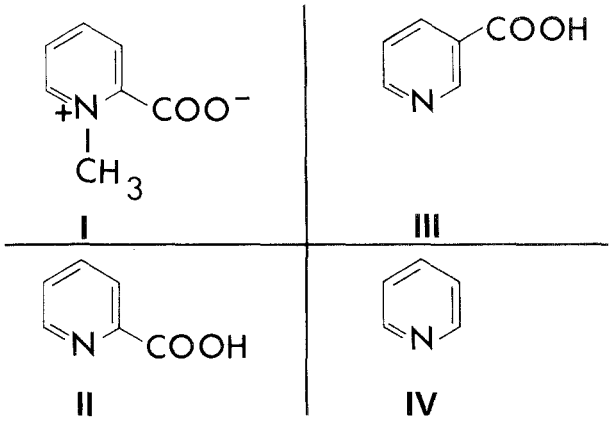

FIG. 2. Homarine (I), picolinic acid (II), nicotinic acid (III), and pyridine (IV).

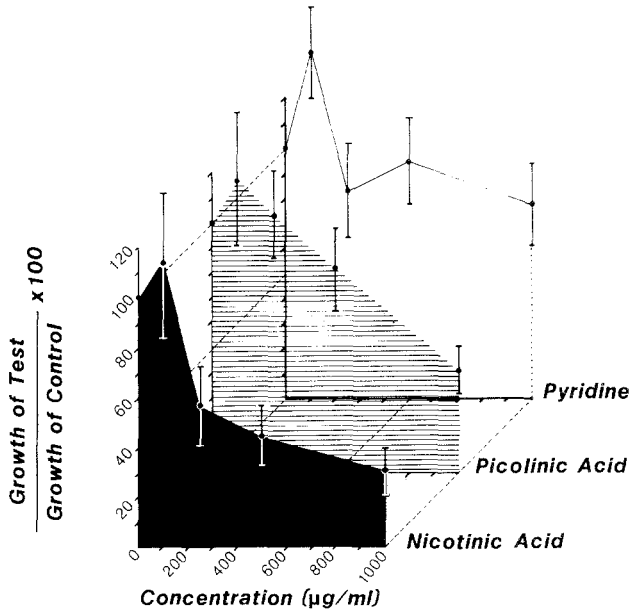

FIG 3. Percentage growth of *Navicula* on test plates relative to control for nicotinic acid, picolinic acid, and pyridine.

between picolinic acid and homarine is that the pyridine nitrogen in picolinic acid is not methylated; consequently, the molecule is not a zwitterion.

Nicotinic acid test plates showed significant decreases relative to control at concentrations $\geqslant 250$ μg/ml (Figure 3). Like picolinic acid, the pyridine nitrogen in nicotinic acid is not methylated. The carboxyl group, however, is located at the 3 position instead of the 2 position.

Pyridine significantly enhanced diatom growth relative to the control at 100 μg/ml. It significantly reduced diatom growth only at concentrations $\geqslant 1000$ μg/ml (Figure 3).

DISCUSSION

Secondary metabolites in gorgonians may function as feeding inhibitors, fouling inhibitors, pheromones or, in some gorgonians, as agents to preserve species specificity in gorgonian–zooxanthellae associations (Bakus, 1981; Burkholder, 1973; Ciereszko and Karns, 1973; Fenical et al., 1981; Sieburth, 1968; Tursch, 1976; Tursch et al., 1978). Predation by fish, mollusks, or polychaetes and encrustation by other corals are minor causes of mortality in gorgonians (Bayer, 1961; Grigg, 1977; Patton, 1972; Preston and Preston, 1975). When the gorgonian colony substratum is weakened by the action of boring organisms, or when sand moves down sand channels, colonies are toppled. Mortality is caused primarily by abrasion and burying of branches of toppled colonies by sand (Grigg, 1977; Kinzie, 1973). Because homarine occurs widely in marine invertebrates including corals, jellyfish, sea anemones, crabs, worms, sea urchins, sponges, and mollusks (Baker and Murphy, 1976; Gasteiger et al., 1955; Halstead, 1965; Weinheimer et al., 1973; Welsh and Prock, 1958), but not in invertebrates from fresh water, it has been suggested that homarine and other nitrogenous bases may serve an osmoregulatory function (Gasteiger et al., 1955). Our investigation shows that homarine inhibits growth of potential fouling diatoms on the gorgonians, *Leptogorgia virgulata* and *L. setacea*, which do not contain symbiotic zooxanthellae (Patton, 1972).

At concentrations found in *L. virgulata* and *L. setacea*, homarine and the water-soluble extracts inhibited growth of the benthic pennate diatom, *Navicula salinicola* Hust. Compounds structurally related to homarine (Figure 2) including picolinic acid, nicotinic acid, and pyridine also reduced the growth of *N. salinicola*. The compounds (ordered from most to least active) are nicotinic acid, picolinic acid, homarine, and pyridine. Since nicotinic and picolinic acids are more active than homarine, *N*-methylation of the pyridine nucleus is not required to inhibit fouling. The presence of a carboxyl group on the pyridine ring instead of a carboxylate moiety is important for activity, however. The pH of certain inshore waters where the

Leptogorgia spp. can occur ranges from 5.5 to 6.7. At the more acidic pH homarine may coexist with homarine HCl, thereby increasing its diatom-inhibiting activity.

Picolinic acid is the probable biogenetic precursor of homarine in analogy with nicotinamide and *N*-methyl nicotinamide (Dagley and Nicholson, 1970). All compounds tested by us may be derived biogenetically from tryptophan (Lehninger, 1970). It is not known whether the *Leptogorgia* species biosynthesize homarine or obtain if from their food. In the former case, it would clearly be advantageous for *Leptogorgia* to use nicotininc or picolinic acid rather than homarine as an antifouling agent because the activity is greater and the metabolic costs would be lower.

Homarine and its analogs are not unique in inhibiting the growth of diatoms and other unicellular algae. At very low concentrations, several terpenoids from soft coral and gorgonian species inhibit growth of pelagic unicellular algae (Tursch, 1976; Tursch et al., 1978). Phenols and brominated phenols, some from marine red algae (McLachlan and Craigie, 1966), and extracellular phenolic substances from the brown marine algae *Fucus vesiculosus* (McLachlan and Criagie, 1964), probably phloroglucinol polymers (Geiselman and McConnell, 1981), also inhibit growth of pelagic unicellular algae. Extracts from a seagrass species and numerous species of unicellular algae inhibit growth of pennate and centric diatoms (Chan et al., 1980; Harrison and Chan, 1980). The allelopathic agents from the seagrass species are probably phenolic acids (Zapata and McMillan, 1979).

In addition to the chemistry of the substrate, several factors including seawater chemistry, water currents or turbulence, temperature, light, nutrient availability, texture of substrate, and exposure time influence the formation of primary films and biofouling layers (Marszalek et al., 1979; Osman, 1977). However, it is the chemical nature of the substrate that is particularly important because it influences the microbiota initially attracted to the surface and may continue to influence the formation of the primary film and subsequent fouling layers (Marszalek et al., 1979). For example, Corpe et al. (1976) were able to enhance the rate of settlement of bacteria by coating glass slides with bacterial mucopolysaccharides. A positive chemotactic response of motile marine bacteria to surfaces covered with a primary organic film was observed by Young and Mitchell (1973). In contrast to glass surfaces, surfaces of copper–nickel alloys are selected by bacteria that produce copious amounts of extracellular mucoid material which may protect the bacteria from the metal (Marszalek et al., 1979). Terpene hydroquinones from the brown marine alga *Sargassum tortile* induce larval settling of epiphytic hydrozoa (Kato et al., 1975a,b). Tannin-like material from young branch tips of brown marine algae from the Sargasso Sea, *Sargassum natans* and *S. fluitans*, which are free from fouling, exhibits antibacterial activity against *Vibrio*, the

dominant marine bacteria on *Sargassum*, and immobilize and kill a variety of epifauna from *Sargassum* (Conover and Sieburth, 1964; Sieburth and Conover, 1965). Sulfuric acid-filled capsules and vanadium-rich surface deposits, which are formed by degenerating vanadocytes in the test of the ascidian, *Ascidia nigra*, are lethal to the larvae of two potential foulers, another ascidian and a hydroid (Stoecker, 1978). Fouling larvae do not avoid the test surface; rather, they are inactivated or killed after settling. Settlement by a worm on parts of the brown marine alga, *Laminaria* sp. and by a bryozoan on sections of another bryozoan, *Flustra foliacea* (L.), correlates inversely with the presence of antibiotics in *Laminaria* sp. and *F. foliacea* (Al-Ogily and Knight-Jones, 1977). The chemical nature of the antibiotics is unknown, however.

Gorgonians may employ chemical defense, mechanical defense, or both against fouling organisms competing with them for space. In this study, we showed that at concentrations present in *Leptogorgia virgulata* and *L. setacea*, homarine and the polar extracts containing homarine significantly inhibited growth of a potential fouling diatom. When maintained in the laboratory, *L. virgulata* sheds thin sheets of spicule-containing material (Patton, 1972). Under both field and lab conditions, the gorgonian *Pterogorgia citrina* sheds transparent, noncellular organic membranes that have spicules stuck to them and are fairly well covered with diatoms and other microorganisms (Patton, 1972). *Pterogorgia citrina* may contain compounds structurally related to the fatty acid-derived butenolides, which exhibit antibiotic activity, from *P. anceps* and *P. guadalupensis* (Schmitz and Lorance, 1971). *Leptogorgia* and *Pterogoria* may effectively rid themselves of fouling organisms by exposing them to secondary metabolites, thereby inhibiting their growth until the outer surface is ablated or sloughed off and all fouling organisms are removed. This mechanism requires that the secondary metabolites are either biosynthesized at the surface of the animal or transported to the surface after being synthesized internally. The outer surface serves as a matrix that contains the active secondary metabolites.

Other coral species and some species of algae may employ similar combined mechanisms of defense against fouling organisms and predators. The soft coral (Alcyonaria) *Cespitularia* sp. aff. *subviridis* produces an abundant quantity of mucus when even slightly molested (Tursch et al., 1978). The mucus containing the toxic terpene palustrol is lethal to a variety of small crustaceans. The sea plume gorgonian *Pseudopterogorgia americana*, which contains several sesquiterpenes (Weinheimer et al., 1968), a dimethylimidazole acetic acid betaine named norzooanemanin (Weinheimer et al., 1973), homarine, and the structurally related compound trigonelline (Weinheimer et al., 1973), secretes a copious slime which may serve a defensive function (Ciereszko et al., 1973). The slime, comprising 7% of the dry weight of *P.*

americana, consists of a sulfated polysaccharide containing arabinose, fucose, and galactose (Ciereszko and Karns, 1973).

An important factor in the regulation of the fouling on some seaweed surfaces is the proteinaceous cuticle (Sieburth, 1975). Some algal surfaces are cleansed by the microzonal sloughing of this cuticle or a similar layer. The perennial brown marine alga *Ascophyllum nodosum* sheds its epidermis periodically and consequently rids itself of potential epiphytes including diatoms (Filion-Myklebust and Norton, 1981). Sieburth (1975), however, cites the red marine alga, *Chondrus crispus*, as an example of an alga that regulates fouling by using ablative surfaces and *A. nodosum* as an example of an alga that regulates fouling by using inhibitory substances, probably phloroglucinol polymers (Geiselman and McConnell, 1981); perhaps both regulatory mechanisms are used in *A. nodosum*. Similarly, both regulatory mechanisms may be employed in *Leptogorgia* species. Our study strongly suggests that a chemical defense against fouling organisms is operative in *Leptogorgia* species.

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MALE-PRODUCED AGGREGATION PHEROMONE OF THE MAIZE WEEVIL, *Sitophilus zeamais*, AND INTERSPECIFIC ATTRACTION BETWEEN THREE *Sitophilus* SPECIES[1]

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Abstract—A dual-choice pitfall bioassay was used to demonstrate the existence of a male-produced aggregation pheromone in the maize weevil. Both males and females showed a highly significant preference for extracts of disks exposed to wheat-feeding males over extracts of disks exposed to wheat kernels only. Neither sex responded significantly to extracts from females. Mating did not reduce pheromone release by males. Response by virgin females to pheromone was significantly higher than response by mated females, but males of either mating status responded equally well. There was no apparent daily periodicity in the responsiveness to pheromone. Rice and maize weevils showed a strong interspecific cross-attraction. Granary weevils of both sexes responded well to maize weevil extracts, but only females showed a significant response to rice weevil extracts. Neither maize nor rice weevils responded significantly to granary weevil extracts.

Key Words—*Sitophilus zeamais*, *Sitophilus granarius*, *Sitophilus oryzae*, maize weevil, granary weevil, rice weevil, Coleoptera, Curculionidae, aggregation pheromone, interspecific attraction.

INTRODUCTION

The maize weevil, *Sitophilus zeamais* Motsch. has long been recognized as a serious pest of stored grains. In some parts of the world, infestations may

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extend to field crops as well. Both adults and larvae can survive on a wide variety of food substances, but gain notoriety as a pest species primarily from their infestations of corn, wheat, rice, and sorghum.

Although Motschulsky first described and named *S. zeamais* in 1855, its status as a distinct species has only recently been recognized (Floyd and Newsom, 1959; Kuschel, 1961). It was previously referred to as the "large strain" of the rice weevil, *Sitophilus oryzae* (L.). The two weevils can be separated on the basis of aedeagal characteristics (Halstead, 1962), differences in thoracic indentations (Boudreaux, 1969), and by differences in their mycetomal symbiotes (Musgrave and Homan, 1962).

Because immatures develop entirely within a grain kernel, control must be aimed almost entirely at the adult. To date, control with insecticides and/or fumigation of grain stores, coupled with sanitary storage conditions and other cultural practices, has generally been effective. However, such chemical treatments are often not used in developing countries due to high cost or lack of availability of equipment. In addition, insecticide resistance has been reported (Champ and Dyte, 1976). The development of a novel, inexpensive method for monitoring and control would help reduce losses due to this pest.

Evidence for the existence of a male-produced aggregation pheromone in both the rice weevil (Phillips and Burkholder, 1981) and the granary weevil, *Sitophilus granarius* (L.) (Faustini et al., 1982) has been reported. We suspected the existence of a male-produced aggregation pheromone in *S. zeamais* and herein present evidence for its presence. In addition, we examined the relationships between these three important *Sitophilus* species by observing their interspecific responses to pheromone.

METHODS AND MATERIALS

Culturing. Maize weevils were obtained from laboratory stock cultures. Cultures were set up by adding two to three hundred 2- to 4-week-old mixed sex weevils to approximately 500 ml of soft spring wheat (moisture content 13%). Weevils and grain were held in 1-liter jars fitted with filter paper-lined screen caps. Cultures were maintained on a 16:8 light–dark photoperiod at 27 ± 1°C and 65 ± 5% relative humidity. After 10 days the adults were removed by sieving, and progeny emergence began in approximately 4 weeks. Insects for experimental use were collected by sieving the wheat daily to obtain adults of known age. Newly eclosed adults were immobilized under CO_2 and sexed using dimorphic rostrum characteristics (Halstead, 1962). Since mating was never observed the first day after emergence, we assumed that they were virgins. Rice weevil and granary weevil rearing techniques were described in previous publications (Phillips and Burkholder, 1981; Faustini et al., 1982).

Pheromone Collections. Pheromone collections were made by placing adult weevils (virgin males, except as indicated) singly in 1-dram vials, each provided with a 1.25-cm absorbent assay disk and one cracked wheat kernel. Vials were capped and held for 10 days, with three intermittent aerations to alleviate stress due to lack of oxygen. Control vials each contained a disk and a cracked wheat kernel only, and were also aerated three times.

A standard pheromone or control collection was made by pooling the disks and extracting them in redistilled hexane. A ratio of 50 disks per 30 ml of solvent was used. Disks were soaked for 2 hr, and then solvent was removed using a pipet. After filtration through glass wool, the sample was concentrated first by rotary evaporation and finally under purified nitrogen gas. Samples were held under nitrogen in a −40° C freezer. Three insect day equivalents (IDE; Phillips and Burkholder, 1981) or grain day equivalents (GDE) were used in each assay receptacle, except as indicated.

Bioassay. The dual-choice pitfall bioassay developed by Phillips and Burkholder (1981) was used to test the attractancy of collected samples. Briefly, insects were added to an inverted funnel, the stem of which fit through a hole in an inverted crystallizing dish. This unit rested on a sanded glass base. When the funnel was raised and test insects released, they could respond by crawling into either of two sample receptacles through holes drilled in the glass base (see Figure 1 in Phillips and Burkholder, 1981).

For bioassays, 10 insects of known age, sex, and mating status were added to each funnel and conditioned for 30 min in the dark. Insects were 7- to 8-day-old virgins, unless otherwise indicated. From 10 to 30 bioassay setups were run concurrently. Samples were prepared for bioassay during the conditioning period. Using 10-μl pipetes, pheromone and control extracts were applied to 1.25-mm bioassay disks placed singly in 5-dram vials. Concentrations were adjusted so that 10 μl of solvent were always applied. After application of the samples, the vials were covered with Parafilm M® and held at room temperature until bioassayed. Samples were added to the bioassay chamber vials at the end of the conditioning period, and bioassays were conducted as described by Phillips and Burkholder (1981), except that they lasted 20 min, instead of 15. To terminate the bioassay, room lights were switched on, and the chambers were quickly removed from the vials to prevent additional insects from falling into the sample receptacles. The numbers of weevils responding to treatment and control, and those remaining in the chamber, were recorded. Ten replicates of each choice pair were completed for each sex in each experiment. Insects were always discarded after bioassay.

Analysis of Results. Response was defined as the difference between treatment and control (T − C), and could thus range from a high of +10 to a low of −10. Responses were calculated for each pitfall unit and compared using an analysis of variance for a completely randomized design. Means were

separated using Duncan's new multiple-range test at $\alpha = 0.05$ (Steel and Torrie, 1960).

Significance of the response to each treatment was determined by using the Student's *t*-test for paired data (Steel and Torrie, 1960). Differences at the 5% level were accepted as significant.

Establishing Presence of a Pheromone. Test samples were prepared from extractions of disks from single vial collections from virgin males, females, and wheat controls. Ten IDE or GDE were used for bioassay, and test insects were 4–6 days old.

Groups of either sex were bioassayed in pitfall chambers containing one of the following pairs of extract choices: male or wheat, female or wheat, or wheat in both vials.

Effect of Time of Day on Response to Pheromone. Initially, a bioassay was run 3 hr into scotophase to ensure the potency of the extract, and the activity level of the insects. Subsequently, bioassays were run at 4-hr intervals throughout the 24-hr period beginning at 3 PM and ending at 11 AM the following day. Thus, responsiveness of both males and females was observed at each of six times throughout a 24-hr period. The experiment was repeated several days later and results were pooled for analysis.

The bioassay developed by Phillips and Burkholder (1981) was conducted in the dark. It was therefore decided that all conditioning periods and bioassays would be run in the dark, even for insects in photophase. The insects remain active in the light, even after 30 min conditioning. Responses elicited by quiescent (i.e., dark-conditioned) insects would therefore be more valid.

Effect of Mating Status on Response to Pheromone. To prepare the test insects, newly emerged weevils were sexed and placed in jars with wheat at a ratio of either 2:1 males–females or the converse. The excess number of insects was added to ensure the mating of the opposite sex. Virgin weevils of both sexes were maintained separately for controls. Weevils were held for 10 days, allowing the pairs adequate time to mate. They were then removed from the grain and sexed under CO_2 gas. The most abundant sex from each jar was discarded, whereas the remaining sex was returned to grain and held for bioassay. Virgin weevils were "sham" sexed by placing them under CO_2 for 10 min, the approximate time of exposure of the paired weevils. They too were then returned to the grain, and all jars were held overnight to ensure complete recovery from the CO_2 exposure. Bioassays were run on the following day.

Effect of Mating Status on Pheromone Release. Newly emerged maize weevils were sexed and paired 3:1 females–males. Each group of four weevils was placed in a screen-capped 5-dram vial with 10 ml of wheat. Thirty such vials were set up. For controls, four virgin males were placed together in each of eight 5-dram vials, also containing 10 ml of wheat. All vials were held in the rearing chamber under standard conditions for 9 days to allow the males

sufficient time to mate. The male weevils were then separated from the females under a magnifying glass, without CO_2 anesthesia. Mated males were placed singly in 1-dram vials with a disk and cracked grain and were held for 7 days. Single-vial collections of virgin males and wheat controls were set up concurrently. At this time all disks were extracted and concentrated. Both males and females were bioassayed.

Interspecific Response. Samples for bioassay were obtained from single vial collections using newly emerged virgin males from each species, as well as wheat controls.

Both sexes of each species were bioassayed using extracts from males of the other two species, as well as their own. Thus 18 pitfall units were required to complete one replicate. The 18 units were randomized each day, and the 10 replicates were completed over a 10-day period. Three IDE and GDE were used to test maize and rice weevils. However, granary weevils were not responsive to such low concentrations (Faustini et al., 1982) so 10 IDE or GDE of each sample were used. Total solvent applied to test disks was always 10 μl.

RESULTS

Both sexes of the maize weevil showed a highly significant attraction ($P < 0.001$) to extracts of disks exposed to males (Table 1). Neither sex responded significantly to extracts of disks exposed to females. All responses by both sexes to female or wheat control extracts were significantly lower than responses to male extracts ($P < 0.05$).

Responses by males and females were statistically equivalent at all six

TABLE 1. RESPONSE OF VIRGIN MAIZE WEEVILS TO VIRGIN MALE, FEMALE, AND WHEAT EXTRACTS

Sex tested	Treatment	Control	Mean response[a] (T − C) ± SE[b]
M	M	W	7.0 ± 0.7 a ***[c]
F	M	W	6.9 ± 0.8 a ***
M	F	W	−0.7 ± 0.7 bc NS
F	F	W	0.7 ± 1.0 bc NS
M	W	W	−1.4 ± 1.0 c NS
F	W	W	1.3 ± 0.5 b *

[a] Means followed by the same letter do not differ significantly ($P > 0.05$) according to Duncan's new multiple range test.
[b] (Treatment − control) ± standard error.
[c] *** $P < 0.001$; * $P < 0.05$; NS, not significant; Student's t test for paired data.

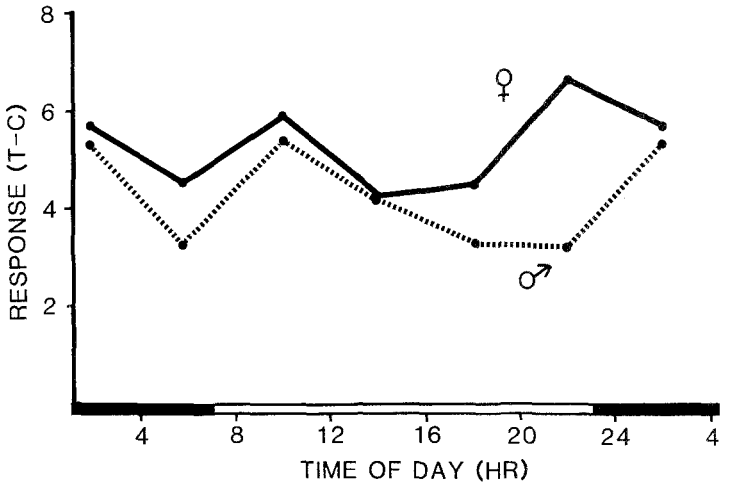

FIG. 1. Effect of time of day on response of maize weevils to pheromone produced by males.

times tested (Figure 1). The fluctuations were nearly identical for the two sexes, except just prior to the onset of scotophase. There, female response reached its highest mean value of 6.6, whereas male response was at its lowest, 3.2. This difference was not statistically significant.

Virgin males and females showed statistically equivalent mean responses of 5.5 and 4.6, respectively (Table 2). Response by mated males was not significantly different from that by virgin males. The responses by mated males and females were statistically equivalent, but virgin females were more strongly ($P < 0.05$) attracted to pheromone than were mated females.

TABLE 2. EFFECT OF MATING STATUS ON RESPONSE OF MAIZE WEEVILS TO PHEROMONE PRODUCED BY VIRGIN MALES

Sex	Mating status	Mean response[a] (T − C) ± SE[b]
M	Virgin	5.5 ± 0.54 a ***[c]
M	Mated	4.2 ± 1.22 ab *
F	Virgin	4.6 ± 0.69 a ***
F	Mated	1.9 ± 0.80 b *

[a] Means followed by the same letter do not differ significantly ($P > 0.05$) according to Duncan's new multiple-range test.

[b] (Treatment − control) ± standard error.

[c] *** $P < 0.001$; * $P < 0.05$; Student's t test for paired data.

Extracts from virgin and mated males proved equally attractive to virgin weevils of both sexes. All responses were highly significant ($P < 0.01$). Mating did not have a significant effect on pheromone release.

Males and females of all three species responded significantly ($P < 0.05$) to extracts from male conspecifics (Table 3). Granary and rice weevils of both sexes showed a highly significant ($P < 0.01$) attraction to extracts from maize weevil males. Granary weevil females were significantly attracted to rice weevil male extracts ($P < 0.05$), but males were not. Both sexes of the maize weevil were strongly attracted to rice weevil male extracts ($P < 0.01$), but did not show a significant response to the granary weevil. Rice weevils did not respond significantly to extracts from male granary weevils.

DISCUSSION

Evidence for the existence of a male-produced aggregation pheromone in *Sitophilus zeamais* is convincing. Both males and females were strongly attracted to extracts of disks exposed to males, whereas extracts from females proved unattractive. These results agreed with those obtained with other *Sitophilus* species by Phillips and Burkholder (1981) and Faustini et al. (1982).

The function of the pheromone is probably complex. We suspect pheromone release occurs in the presence of food and oviposition sites, causing aggregation of conspecifics at optimum locations. Initiation of feeding and/or mating behavior may follow aggregation.

Evidence for both sexual and feeding functions of the pheromone are apparent in our results. The sexual function is suggested since mated females demonstrated a significantly reduced response as compared to virgins. Maize weevils are known to be polygamous (Richards, 1947), and would thus be expected to respond whether mated or not. That mated males still released pheromone could relate to either a sexual or feeding role of the pheromone. Mated males can mate again, and release of pheromone would continue to attract females for postvirginal mating. In addition, since these insects are long-lived, they need to feed to survive. The task of chewing through the seed coat of a grain kernel requires the expenditure of a great deal of energy. Females require some 30 min or more to make a hole in a wheat kernel large enough to deposit an egg (Cotton, 1920). Personal observations indicated that when a weevil was feeding on a fresh kernel of wheat, one or more companions were often present. Weevils often hollowed an entire wheat kernel before feeding on other, untouched kernels. The pheromone appears to play a role in initiating such feeding aggregations. The aggregations are beneficial to the initiator, since food sources may not be available without the efforts of several weevils. Such aggregations are vital in bark beetles (Birch, 1978).

TABLE 3. MAIZE WEEVIL–RICE WEEVIL–GRANARY WEEVIL INTERSPECIFIC RESPONSE[a]

Test sample	Responding species and sex					
	MW♂	MW♀	RW♂	RW♀	GW♂	GW♀
MW♂	5.7 ± 0.82a-e***	6.2 ± 0.58a-d***	8.2 ± 0.53a***	7.9 ± 0.86ab***	3.6 ± 1.05d-g**	4.5 ± 0.70c-g***
RW♂	4.8 ± 1.20b-f**	7.0 ± 0.45a-c***	7.9 ± 0.64ab***	6.4 ± 0.70a-d***	1.3 ± 0.84g-i^{ns}	3.1 ± 1.17e-g*
GW♂	−0.3 ± 1.36hi^{ns}	2.1 ± 1.13f-h^{ns}	−0.5 ± 1.67hi^{ns}	−1.2 ± 0.92i^{ns}	4.6 ± 1.07c-f**	3.9 ± 1.32c-g*

[a] Means followed by different letters are significantly different ($P < 0.05$) according to Duncan's new multiple-range test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significant; Student's t test for paired data. Values are mean (treatment − control) ± standard error. Ten replicates per treatment.

The consistent attractancy of extracts of virgin or mated males to males of either mating status is intriguing. Nothing appears to daunt male responsiveness to other males. There appears to be no selective advantage for a male to attract another male, a potential mating competitor. Shorey (1973) suggested that aggregation pheromones in Coleoptera arose as mechanisms to cause aggregation at a suitable food source, and implied that the sexual function arose secondarily. It is also possible that, evolutionarily, the males developed the ability to produce sex pheromone first. The ability of a male to perceive the pheromone may have evolved as a secondary sexual strategy. A male could benefit by mating with females already drawn to an area by other males.

No periodicity of responsiveness to pheromone by maize weevils was evident in this study. In contrast, granary weevils appeared responsive only during photophase (Faustini et al., 1982). Maize weevil results suggest several possibilities: (1) pheromone release may be nearly continuous; (2) responsiveness may not be directly correlated with release (i.e., while pheromone may normally be released only at certain times during the photoperiod, response is possible whenever pheromone is introduced); (3) daily temperature fluctuations may affect weevil responsiveness to pheromone, or temperature and light periodicity may interact to affect response. Temperature and relative humidity were held constant in this experiment, so no conclusions can be made in this regard.

In scolytid beetles, males generally tend to be the colonizers in species which are polygamous, and females colonize in monogamous species (Borden, 1974). Bishara (1968) observed that male maize weevils were stronger fliers than females. It is proposed that males are the primary colonizers for *S. zeamais*. They may arrive first at a food source, then attract other weevils for the purposes of mating and feeding.

The relationships between the species have been established primarily on the basis of hybridization studies (Birch, 1944; Floyd and Newsom, 1959) and morphology. There are minor morphological differences between maize and rice weevils, but the fact that the granary weevil has lost its hindwings, and the elytra are fused, sets it apart from the other two. In addition, McLaurin and Downe (1966) showed that all three were serologically distinct, and confirmed that the maize and rice weevils were more closely related to each other than to the granary weevil.

The results of the interspecific attraction experiment reinforced the findings of previous researchers. The strong cross-attraction between maize and rice weevils suggests that the two are very closely related. Male and female granary weevils were strongly attracted to maize weevil extracts, and females responded well to rice weevil extracts. However, neither maize nor rice weevils were significantly attracted to granary weevil extracts. This emphasizes the

close relationship between the maize and the rice weevil and their more distant relationship to the granary weevil.

Because *S. zeamais* extracts were attractive to both of the other species, and none of the other extracts were universally attractive, we propose that the composition of the pheromone of this species is the closest to the primitive condition. As evolution progressed, the three groups diverged, with the rice weevil pheromone remaining very close to the ancestor's, whereas the granary weevil pheromone changed most dramatically. However, there must be some differences between the pheromones of *S. zeamais* and *S. oryzae*, since only females of *S. granarius* were attracted to *S. oryzae* extracts.

In summary, we have presented evidence for the existence of a male-produced aggregation pheromone in *Sitophilus zeamais*. This is the third *Sitophilus* pheromone shown to exist. Interspecific studies confirmed already suspected taxonomic relationships between these three economically important grain weevils. Further work toward deciphering the communication code of these insects will lead to improved methods of monitoring and control.

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IDENTIFICATION OF HOST PLANT ATTRACTANTS FOR THE CARROT FLY, *Psila rosae*[1]

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Abstract—Cold-trapped carrot leaf volatiles were analyzed by gas chromatography with an outlet splitter to a flame ionization detector and to a carrot fly antennogram preparation as the second detector (GC-EAD). Strongest EAD responses were elicited by products whose elution temperatures corresponded to the propenylbenzenes, *trans*-methylisoeugenol (3,4-dimethoxy-1-propenylbenzene) and *trans*-asarone (2,4,5-trimethoxy-1-propenylbenzene) and, to a lesser extent, by-products matching the elution temperatures of the leaf aldehydes hexanal, (*E*)-2-hexenal, and heptanal, and of the terpenes linalool and caryophyllene. The identity of the propenylbenzenes was confirmed by gas chromatography–mass spectrometry. GC-EAD permitted accurate estimation of the olfactory thresholds; it was lowest for *trans*-asarone at 500 attogram (5×10^{-16}g)/ml of air passing over the antenna. Both the leaf aldehydes and propenylbenzenes were attractive when tested individually in the field with yellow sticky traps; fly captures were linearly related to the quantity of propenylbenzenes applied per trap. A combination of *trans*-asarone and hexanal was more attractive than either compound singly, suggesting that the fly is adaptively equipped to respond to a mixture of compounds emanating from carrot foliage. In laboratory choice tests, flies were more attracted by vapors from intact carrot foliage than by that from a nonhost; leaf odor alone also mediated oviposition. We conclude that through the selectivity and sensitivity of its response to foliar volatiles, the carrot fly may achieve host-plant orientation and also at close range, in union with its response to less volatile leaf surface components, selection of an oviposition site.

Key Words—Carrot fly, *Psila rosae*, Diptera, Psilidae, host odor, leaf aldehydes, propenylbenzenes, *trans*-asarone, *trans*-methylisoeugenol, gas chromatography linked electroantennographic detection, attractants, field traps, aldehydes, aromatics, alcohols, terpenes.

[1]Diptera: Psilidae.

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INTRODUCTION

Leaf color and shape and the presence of contact chemostimulants in the cuticular layer of carrot leaves mediate, at least in part, host plant selection by the carrot fly *Psila rosae* (F.) (Städler, 1977); *trans*-methylisoeugenol, a propenylbenzene isolated from carrot leaves, is an oviposition stimulant for the fly (Berüter and Städler, 1971; Städler, 1972). It has been suggested that olfactory cues do not influence host finding in this species (Bohlen, 1967; Städler, 1972). This is improbable, however, since this oligophagous pest is attracted by Umbellifer-associated propenylbenzenes in the field (Guerin and Städler, 1980). Indeed, the olfactory system of the carrot fly is sensitively tuned to the perception of a group of generally occurring green leaf volatiles and some more host-specific products (Guerin and Visser, 1980). Here we identify the principal attractants in carrot foliage for the carrot fly and demonstrate their role in field attraction, host selection, and oviposition.

METHODS AND MATERIALS

Laboratory Bioassays

Cages, 50 × 50 × 50 cm, as described by Städler (1977) were used routinely with uniform illumination throughout and maintained at 21° C, 75% relative humidity with 16 hr light. One hundred females and 50 males between 3 and 6 days old (peak oviposition age) were obtained from a laboratory culture and introduced into the bioassay cage 24 hr before tests. Prior to this, flies were provided with an apple seedling (40 cm high) as a copulation site and with carrot leaves for oviposition.

Host Selection and Oviposition. Flies were offered the vapor over carrot or fern, *Nephrolepis exaltata* (L.) (nonhost), to determine the influence of volatiles on host selection. The foliage of each plant (20 g) with stems in water was enclosed within a black Plexiglas cylinder (14 × 20 cm high) and placed at diagonally opposite corners of the bioassay cage. Bronze gauze (1-mm mesh) sealed the opening to the cylinder from above, leaving a clearance over the foliage. The number of females alighting and probing the gauze with the ovipositor was counted every 15 min during the daily peak in oviposition (last 3 hr of light); females were blown off and cylinder positions reversed at each count.

The effect of plant vapor on oviposition was measured by daily counts of egg numbers deposited on two moistened oviposition substrates (5 cm diameter) (cf. Städler, 1977) supported immediately beneath the bronze gauze. To avoid any bias due to repellents from fern, tests were also made with moist filter paper as control.

Oviposition Stimulants. Host-plant volatiles eliciting strong electroantennogram (EAG) and behavioral responses were examined for their effects on oviposition. A 5-mg sample of the substance in methylene chloride (Fluka, puriss. grade) was applied to an artificial leaf made of a double strip of chemically pure filter paper (5 × 15 cm) cut pinnatedly; two replicates of the impregnated surface were attached to oviposition substrates (cf. Städler, 1977) and placed in the bioassay cage with controls to which solvent only had been applied. Eggs were counted and positions fully randomized daily for a minimum of 3 days.

Chemistry

Foliar Extract. Paraffin oil was used as a slow release medium for foliar volatiles. Some 50 g of carrot foliage frozen at −20° C and ground in a coffee blender was dissolved in 50 ml paraffin oil (Merck, Uvasol grade) with 25 g Na_2SO_4. After blending (1 min) the slurry was centrifuged for 10 min at 8000*g* and the oil plus dissolved volatiles was filtered over Na_2SO_4 on Whatman No. 1 filter paper in a pressure funnel under N_2 at 10 psi. From a 10^{-2} dilution of the extract (v/v in paraffin oil) 1 ml was placed in a pill glass beneath the bronze gauze of the oviposition device, described above, with paraffin oil alone as control at the opposite corner of the cage. Eggs were counted, positions reversed, and odor source replaced daily.

Collection and Analysis of Foliar Volatiles. Some 100 g of carrot foliage (stems plus leaves) were shredded into a 1-liter gas-wash bottle. Pressurized air was driven over charcoal through the bottle at 1 liter/min for 5 min and subsequently through a U tube (30 × 0.5 cm ID) immersed in acetone and dry ice in a Dewar flask; three such samples were taken in separate U tubes. The volatiles in each cold trap were dissolved in 0.5 ml pentane (Fluka, puriss. grade) and the combined washings were dried by placing the extract in a glass vial at −20° C where the remaining water droplets froze on the walls.

The extract was analyzed by gas chromatography (GC) on a SP-1000 glass capillary column (15 m × 0.3 mm ID) with outlet splitter to a flame ionization detector (FID) and an electroantennogram preparation, termed the electroantennographic detector (EAD) in a ratio of 2:1. The EAD detector incorporated a female carrot fly antenna mounted in the manner already described (Guerin and Visser, 1980) connected to a differential amplifier (BB 3670) with high input impedance (10^{13} Ω) and low bias current (<10 pA). The unit required minimal shielding and was connected to the recorder over a high-pass filter with a corner frequency of 0.003 Hz.

The identity of the compounds evoking strongest EAD responses was confirmed by gas chromatography–mass spectrometry (GC-MS) employing a Finnigan 4000 equipped with a 6111 data system and a 50-m SP 1000 glass capillary column.

EAG Screening

The sensitivity spectrum of the carrot fly olfactory system was observed by means of the EAG to 50 previously unexamined volatiles of Umbelliferae which were available in sufficient quantity and purity from commercial sources (≥95%, GC). The odor delivery system and recording technique has been described (Guerin and Visser, 1980). The oviposition stimulant, *trans*-methylisoeugenol, was employed as standard since the antenna is sensitive to the product and its field attraction for the fly had already been established (Städler, unpublished data). Plant volatiles showing EAG activity greater than that of *trans*-methylisoeugenol at 10^{-3} (v/v in paraffin oil) were subjected to behavioral assays.

Field Tests

Experimental Design. Attraction to individual volatiles obtained from commercial sources and whole essential oil extracts (as supplied by J. Chiquet, Basel) from six host plants of the carrot fly was field tested during the second generation flight in 1979 and 1980. *trans*-Asarone was supplied by Sigma Chemical Company, St. Louis, Missouri; *trans*-methylisoeugenol by K & K Labs Division, Plainview, New York; and the aldehydes hexanal, (*E*)-2-hexenal, and heptanal by Fluka AG, Buchs, Switzerland. Tests were undertaken in large fields of mature carrots in areas under intensive cultivation and supporting large populations of the fly at Tägerwilen (Canton Thurgau), in the Berner Seeland (Cantons Bern and Fribourg) and at Wädenswil. The volatiles were tested in conjunction with a yellow color trap (a sheet of Plexiglas 20 × 20 cm, ICI-229) coated with Tanglefoot® glue; this trap is an established tool for monitoring the carrot fly (Freuler et al., 1982). The traps were attached to timber poles 60 cm above the ground which, depending on crop growth, gave a clearance of between 10 and 30 cm above the carrot foliage. Where possible, the experiments were laid out in a Latin square with a minimum of 5 replicates per treatment and 5 m between individual traps. Otherwise, randomized blocks were used. Insect captures were counted at three-day intervals or more frequently as population densities warranted.

Statistical Analysis. Trap captures were compiled in a contingency table and tested for homogeneity by means of χ^2. Significance between treatments was established using a single classification χ^2 and comparisons between individual treatments was made using the formula $u = (a - b)/N/2$ based on the multinomial distribution and normal approximation; a and b are the pooled totals for the treatments being compared and N the grand total summing through all treatments. This test is highly efficient (Berchtold, personal communication) and can be carried out with ease.

Dispensers. Fly capture was measured as a function of the release rate of *trans*-methylisoeugenol from silicone tubing (3 mm ID × 6 mm OD); the quantity given off is linearly related to the length of the tube: 0.5-, 5.0-, 50-, 125-, and 250-cm pieces release a mean of 1.0, 12, 106, 244, and 560 mg/day ($r = 0.997$, $P < 0.001$ with three degrees of freedom). These different lengths of tubing, filled with the product and stoppered at each end, were stretched between clamps across the top of the trap. *trans*-Asarone, being crystalline, was uniformly applied using a pipet to the glue in pentane (Fluka, purum grade) at 5.0, 50 and 500 mg/trap (a half to each face). A polyethylene capsule, 30 × 15 mm diameter with four 1-mm holes pierced in the lid and attached to the top of the trap was employed for the C_6 and C_7 green leaf alcohols and aldehydes; this dispenser releases (*E*)-2-hexenal at 15 mg/day in warm weather (midday temperatures at 24–28° C). The same dispenser sufficed for all the other volatiles while a 20-cm length of polyvinylchloride tubing (3 mm ID × 5 mm OD) dispensed the essential oil extracts.

RESULTS

Analysis of Foliar Volatiles

GC-EAD. Based on the chromatogram, the cold-trapped volatiles may be divided into three sections: (1) low-boiling-point compounds eluting with or on the tail of the solvent peak (30–60° C); (2) those eluting midway through the temperature program (100–130° C); and (3) two high-boiling-point compounds eluting in the higher temperature range (>165° C) (Figure 1). Distinct EAD responses were obtained in each of the three sections coinciding with peaks whose elution temperatures match those of known standards. EAD responses within the solvent peak correspond to hexanal (34° C) and to (*E*)-2-hexenal and heptanal, coeluting at 44° C (Figure 1). The presence of large amounts of these volatiles was confirmed in the absence of solvent by taking a 0.5-ml sample of air over the shredded foliage in a gas-tight syringe and injecting it directly onto the column (Guerin and Stadler, 1982). In section 2, the peaks evoking strong EAD responses at 102° C and 104° C correspond with the elution temperatures of linalool and *cis*-caryophyllene, respectively; none of the other peaks evoking strong EAD responses in this section of the chromatogram have as yet been examined (Figure 1). The most pronounced EAD responses were obtained in section 3, at 169.5° C, corresponding to the elution temperature of *trans*-methylisoeugenol and 5.5 min later at 180° C (upper temperature limit of the program) matching *trans*-asarone (Figure 1).

While *trans*-methylisoeugenol was represented by a distinct peak on the chromatogram, the quantity of *trans*-asarone was below the detection limit of

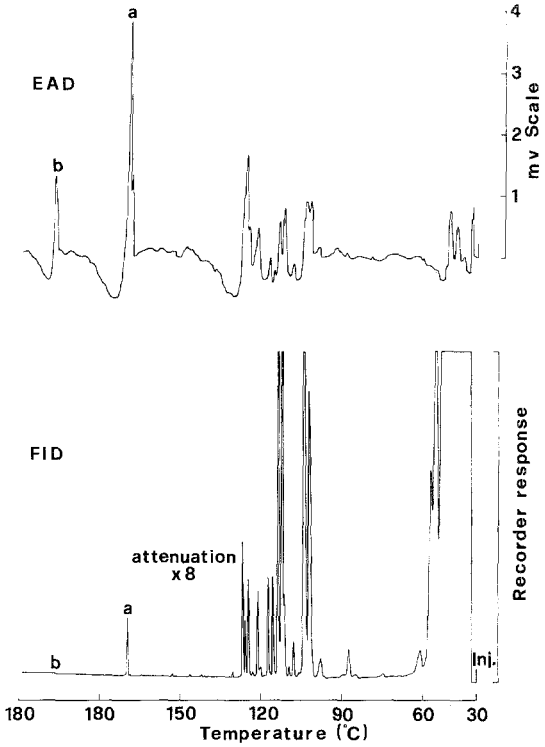

FIG. 1. Gas chromatograms with (1) EAD responses of a carrot fly antenna and (2) FID responses to cold-trapped volatiles of carrot foliage. Splitless injection, 1 min at 30°, 10°/min to 60° and 5°/min to 180°C with He as carrier gas. For further specifications see text.

the FID. However both products were present in sufficient quantity for confirmation by GC-MS (dimethoxy—propenylbenzene: m/z 178, M^+; 163, M^+-CH_3; 147, M^+-OCH_3; 135, M^+-CH_3-CO; and trimethoxy-propenylbenzene: m/z 208, M^+; 193, M^+-CH_3; 177, M^+-OCH_3; 165, M^+-CH_3-CO). Evidence was also obtained for the presence of *cis* isomers of both products in the extract.

The amount of *trans*-methylisoeugenol and *trans*-asarone present was estimated at 343 ng and 75 ng, respectively, based on comparisons of EAD responses obtained with the extract to those established by injecting known quantities of standards. Since the 15 liter of air drawn over the 100-g shredded foliage can be expected to have carried off but a fraction of the volatiles, we may assume that an average carrot leaf weighing 5 g contains, at a minimum, 17.2 ng *trans*-methylisoeugenol and 3.75 ng *trans*-asarone.

Response Thresholds. The response curves obtained by passing successive dilutions of the two propenylbenzenes simultaneously over the FID and EAD demonstrates the relative sensitivity of the antenna to the products and permits the estimation of perception thresholds (Figure 2). The lowest injected quantity evoking a distinct EAD response was 100 pg *trans*-methylisoeugenol and 100 fg *trans*-asarone (Figure 2). Taking into account the split ratio between the two detectors, the rate of air flow over the antenna and the manner in which the compounds elute from the column (FID peak shape), the perception threshold (expressed as the quantity of compound/ml of air passing over the antenna), is 1 pg/ml (3.4×10^9 molecules) for *trans*-methylisoeugenol and 2000 times less at 500 attogram/ml (1.4×10^6 molecules) for *trans*-asarone [1 attogram (ag) = 10^{-18}g].

EAG Screening. The responses evoked by umbelliferous volatiles in the EAG confirm the results obtained by GC-EAD analysis of the foliar extract. The responses detailed here are at a 10^{-3} dilution of each product in paraffin oil, a level more indicative of the selectivity of the olfactory system than higher concentrations. Since the sensitivity of an antenna drops during the test period the EAGs are expressed as a percentage of the response to a standard (*trans*-methylisoeugenol at 10^{-2}) which was applied at regular intervals.

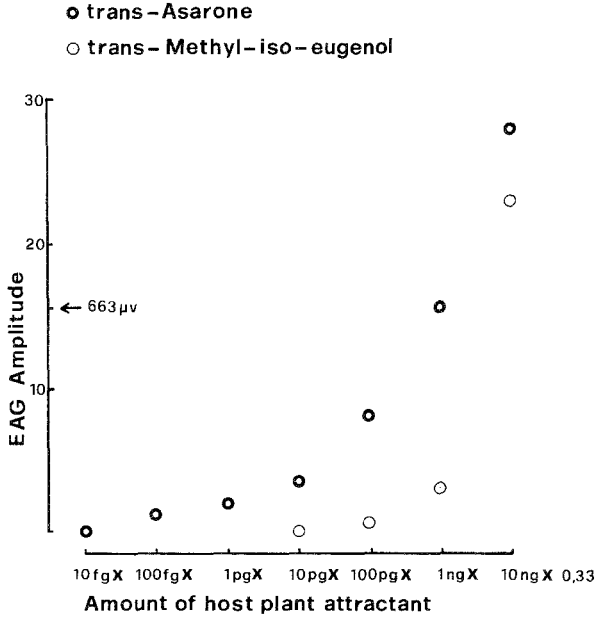

FIG. 2. Responses of a carrot fly antenna to propenylbenzenes obtained by employing combined gas chromatography and electroantennographic detection (GC-EAD).

Strong EAG responses were evoked by three groups of compounds: (1) the leaf aldehydes such as heptanal (37.2 ± 0.2), octanal (31.0 ± 7.5), nonanal (19.8 ± 4.7), and the leaf ester hexyl acetate (44.1 ± 13.0); (2) the acyclic terpene linalool (47.3 ± 17.1), and sesquiterpene *cis*-caryophyllene (49.8 ± 13.8); and (3) to the allyl- and propenylbenzenes listed in Table 1.

trans-Asarone evoked the highest EAG response (146.7 ± 13.5), four times higher than its geometric isomer *cis*-asarone (39.3 ± 7.0) and 10 times that of its analog, *trans*-methylisoeugenol (13.7 ± 1.3) (Table 1 and Figure 3); the related benzaldehyde, asaronaldehyde, elicited no response. The presence of an extra methoxy group in *trans*-asarone apparently accounts for the 10-fold difference in response over its dimethoxy analog, *trans*-methylisoeugenol. Male and female responses to *trans*-asarone and *trans*-methylisoeugenol were equal both relative to the standard and in absolute amplitude.

It may be of interest to compare the responses evoked by the allyl- and propenylbenzenes from the viewpoint of structure activity relationships. Only the di- and trimethoxy-propenylbenzenes *trans*-methylisoeugenol and *trans*-asarone elicited a significantly higher response than their allylbenzene counterparts, methyleugenol (3,4-dimethoxy-1-allylbenzene) and elemicine (3,4,5-trimethoxy-1-allylbenzene) (Table 1 and Figure 3). The combination of three methoxy units on the benzene ring with the stereochemistry of the propenyl group apparently accounts for the unique EAG activity evoked by *trans*-asarone (Figure 3). Both the propenylbenzenes, anethole (4-methoxy-1-propenylbenzene) and isosafrole (3,4-methylenedioxy-1-propenylbenzene), elicited as high a response as *trans*-methylisoeugenol but no higher than their allylbenzene analogs, methylchavicol and safrole (Table 1). As contrasted

TABLE 1. EAG RESPONSES OF FEMALE CARROT FLIES TO PROPENYL- AND ALLYLBENZENES AS PERCENTAGE OF RESPONSE TO *trans*-METHYLISOEUGENOL AT 10^{-2} [a] (± ONE STANDARD ERROR; $N = 6$ IN EACH CASE)

Propenylbenzenes 10^{-3}		Allylbenzenes 10^{-3}	
trans-Asarone	146.7 ± 13.5	Elemicine	7.0 ± 3.2
cis-Asarone	39.3 ± 7.0		
trans-Methylisoeugenol	13.7 ± 1.3	Methyleugenol	6.3 ± 2.2
Anethole	12.2 ± 1.0	Methylchavicol	15.0 ± 2.7
4-Methyl-1-propenylbenzene	13.3 ± 2.0		
Isosafrole	10.5 ± 2.0	Safrole	14.5 ± 0.9
Isoeugenol	1.2 ± 0.8	Eugenol	1.9 ± 1.1
		Myristicin	10.6 ± 2.3
		Apiole	2.6 ± 2.6

[a] Concentration of the compound in paraffin oil expressed on a volume/volume basis.

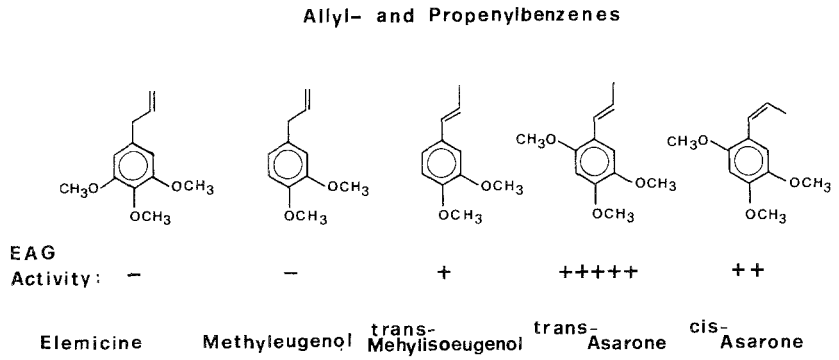

FIG. 3. Structures of some allyl- and propenylbenzenes and their relative strengths as olfactory stimulants for the carrot fly.

with the propenylbenzenes, increasing methoxylation of allylbenzenes apparently reduces EAG activity since methylchavicol was significantly more active than methyleugenol, and elemicine and safrole were more active than the mono- and dimethoxy-methylenedioxy analogs, myristicin and apiole (Table 1). Both the hydroxy products, isoeugenol (4-hydroxy-1-propenylbenzene), and the allylbenzene, eugenol, evoked little or no response (Table 1).

Behavioral Observations

Host Odor Discrimination. Volatiles from carrot foliage attract the carrot fly; 10 times as many insects alighted in the vapor over foliage from the host plant as compared with fern (Table 2). Under these test conditions, apparently only females were attracted since all alighting insects displayed the typical behavior response of probing the metal gauze with the ovipositor. This response by gravid females was strong considering the rigors of blowing off the responding insects and reversing the positions of the test materials every 15 min. Females deposited significantly higher egg numbers over the foliage of carrot than over fern. Similar results were obtained with moist filter paper as control, thus eliminating any bias arising from possible repellency of the nonhost (Table 2). Flies also showed a preference for the vapor over a paraffin oil extract of carrot foliage as a site for oviposition, demonstrating that the pertinent volatiles were extractable and sufficiently stable for analysis.

Oviposition Stimulants. Significantly higher egg numbers were deposited near artificial substrates treated with *trans*-asarone (139.6 $\pm$ 12.1) than on controls (90.4 $\pm$ 9.8) ($P < 0.0005$, Friedman test); a similar observation was made with *trans*-methylisoeugenol in an earlier study (Berüter and Städler, 1971). No such effect was observed for *cis*-asarone, asaronealdehyde,

TABLE 2. LABORATORY BEHAVIOURAL OBSERVATIONS WITH CARROT FLIES

Behavior		N	Significance level (P)
Female landings in the headspace vapor over			
Carrot foliage	Nonhost		
5.0 ±0.7	0.5 ± 0.8	12	<0.003[a]
Egg numbers deposited in the headspace over			
Carrot foliage	Blank control		
89.0 ± 20.8	42.3 ± 13.4	4	<0.005[b]
Carrot Foliage	Nonhost		
171.8 ± 28.0	82.5 ± 8.4	4	<0.025[b]
Carrot leaf extract	Blank control		
134.6 ± 13.3	79.5 ± 10.3	14	<0.001[a]

[a] Wilcoxon test.
[b] Friedman test.

safrole, or *cis*-caryophyllene in this study; a number of other host-related volatiles were screened by Städler (1972).

Field Responses

Individual Volatiles. *trans*-Asarone and *trans*-methylisoeugenol proved strong field attractants, fly captures were linearly related to the dose applied per trap and release rate from the dispenser per day, respectively (Figures 4 and 5). The release rate of *trans*-asarone from the glue was very low; 100 mg of the neat product in a Petri dish lost on average 370 μg/day in the open in warm weather (peak daily temperature reaching 28° C) and crystals of the product were observable in the glue of treated traps after many months of storage at room temperature. Applying 50 mg of *trans*-asarone/trap doubled the efficiency of the color trap alone (control), whereas a release rate of 500 mg/trap/day was required to achieve a similar effect with *trans*-methylisoeugenol (Figures 4 and 5); *trans*-asarone was clearly the superior attractant. The proportion of immature females (characterized as possessing eggs without a striated chorion) captured in traps with 500 mg *trans*-asarone/trap was significantly higher than with lower doses of the product ($\chi^2 = 10.25$, $P < 0.05$ with four degrees of freedom). No sex-specific effects of the propenylbenzenes were observed.

The aldehyde components of the green leaf volatile complex, hexanal and heptanal, proved strong attractants for the carrot fly; the corresponding leaf alcohols, hexanol and heptanol, were unattractive (Figure 6). Hexanal was

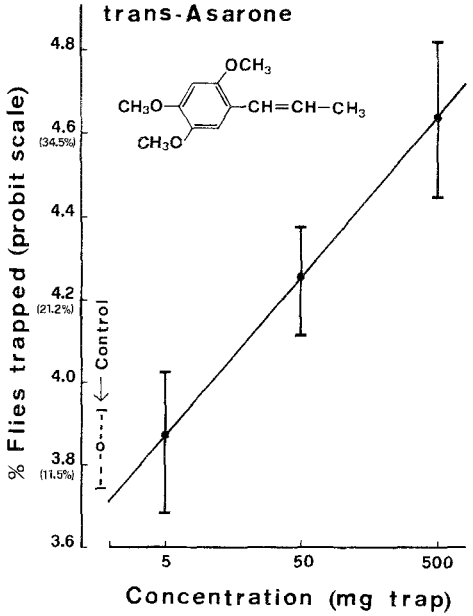

FIG 4. Carrot fly field responses to *trans*-asarone applied to glue-covered yellow traps.

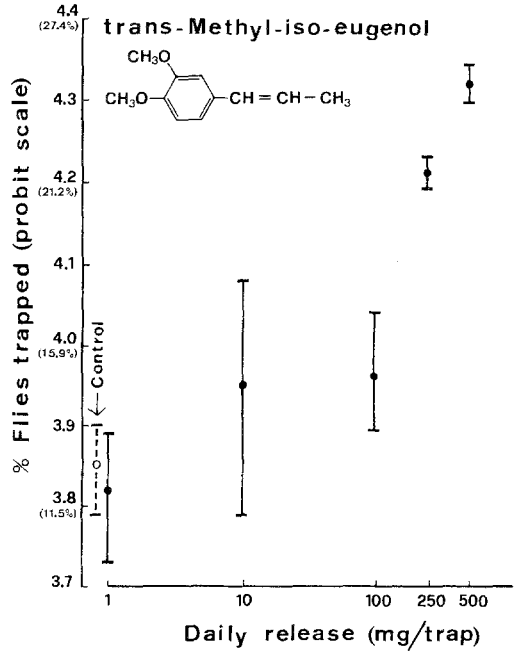

FIG. 5. Carrot fly field responses to *trans*-methylisoeugenol released from silicone dispensers on glue-covered yellow traps.

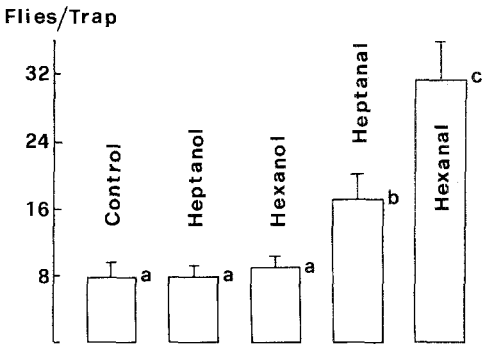

FIG. 6. Carrot fly field responses to C_6 and C_7 green leaf alcohols and aldehydes in polyethylene dispensers on glue-covered yellow traps. Dissimilarly labeled treatments are significantly different at $P < 0.001$; $N = 10$.

almost twice as attractive as heptanal. The related monoene, (*E*)-2-hexenal, was also attractive (Table 3), increasing the efficiency of the color trap alone (control) 13.4 times; the corresponding factor for hexanal was 4.5. Fly captures/trap/day were 11.1 for (*E*)-2-hexenal versus 0.8 for control and 4.2 for hexanal versus 0.9 for control.

The following volatiles were unattractive: hexyl acetate, 6-methyl-5-hepten-2-one, (*E*)-2-nonenal, decanal, dodecanal, geranyl acetone, geranyl acetate, (+)-carvone, *cis*-caryophyllene, methylchavicol, carvacrol, safrole, and anisole (Table 3). None of the commercial essential oils of seven family members of the Umbelliferae were attractive, namely: anise, caraway, carrot, celery, coriander, fennel, and parsnip. Calamus oil, a known source of *cis*-asarone, also proved unattractive.

Attractant Mixtures. Since the generally occurring green leaf volatiles and relatively host-specific propenylbenzenes represent different chemical classes, possible interactions on attraction of carrot flies were examined. A

TABLE 3. CARROT FLY FIELD RESPONSES TO FOUR VOLATILES ASSOCIATED WITH UMBELLIFERAE

Compound	Flies/trap[a]
(*E*)-2-Hexenal	44.2 ± 10.0 A
Hexyl acetate	3.3 ± 1.2 B
Anisole	3.6 ± 0.9 B
Safrole	2.9 ± 0.8 B
Control	3.3 ± 1.1 B

[a] $N = 10$ in each case; means with dissimilar letters are significantly different ($P < 0.001$).

TABLE 4. CARROT FLY FIELD RESPONSES TO CARROT LEAF VOLATILES AND MIXTURES ($N = 10$ IN EACH CASE)[a]

Experiment	Treatments			
	Control	*trans*-Asarone	*trans*-Asarone and Hexanal	*trans*-Asarone and *trans*-methylisoeugenol
I	4.2 ± 0.9 A	8.6 ± 1.1 B	13.9 ± 2.3 C	—
II	2.3 ± 0.5 D	6.6 ± 1.7 E		3.3 ± 0.7 D

[a] Means on the same line with dissimilar letters are significantly different ($P < 0.001$). Experiment I: *trans*-asarone, 250 mg/trap; hexanal released at 15 mg/trap/day. Experiment II: *trans*-asarone, 50 mg/trap; *trans*-methylisoeugenol released at 100 mg/trap/day.

combination of *trans*-asarone and hexanal led to a significant increase in trap catch over that observed with *trans*-asarone alone (Table 4); by contrast, combining *trans*-asarone with *trans*-methylisoeugenol led to a signficiant reduction in catch (Table 4). These results are in accordance with EAG data showing that air samples from mixtures (50:50) of *trans*-asarone and hexanal evoke EAG responses greater than the effect of either product singly (at 10^{-2} and 10^{-3} they are synergistic) while similar mixtures of *trans*-asarone and *trans*-methylisoeugenol have no such effect (Table 5).

Interaction of Trap Color and Chemical Components. The efficacy of both color and chemical components on trap catch were demonstrated by comparing the color trap alone, a colorless Plexiglas trap plus *trans*-methylisoeugenol, and a colorless trap as control (Table 6). The color trap was as effective as attractant as the colorless trap incorporating *trans*-methyl-

TABLE 5. EAG RESPONSES OF CARROT FLY TO CARROT LEAF VOLATILES AND MIXTURES AS PERCENTAGE OF RESPONSE TO *trans*-METHYLISOEUGENOL AT 10^{-2}

	Concentration		
	10^{-3}	10^{-2}	10^{-1}
Hexanal	14.0	63.6	456.0
trans-Methylisoeugenol	21.1	100.0	240.0
trans-Asarone	115.8	308.6	384.0
Hexanal and *trans*-asarone	147.4	405.7	683.3
trans-Methylisoeugenol and *trans*-asarone	126.3	291.4	358.3

[a] Concentration of the compound in paraffin oil on a volume/volume basis.

TABLE 6. TRAP EFFICACY—DEMONSTRATED EFFECT OF CHEMICAL AND COLOR COMPONENTS ON ATTRACTION OF CARROT FLIES

	No. of flies captured per trap[a]
Colorless Plexiglas	0.7 ± 0.3B
Colorless Plexiglas and *trans*-methylisoeugenol dispenser[b]	3.2 ± 0.73A
Yellow Plexiglas "229"	4.2 ± 0.9A

[a] $N = 10$ in each case; means with dissimilar letters are significantly different ($P < 0.001$).
[b] *trans*-Methylisoeugenol released at 200 mg/trap/day.

isoeugenol at a release ratio of 200 mg/day, and both traps were significantly superior to the colorless control (Table 6). This experiment also established *trans*-methylisoeugenol as a true attractant—operative in the absence of any color stimuli.

DISCUSSION

trans-Asarone is the dominant olfactory stimulant and field attractant for the carrot fly. Its presence in the headspace vapor over carrot foliage together with the propenylbenzene analog, *trans*-methylisoeugenol, has been confirmed by GC-EAD. The perception threshold for *trans*-asarone at 500 ag/ml of air (1.4×10^6 molecules/ml) is lower than that recorded for any other insect to a plant volatile; the threshold for (*E*)-2-hexanal, the most effective stimulant for sensilla coeloconica on the antenna of the locust, *Locusta migratoria* (L.), was calculated at 0.5×10^8 molecules/ml of air (Kafka, 1970), which is approximately in the same order as that estimated for (*E*)-2-hexen-1-ol, the most effective EAG stimulant for the Colorado beetle, *Leptinotarsa decemlineata* (Say) (Visser, 1979). The cabbage root fly has an electrophysiological threshold of 10^{-14} g/ml of air for allylisothiocyanate (Finch, 1980); this is some 20 times higher than the threshold of the carrot fly to *trans*-asarone. The sensitivity of the carrot fly to this compound is surprisingly high, matching that of Lepidoptera to their sex pheromones. As an analogy, Arn et al. (1975), employing a GC-EAD apparatus similar to that used here, observed that the minimum quantity of sex attractant required to evoke a discernible EAD response in *Lobesia botrana* (Schiff.) was 0.1 pg of its sex attractant (*E*,*Z*)-7,9-dodecadienyl acetate. This is just five times less than the quantity of *trans*-asarone required to evoke a similar EAD response in the carrot fly. Thus, GC-EAD provides a powerful tool for the analysis of

frequently complex mixtures of volatiles of plant origin, this being the first report of its use in the identification of host-plant attractants.

The behavioral threshold of the carrot fly to *trans*-asarone was estimated by means of comparing its field attractancy with that of its analog, *trans*-methylisoeugenol. The perception threshold for the latter was found to be 2000 times higher than that of *trans*-asarone. In the field, a behavioral difference of approximately the same order was observed: a significant increase in trap catch (over the blank control) was effected by *trans*-methylisoeugenol at a release rate of 100 mg/trap/day as compared with a similar effect by *trans*-asarone at 100 μg/trap/day. The latter value is but 25% of the threshold level of *n*-propyl disulfide required to effect significant trap catches of the onion fly, *Delia antiqua* (Meigen) (Dindonis and Miller, 1981). *trans*-Methylisoeugenol appears to be the least specific of the two propenylbenzene attractants, since traps baited with this product capture a variety of other insects, while *trans*-asarone attracts few but the carrot fly.

Phenylpropanoid receptors occur in a number of other insects, particularly among Tephritidae. Methyleugenol is a male attractant for the oriental fruit fly *Dacus dorsalis* (Hendel) (Steiner, 1952) and also for two Australian *Dacus* species, *D. tryoni* (Frogatt) (Fletcher et al., 1975) and *D. opillae* (Drew and Hardy) (Fitt, 1981), and this product has been isolated from blossoms, fruits, and leaves of plants in different families to which these insects are attracted (Metcalf et al., 1975; Fletcher et al., 1975). Male Oriental fruit flies are also attracted by *cis*-asarone, a major constituent of Indian calamus oil (Jacobson et al., 1976), thus affording an example of an insect attracted by both an allyl- and propenylbenzene. Asaronaldehyde (2,4,5-trimethoxy-benzaldehyde), another constituent of Indian calamus oil, is a strong attractant for both sexes of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) and female Oriental fruit flies and its positional isomer, 3,4,5-trimethoxy-benzaldehyde, is attractive to female melon flies, *Dacus cucurbitae* (Jacobson et al., 1976). By contrast, asaronaldehyde evokes no EAG response in the carrot fly, is unattractive in the field, and inactive in oviposition assays. It is interesting that traps baited with the highest dose of *trans*-asarone (500 mg) were more attractive to immature than gravid females. This age-related reaction to the attractant requires further study.

The generally occurring green leaf volatiles were estimated to occur at levels 10^3 times that of the more host specific propenylbenzenes in the headspace over diced carrot foliage (Guerin and Städler, 1982). Assuming that the relative proportion of these volatiles is similar over intact and diced foliage, it would appear that the olfactory system of the carrot fly compensates for these differences by being at least 10^3 times more sensitive to *trans*-asarone than to the generally occurring green leaf aldehyde, (*E*)-2-hexenal. This is in accordance with the supposition that compounds with a more specific

influence on insect behavior should have higher molecular weights (Bossert and Wilson, 1963).

EAG screenings demonstrated that the carrot fly is more sensitive to green leaf aldehydes than the corresponding alcohols (Guerin and Visser, 1980). The functional significance of this has been established here as the aldehydes are the only components of the green leaf volatile complex to elicit a behavioral response in the field. The most effective of these attractants, (*E*)-2-hexenal, has been identified in the headspace vapor of parsley leaves, *Petroselinum crispum* (Hoffmann) (Kasting et al., 1972). Since the relative proportion of the constituents comprising the green leaf volatile complex will vary between plant species, the ability of the carrot fly to discriminate between host and nonhost odor is probably augmented by sensitivity to components of the complex predominating in carrot. Correspondingly, the Colorado beetle is most sensitive to leaf alcohols predominating in potato leaf (Visser, 1979).

No recordings from individual receptors on the antenna of the carrot fly have been made. However, the elevated EAG responses evoked by mixtures of *trans*-asarone and hexanal suggest that two functionally different receptor types exist. Furthermore, a mixture of these two products is more attractive than either singly. By contrast, the lack of an elevated EAG response to a mixture of the chemically similar propenylbenzenes, *trans*-asarone and *trans*-methylisoeugenol, suggests competition for the same receptor site; in the field this mixture leads to inhibition of trap catch as compared with *trans*-asarone alone. With just two receptor types tuned to leaf aldehydes and propenylbenzenes, the carrot fly would already be equipped with considerable powers of discrimination. However, for the perception of a complex odor such as that of carrot foliage, some more receptor sites may be involed.

Host finding by pest insects is probably enhanced in monocultures due to the combined effect of chemicals emanating from the many plants in a crop (Feeny, 1976). An average carrot leaf (weighing 5 g) is estimated to contain, at a minimum, 17 ng *trans*-methylisoeugenol and 4 ng *trans*-asarone, and Umbelliferae possess specialized schizogenous canals for the release of these and other aromatic products from roots stems and leaves (French, 1971). The contribution of volatiles from the hitherto unmentioned root system of carrots is probably substantial, since vapor samples over carrot plants (foliage plus roots) evoke significantly higher EAG responses than that from the foliage alone (Guerin and Städler, 1982). An assessment of the "active space" (Bossert and Wilson, 1963) or zone of influence of a carrot crop for the carrot fly must await estimation of the release rates of attractants from carrot plants.

trans-Asarone-baited traps catch significantly fewer flies when placed in fallow ground than in a stand of host plants. It seems plausible to suggest that the superiority of the *trans*-asarone traps in the host crop is due an interaction between the trap released volatile and leaf vapors, since mixtures of functionally different types of volatiles prove most attractive. Alternatively,

host leaves have an arresting effect on the flies, thus increasing the number of possible respondents to the traps within the crop. The strong EAD responses (relative to FID peak height) evoked by some unidentified peaks midway in the chromatogram of cold-trapped foliar volatiles of carrot suggests that the best attractant for the fly is most probably a complex of volatiles. Some compounds which proved unattractive on their own in the field may well contribute to such a blend.

A number of phenylpropanoids (allyl- and propenylbenzenes) are associated with the Umbelliferae (Harborne, 1971; Hegnauer, 1973; Starkovsky, 1962). *trans*-Asarone has been isolated from carrot seed oil (Asahina and Tsukamoto, 1926), but this is the first report of its presence in the foliage. The latter product and its analog, trans-methylisoeugenol, are found in the waxy coating of carrot leaves and also in celery leaves (Städler, Lund, and Buser, unpublished data). These lipid-soluble products have also been identified as natural substituents of species from other plant families and as such have received considerable attention due to their psychoactive and carcinogenic properties (Oswald et al., 1974; Wulf et al., 1978). Indian calamus oil, derived from the root of *Acorus calamus* (L.), contains 90% asarone (*cis* and *trans* forms) (Hall, 1973), but this essential oil is unattractive to the carrot fly, most probably due to some repellent constituents. Asarone also occurs in several species of *Asarum* and *Asiasarum* genera such as *Asarum europaeum* (L.), from which its name is derived (Hall, 1973). The occurrence of *trans*-asarone in these plants, some of which occur within the same geographical range as the carrot fly, shows that the perception of propenylbenzenes alone could not explain the host-plant specificity of this species.

Low EAG responses evoked by phthalide derivatives typifying celery such as sedanolde, sedanenolide, and 3-*n*-butylphthalide (Wilson, 1970; Bjeldanes and Kim, 1977; Lund, 1978) suggest that the carrot fly is adapted to the perception of products typical of Umbelliferae rather than to species specific products of its family members.

At close range, the odor over host foliage has an arresting effect on the locomotion of the carrot fly leading to oviposition. Arrestment on the leaves in nature facilitates the perception of nonvolatile contact chemostimulants present in the epicuticular waxy layer of carrot foliage (Städler, 1977; Städler and Buser, 1982). This further underlines that host-plant selection involves a complex of specific stimuli, representing different modalities (attractants, arrestants, contact chemostimuli, visual and proprioceptive factors), all acting in unison.

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STEREOSELECTIVE SYNTHESIS OF (*Z*)-13-HEXADECEN-11-YN-1-YL ACETATE, THE MAJOR COMPONENT OF THE SEX PHEROMONE OF THE PINE PROCESSIONARY MOTH (*Thaumetopoea pityocampa*)

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Abstract—A short and stereoselective synthesis of (*Z*)-13-hexadecen-11-yn-1-yl acetate is described. The main feature is a low-temperature Wittig reaction of a triphenylpropylphosphonium bromide with a long-chain alkylated propargyl aldehyde.

Key Words—(*Z*)-13-Hexadecen-11-yn-1-yl acetate, pine processionary moth, *Thaumetopoea pityocampa*, *Thaumetopoea wilkinsoni*, Lepidoptera; Notodontidae, sex pheromone, synthesis, biological activity, ester.

INTRODUCTION

The pine processionary moth *Thaumetopoea pityocampa* Denis and Schiff. is a major forest pest in the Mediterranean region, and its life cycle has been described (Demolin, 1969). The moth *Thaumetopoea wilkinsoni* Tams, which was described in Cyprus in 1925 (Tams, 1925) and attacks pine trees in Israel and some neighboring countries is considered to be an ecotype of *Th. pityocampa* (Halperin, 1970).

The main component of the sex pheromone of the female *Th. pityocampa* has been recently isolated and identified as (*Z*)-13-hexadecen-11-yn-1-yl acetate (I) (Guerrero et al., 1981). This discovery might solve the question of ecotype vs. separate species of the *Th. wilkinsoni* in relation to *Th.*

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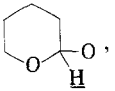
pityocampa and enable us to study the potential use of pheromone in monitoring and hopefully in actual control of *Th. wilkinsoni* in Israel. We accomplished the synthesis of I and supplied it for field tests in the autumn of 1981; it was found to be active towards *Th. wilkinsoni* (Halperin et al., 1981).

There are several approaches available for construction of the enyne unit as part of a long-chain acetate. Different syntheses have been recently published (Camps et al., 1981; Michelot et al., 1982), which demonstrate some of these approaches.

METHODS AND MATERIALS

General. The crude product, after each chemical transformation, was used, without further purification, for the next step. IR and NMR spectra were used to check product identity and purity. IR spectra were run neat on a Perkin-Elmer 377 instrument. NMR spectra were recorded on Varian XL-100 in CCl_4, chemical shifts are in δ, downfield from TMS as internal reference. GC analyses were obtained on a Packard 417 equipped with an FID detector on a 6-ft × 1/8-in. ID, 5% FFAP on Chromosorb W, SS column. Mass spectra were obtained on a Varian MAT-112 GC/MS, EI mode, 70 eV with SE-30 6-ft × 1/4-in. column.

Solvents. Petroleum ether was distilled (60–80). Ether was dried over Na and distilled before use. Pyridine was distilled and kept over CaH_2 prior to use. Tetrahydrofuran (THF) was dried over KOH, distilled, and stored over CaH_2, then distilled over $LiAlH_4$ immediately before use.

Preparation of 13-THP-Tridec-2-yn-1,13-diol (IV). A solution of propargyl alcohol (II), 6.1 g (0.11 mol) in 100 ml of THF, was added dropwise with stirring to a suspension of lithium amide (0.24 mol), prepared from 1.68 g of Li and a crystal of $Fe(NO_3)_3 \cdot 9H_2O$ in 250 ml of liquid NH_3. After 30 min, 32 g (0.1 mol) of 10-bromodecan-1-ol-THP (III) (Kondo et al., 1974) was added and the mixture was stirred overnight. Saturated NH_4Cl solution (10 ml) was added, and the reaction mixture was extracted with petroleum ether (3× 50 ml), the petroleum ether was washed with saturated NaCl solution and dried over K_2CO_3. Evaporation of the solvent left 30 g of crude product; NMR 4.46 (1H,  , t, poorly resolved), 4.02 (2H, —C≡CC<u>H</u>$_2$OH, t, poorly resolved), 3.1–3.9 (4H, C<u>H</u>$_2$O—, m), 2.18 (2H, —C<u>H</u>$_2$—C≡C—, t, J=7 cps), 1.3–1.6 (22H). IR 3500, 2260, 2200 cm^{-1}.

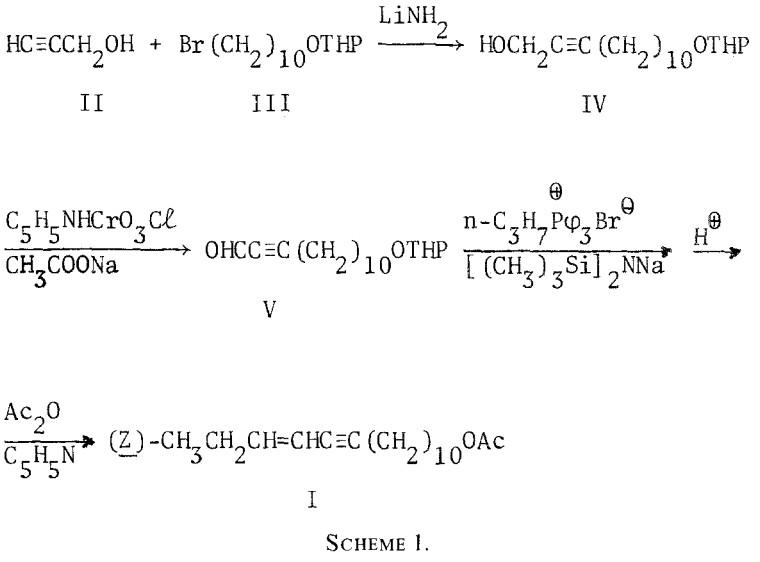
Oxidation of IV to Aldehyde V. To a base-washed flask, containing 13.3 g (0.045 mol) of IV and 0.02 g of sodium acetate in 120 ml CH_2Cl_2 (that had been passed through a column of 5 g basic alumina), 10 g (0.045 mol) of pyridinium chlorochromate were added and the mixture was stirred for 2 hr at

room temperature. Ether (150 ml) was added, and the crude mixture was filtered through a 30-g column of Florisil to yield 11.3 g of V (86%). NMR 8.96 (1H, —CHO), 4.46 (1H,  , t, poorly resolved), 3.1–3.8 (4H, —CH_2O—, m), 2.38 (2H, —$CH_2C{\equiv}C$—, t, J = 7 cps). 1.2–1.6 (22H). IR 2720, 2260, 2200, 1670 cm^{-1}.

Preparation of (Z)-*13-Hexadecen-11-yn-1-yl Acetate* (*I*). Hexamethyldisilazane sodium salt (0.04 mol) was prepared from 6.5 g (0.04 mol) of 1,1,1,3,3,3-hexamethyldisilazane and 1.76 g (0.045 mol) of $NaNH_2$ in 65 ml THF and under a N_2 atmosphere (Bestman et al., 1976). To this suspension, 18.2 g (0.047 mol) of triphenylpropylphosphonium bromide in 120 ml THF was added to yield an orange solution which was refluxed for 3 hr. After cooling to −90° C, 11.3 g (0.038 mol) of aldehyde V was added, the mixture was held at −90° for 1 hr and then allowed to reach room temperature and stirred overnight. The solution was evaporated, petroleum ether (100 ml) was added, the organic mixture was washed with H_2O, then with 10 ml DMSO–H_2O (9:1), saturated NaCl solution, and dried over $MgSO_4$. The crude product was hydrolyzed in 30 ml CH_3OH, 3 ml H_2O, and 1 drop of concentrated HCl at 50° for 3 hr. The methanol was evaporated and the residue was poured into water (50 ml) and extracted with petroleum ether (3 × 30 ml). The petroleum ether was washed with H_2O, dried over $MgSO_4$, evaporated and the remaining crude oil was then acetylated by 10 ml acetic anhydride and 10 ml pyridine overnight at room temperature. The reaction mixture was poured into ice, extracted with petroleum ether (3 × 20 ml), washed with 5% HCl, saturated NaCl solution, dried over $MgSO_4$ and distilled at 110–115°/0.03 mm to yield 4.4 g of pure I (42%). MS, *m/e*, 278 (M^+), 135, 121, 107, 105, 94 (100%), 93, 91, 79. NMR 5.66 (1H, —CH=CH—C≡C—, dt, J = 11 and 7 cps), 5.22 (1H, —CH=CHC≡C—, d, J = 11 cps), 3.94 (2H, —CH_2OAc, t, J = 7 cps), 2.22–2.40 (4H, allylic hydrogens, m), 1.94 (3H, CH_3CO, s). 1.2–1.7 (16H), 1.00 (3H, CH_3CH_2, t, J = 7 cps). IR 2200, 1735 cm^{-1}.

RESULTS AND DISCUSSION

Our synthesis is based on two building units; each introduces one of the unsaturation functionalities. Propargyl alcohol is used to introduce the triple bond and a stereoselective Wittig reaction (Bestmann et al., 1976) to introduce the *Z* double bond in better than 96–97% purity. No *E* isomer could be detected in the NMR spectrum (Shani, A., 1979). The total synthesis is outlined in Scheme 1.



SCHEME 1.

Thus, alkylation of propargyl alcohol is run in liquid ammonia to yield IV in almost quantitative yield, oxidation to V is conducted in buffered solution to afford crude aldehyde in 86% yield and stereoselective Wittig reaction produces I in 42% yield. This short sequence of reactions is convenient as no purification is done during the synthesis, eliminating loss of materials. A drawback for industrial synthesis is the low temperature conditions of the Wittig reaction, which is essential for the stereoselective *cis*-olefination; however, the Wittig reaction can be run on a large scale in the laboratory.

The biological activity of the synthetic compound (I) towards *Th. wilkinsoni* was tested in infested pine forests and was found to be active both at low and high population densities. Moths were found in traps located as far as 1400 m from highly infested forest. Up to 183 moths per night were caught in a single trap when placed in the forest (Halperin et al., 1981).

The biological activity of the synthetic sex pheromone of *Th. pityocampa* toward *Th wilkinsoni* raises the question whether *Th. wilkinsoni* is really a different species from *Th. pityocampa*. Entomological studies might solve this problem.

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SIMPLE AND STEREOSELECTIVE SYNTHESIS OF SEX PHEROMONE OF PROCESSIONARY MOTH *Thaumetopoea pityocampa* (DENIS AND SCHIFF.)

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Abstract—A simple and stereoselective synthesis of (*Z*)-13-hexadecen-11-yn-1-yl acetate, the major component of the sex pheromone of *Thaumetopoea pityocampa* (Denis and Schiff.) is described. The procedure essentially involves formylation of a terminal acetylene to the corresponding aldehyde followed by a stereochemically controlled Wittig reaction, which has been studied under a variety of conditions.

Key Words—Sex pheromone, processionary moth, *Thaumetopoea pityocampa*, Lepidoptera, Notodontidae, (*Z*)-13-hexadecen-11-yn-1-yl acetate, ester, synthesis.

INTRODUCTION

The major component of the sex pheromone of the processionary moth *Thaumetopoea pityocampa* (Denis and Schiff.), an economically important pine pest in northern Africa and southern Europe, has been identified recently as (*Z*)-13-hexadecen-11-yn-1-yl acetate (I) (Guerrero et al., 1981) and its synthesis by different approaches reported thereafter (Camps et al., 1981; Michelot et al., 1982). The synthetic compound has exhibited an excellent activity in the field, and its remarkable persistency is noteworthy (Cuevas et al., 1982) as far as its potential application in pest-control programs is concerned. Since the requirement of complete isomeric purity to elicit biological activity does not appear to be crucial in the present case (Cuevas et al., 1983), we have developed an alternative, short, and stereoselective

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synthesis of compound I. The synthetic scheme involves a stereochemically controlled Wittig reaction of the appropriate acetylenic aldehyde with a nonstabilized phosphorane. The necessary tetrahydropyranyloxy acetylenic aldehyde has been prepared through a simple approach from readily available starting materials.

METHODS AND MATERIALS

Boiling points are uncorrected. IR spectra were recorded in CCl_4 or $CHCl_3$ solution on Perkin Elmer 257 and 399B grating spectrometers. [^{1}H]NMR were determined in CCl_4 solution on a Perkin Elmer R12B 60 MHz and Bruker WP80SY 80 MHz and absorptions expressed in δ scale relative to TMS. [^{13}C]NMR spectra were recorded on a Bruker WP80SY 20 MHz in $CDCl_3$ solution and the values expressed in ppm relative to TMS. GLC analyses were performed on a Carlo Erba 2350, operating with a FID detector and using 3% OV-101 and 5% FFAP glass columns 2 m × 1/8 in. ID on Chromosorb W (20 ml/min of nitrogen as carrier gas).

10-Bromodecan-1-ol (*II*). The compound was prepared in 79% yield after distillation as previously described (Pattison et al., 1956), b.p. 98–101° C (0.07 torr). The compound appeared to contain 7% of the corresponding 1,10-dibromodecane by GLC analysis.

10-Bromo-1-tetrahydropyranyloxydecane (*III*). The compound was obtained from compound II in 75% yield after distillation by a standard procedure (dihydropyran/*p*-TsOH), bp 125–127° C (0.02 torr).

12-Tetrahydropyranyloxydodec-1-yne (*IV*). This was prepared by a procedure similar to that described by Maurer and Grieder (1977). A rapid flow of KOH-dried acetylene was passed through anhydrous liquid NH_3 (450 ml), and lithium (2.65 g, 0.38 g atm), cut into small pieces, was added. When the lithium was dissolved, compound III (48.2 g, 0.155 mol) in anhydrous DMSO (225 ml) was added at −30° C. The reaction mixture was maintained at this temperature for 60 min and allowed to warm up to room temperature to evaporate the ammonia, working-up was carried out by addition of 750 ml of hexane–diethyl ether (1 : 1) and ice water. The organic phase was decanted and the aqueous layer extracted with hexane–diethyl ether (1 : 1) (3 × 100 ml). The combined organic phases were washed with water and dried ($MgSO_4$). Evaporation of the solvent afforded a residue which, after distillation, yielded compound IV (39.0 g, 95%), bp 130–133° C (0.1 torr). IR 3320, 2120 cm^{-1}. [^{1}H]NMR δ 4.44 (b, 1H, OC<u>H</u>O), 3.1–3.9 (m, 4H, C<u>H</u>$_2$O), 2.1 (m, 2H, C<u>H</u>$_2$C≡), 1.8 (t, 1H, J = 3 Hz, HC≡C), 1.1–1.7 (b, 22H, —C<u>H</u>$_2$—).

13-Tetrahydropyranyloxytridec-2-ynal (*V*). To a solution of IV (19 g, 0.071 mol) in 80 ml of anhydrous THF was added under nitrogen 0.073 mol of *n*-butyllithium (69.5 ml of a 1.05 M hexane solution). After the addition,

stirring was maintained for 30 min and distilled ethyl formate (9.0 g, 0.146 mol) in 10 ml of anhydrous THF slowly added at −30° C (Hauptmann and Mader, 1978). Stirring was kept up for 45–60 min and the reaction quenched by pouring into 100 ml of ice water, which contained acetic acid (7.0 g, 0.077 mol) and a small amount of hydroquinone to prevent polymerization of the aldehyde. The reaction mixture was thoroughly extracted with diethyl ether (5× 120 ml), washed with brine, and dried ($MgSO_4$). Evaporation of the solvent and distillation afforded aldehyde V (12.1 g, 60%), bp 140–145° C (0.1 torr). IR 2260, 2200, 1670 cm^{-1}. [^{1}H]NMR δ 9.2 (s, 1H, OC<u>H</u>O), 3.1–3.9 (m, 4H CH_2O), 2.3 (t, 2H, J = 6 Hz, C<u>H</u>$_2$C≡C), 1.1–1.8 (b, 22H, —C<u>H</u>$_2$—).

(Z)-*13-Hexadecen-11-yn-1-ol* (*VI*). The compound was prepared under different conditions by Wittig reaction of aldehyde V with the corresponding propylidentriphenylphosphorane.

1. Using potassium *t*-butoxide as base (entry 1), to a suspension of dry *n*-propyltriphenylphosphonium bromide (0.814 g, 2 mmol) in anhydrous THF (15 ml) under nitrogen was added freshly sublimed potassium *t*-butoxide (0.235 g, 2 mmol). The mixture was stirred for 30 min at room temperature and the aldehyde V (0.49 g, 1.6 mmol) added. Stirring was continued for 2 hr and the reaction quenched by adding a small volume of water. The mixture was poured into 40 ml of a mixture of diethyl ether–brine (1 : 1), decanted, and the aqueous layer extracted with diethyl ether (3× 15 ml). The combined organic extracts led to a residue which was taken up in hexane, cooled to −20° C, and filtered. The solvent was stripped off to give the expected tetrahydropyranyl derivative of VI (0.44 g, 83%). Hydrolysis was conducted by dissolving the residue in CH_3OH (20 ml) in the presence of small crystals of *p*-toluenesulfonic acid. The solution was heated to reflux for 3 hr, cooled, and neutralized with $NaHCO_3$. After filtration and evaporation of the solvent, water was added and extracted with diethyl ether (4× 10 ml). Removal of the solvent and purification by preparative TLC afforded the desired alcohol VI (0.271 g, 68.5%). IR 3340, 3020, 2220, 1615 cm^{-1}. [^{1}H]NMR δ 5.7 (dt, 1H, J = 11 Hz J' = 7 Hz, CH_2C<u>H</u>=CH), 5.3 (d, 1H, J = 11 Hz, C=C<u>H</u>C≡C), 3.5 (t, 2H, J = 7 Hz, C<u>H</u>$_2$OH), 2.3 (t, 2H, J = 5.7 Hz, C<u>H</u>$_2$C≡C), 1.8 (m, 2H, C<u>H</u>$_2$C=C), 1.35 (b, 16H, —C<u>H</u>$_2$—), 1.0 (t, 3H, J = 7 Hz, CH_2C<u>H</u>$_3$). GLC analysis on 5% FFAP column showed alcohol VI to be a 80 : 20 stereoisomeric mixture of *Z* and *E* isomers.

A shorter reaction time (45 min) under the same experimental conditions (entry 2) improved the stereochemical course of the reaction to 90 : 10 *Z* : *E*, being alcohol VI obtained in 64% yield. Further shortening of the reaction time led to the partial recovery of unreacted aldehyde V.

2. Using *n*-butyllithium as base (entry 3), at −70° C a suspension of the phosphonium salt (0.231 g, 0.6 mmol) in anhydrous THF (5 ml) and anhydrous HMPTA (1.07 g, 6 mmol) was prepared under nitrogen. *n*-Butyllithium (1.6 ml of a 1 M solution) was added and the mixture stirred for

25 min. The aldehyde V (0.15 g, 0.5 mmol) in anhydrous THF (1 ml) was added and stirring maintained for 1 hr at −70° C and 1 hr at room temperature. After the usual work up, the residue was hydrolyzed to afford after purification on TLC alcohol VI (0.075 g, 64%). GLC analysis on 3% OV-101 showed a 94:6 stereoisomeric mixture of *Z*:*E* isomers (Sonnet, 1974).

The use of diethyl ether–DMF (1:1) as solvent (1 hr at room temperature) (entry 4) yielded alcohol VI in 57% in a 90:10 *Z*:*E* isomeric ratio.

3. Using sodium hydride as base (entry 5), a dispersion of 50% sodium hydride in oil (32 mg, 0.6 mmol) was washed with pentane and to the residue was added anhydrous DMF (5 ml) under nitrogen. The phosphonium salt (0.27 g, 0.7 mmol) was added and the mixture stirred for 2 hr at room temperature. Aldehyde V (0.15 g, 0.5 mmol) dissolved in 0.5 ml of anhydrous DMF was added to the ylide and the mixture further stirred for 2 hr. The reaction was worked up in the usual fashion and the crude hydrolyzed to yield alcohol VI (0.066 g, 56%) (90:10 Z:E) after purification on TLC (Bergelson and Shemyakin, 1964).

The use of HMPTA–THF (1:3) as solvent (1 hr at −70° C followed by 2 hr at room temperature) (entry 6) afforded alcohol VI in 56% yield (94:6*Z*:*E*).

4. Utilizing sodium trimethylsilylamide as base (entry 7), the base was prepared according to Wannagat and Niederprum (1961) by heating under nitrogen a mixture of 40% sodium amide (1.17 g, 0.012 mol) in toluene and trimethylsilylamine (1.59 g, 0.012 mol) in anhydrous benzene (15 ml). The product was obtained in 98% and sublimed before use. The ylide was prepared by heating under nitrogen sodium trimethylsilylamide (0.11 g, 0.6 mmol) and the phosphonium salt (0.27 g, 0.7 mmol) in 14 ml of anhydrous THF (14 ml) at reflux for 45 min. The mixture was cooled to −70° C and the aldehyde V (0.15 g, 0.5 mmol) in THF (1 ml) added. Stirring was continued for 1 hr at −70° C and 20 hr at room temperature. Usual work up and hydrolysis yielded alcohol VI (61%) after purification on TLC. GLC analysis on OV-101 showed a 95:5 *Z*:*E* stereoisomeric ratio. The procedure was scaled up (10×) with similar results.

Addition of magnesium bromide, freshly prepared from magnesium and 1,2-dibromoethane in THF, to the ylide in a 1:1 ratio (entry 8), under the conditions stated for entry 7, afforded a lower yield of VI (50%) with a poor stereoisomeric ratio (89:11 *Z*:*E*).

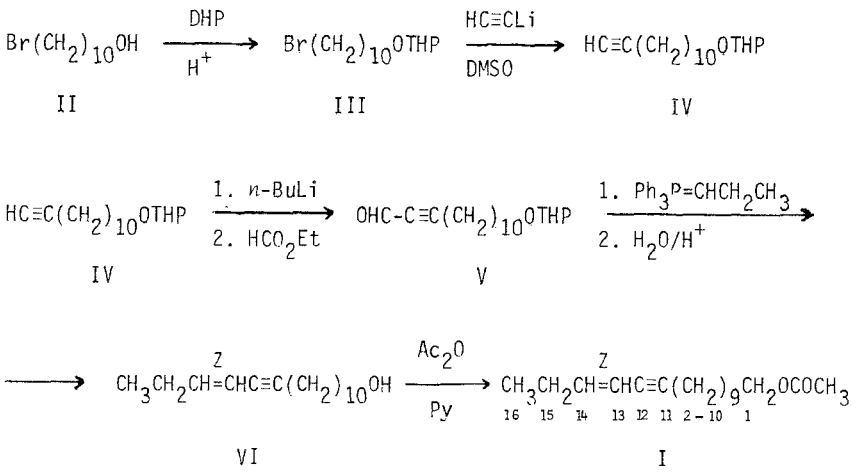
(Z)-*13-Hexadecen-11-yn-1-yl Acetate* (*I*). To a mixture of acetic anhydride (20 ml) and anhydrous pyridine (10 ml) was added alcohol VI (4.5 g, 0.019 mol) in pyridine (10 ml). The solution was stirred at room temperature for 3 hr, methanol (20 ml) was added, and the mixture further stirred for 20 min. The volatile materials were removed under vacuum and the residue extracted with diethyl ether (4× 50 ml), washed with 2 N HCl, saturated solution of $NaHCO_3$, brine, and dried ($MgSO_4$). Evaporation of the solvent

left a crude oil which, after distillation, yielded (*Z*)-13-hexadecen-11-yn-1-yl acetate (I) (4.85 g, 92%), bp 115–120° C (0.1 torr). IR 3020, 2200, 1740, 1615 cm^{-1}. [^{1}H]NMR δ 5.85 (dt, 1H, J = 10 Hz, J' = 7 Hz, $CH_2C\underline{H}{=}C$), 5.4 (d, 1H, J = 10 Hz, $C{=}C\underline{H}C{\equiv}C$), 3.95 (t, 2H, J = 7 Hz, $C\underline{H}_2OAc$), 2.3 (m, 4H, $C\underline{H}_2C{=}C$ and $C\underline{H}_2C{\equiv}C$), 2.05 (s, 3H, $COC\underline{H}_3$), 1.6 (b, 16H, $-C\underline{H}_2-$), 1.0 (t, 3H, J = 7 Hz, $CH_2C\underline{H}_3$). [^{13}C]NMR δ 64.1 (C-1), 28.3 (C-2), 23.0 (C-3), 28.5 (C-4), 28.5 (C-5), 28.7 (C-6), 28.9 (C-7), 29.1 (C-8), 29.1 (C-9), 25.6 (C-10), 77.1 (C-11), 93.9 (C-12), 108.7 (C-13), 143.2 (C-14), 20.4 (C-15), 13.0 (C-16), 19.1 ($OCO\underline{C}H_3$), 170.4 ($O\underline{C}OCH_3$).

RESULTS AND DISCUSSION

The presently reported synthetic scheme for the preparation of (*Z*)-13-hexadecen-11-yn-1-yl acetate (I) is based on two key steps, namely formylation of a terminal acetylene to the corresponding propargylic aldehyde by the low temperature reaction of the lithium acetylide with ethyl formate, and the stereocontrolled Wittig condensation of the aldehyde with propyliden-triphenylphosphorane (Scheme 1).

Condensation of 10-bromo-1-tetrahydropyranyloxydecane (III) with lithium acetylide in liquid ammonia yielded the tetrahydropyranyl ether of dodec-11-yn-1-ol (IV) in nearly quantitative yield. Formylation of the acetylide of IV with ethyl formate at low temperature afforded acetylenic aldehyde V in 60% yield. The Wittig reaction has been undertaken under a variety of conditions and the results are summarized in Table 1.



SCHEME 1.

TABLE 1. WITTIG REACTION OF 13-TETRAHYDROPYRANYLOXYTRIDEC-2-YNAL (V) WITH PROPYLIDENTRIPHENYLPHOSPHORANE

Entry	Base	Solvent	Time (min)	Temp. (°C)	Yield[a] (%)	Isomer ratio $Z:E$[b]
1	*t*-BuOK	THF	120	20	68	80:20
2	*t*-BuOK	THF	45	20	64	90:10
3	*n*-BuLi	THF–HMPT (5:1)	60[c]	−70	64	94:6
4	*n*-BuLi	Et_2O–DMF (1:1)	60	20	57	90:10
5	NaH	DMF	60	20	56	89:11
6	NaH	THF–HMPT (3:1)	60[d]	−70	56	94:6
7	$(Me_3Si)_2NNa$	THF	60[e]	−70	61	95:5
8	$(Me_3Si)_2NNa + MgBr_2$	THF	60[e]	−70	50	89:11

[a] Distilled yield after hydrolisis to the corresponding alcohol VI.
[b] By GLC analysis.
[c] Followed by 60 min at room temperature.
[d] Followed by 2 hr at room temperature.
[e] Followed by 20 hr at room temperature.

As is known, Wittig reaction of nonstabilized alkylidentriphenylphosphoranes with aliphatic aldehydes in nonpolar solvents under "salt-free" conditions yield predominantly *Z* olefins (Vedejs et al., 1981, and references therein). Similarly, when the reaction is carried out in a dipolar aprotic solvent such as DMF, DMSO, or HMPTA, *Z* olefins are also mainly produced, regardless of the presence or absence of inorganic salts. As shown in Table 1, low temperatures and short reaction times afforded a better kinetic control to yield the desired *Z* isomer. On the other hand, different bases gave slight differences in yield and stereochemical purity, under the same reaction conditions. Thus, satisfactory procedures were those shown in entries 3, 6, and 7, being the results in fairly good agreement with those reported by Anderson and Henrick (1975). The use of sodium trimethylsylilamide as base (entry 7) (Bestmann et al., 1976) gave the best *Z*:*E* isomeric ratio of alcohol VI (95:5). It should be emphasized that the procedures shown in entries 3 and 7 can be scaled up in order to prepare the sex pheromone in the quantities required for field tests. Hydrolysis of the tetrahydropyranyl ether of alcohol VI and acetylation under standard conditions yielded the desired acetate I in 33.6% overall yield from III (entry 3). The synthetic compound (*Z*:*E* 95:5) has been successfully applied in mass trapping experiments in Spain during the summer campaign of 1982.

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RESPONSES OF FREE-RANGING COYOTES TO LURES AND THEIR MODIFICATIONS

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Abstract—Several chemical modifications of a synthetic fermented egg (SFE) lure were field tested on free-ranging coyotes (*Canis latrans*) to determine the effects of odor intensity and quality on their behavioral responses. SFE was modified for testing by (1) enhancing one of the four basic odor components (fruity, sulfurous, sweaty, or fishy), (2) deleting one of the basic components, (3) individually testing an odor component, and (4) addition of aldehydes and indoles to SFE. Most behavioral responses, especially visitation, increased with odor intensity. Widely different odors elicited similar visitation. Specific odor quality influenced response rates such as urinating, defecating, digging, scratching, rolling, and pulling.

Key Words—Behavioral responses, *Canis latrans*, coyotes, fermented, lures, odor, synthetic fermented egg.

INTRODUCTION

Few terms embrace such a nondescript chemical definition as the term lure. Even when the specifications are narrowed to include only coyote (*Canis latrans*) lures, the chemical variety seems endless. Specialists in coyote trapping and damage control often classify lures according to the behavioral response that they elicit, under the vague terminology "food, curiosity, passion, and natural." Not only are there combinations of these four classifications, but every imaginable ingredient within, and the same ingredients may be categorized differently depending on the specialist. A given lure often elicits different responses depending on climate, habitat, or season. Years of experience have given many of these specialists an uncanny intuition as to the best recipe for a homemade lure for a particular situation. Yet,

virtually nothing is known about what kinds of compounds elicit what kinds of responses, regardless of the conditions.

The repertoire of coyote responses to odors has been described as licking, chewing, removing (pulling and carrying), digging, sniffing, scent-rubbing, urinating, defecating, and scratching (Timm et al., 1975; Turkowski et al., 1979). It is difficult to find a given chemical that will elicit one specific response. Rather, it appears that odor combinations, in the context of ongoing canid activity, elicits the particular response (Bullard, 1982).

The basis of the present study was to document specific responses to basic odors and their modifications. A synthetic fermented egg (SFE) formulation that had been the best attractant in nearly all tests against other private and commercial lures (Turkowski et al., 1979) was an ideal base material. The altering of odor quality is easily accomplished since SFE is a blend of four odor characters: sweaty, fruity, sulfurous, and fishy (Bullard et al., 1978b). It was developed on the basis of a natural fermented egg product (FEP) which elicits the whole range of responses described above (Bullard et al., 1978a,b; Turkowski et al., 1979). SFE elicits the urinating, defecating, and scratching responses associated with scent-marking in coyotes (Camenzind, 1978; Barrette and Messier, 1980; Bowen and Cowan, 1980) and the scent-rubbing (Reiger, 1979) or rolling response which occurs in response to highly decomposed carrion. It contains the broad spectrum of compounds found as fermentation products in carrion and the skin glands and vaginal secretions of both coyotes and their prey (Bullard et al., 1978a; Bullard, 1982). Thus, food seeking, territorial maintenance, and breeding activities of coyotes are all influenced by SFE. We propose that the deletion or enhancement of certain odor qualities should produce a corresponding change in coyote behavioral responses.

METHODS AND MATERIALS

Coyote Attractancy Field Tests. Briefly, the relative responses to candidate lures were determined by assessing the number of visits as determined by tracks and behavioral "signs" left at scent stations. A scent station was a cleared 0.91-m-diameter circle covered with sifted earth or sand upon which tracks and other marks could be imprinted. Lures were contained in 7-mm-high × 29-mm-diameter round plastic capsules (Lab-Tek Products, Westmont, Illinois, used for biological tissue processing[1]). A capsule was secured in the center of each station by a No. 16 iron box nail pushed through the capsule and into the soil. The quantity of lure was either 1.2 g of powder of 1 ml of liquid absorbed into a piece of cellulose sponge.

[1]Use of product names does not imply endorsement by the U.S. government.

The stations were positioned at 0.48-km intervals within 1.5 m of the edge and on alternate sides of infrequently used unpaved roads. Replicate placement of sample groups continued until there were 100 or more stations per line. Stations were checked daily and recorded as visited if coyote tracks were found. The capsules at all stations were replaced after every fifth exposure day. Behavioral signs recorded were pulling, carrying, digging, scratching, rolling on the station, urinating, and defecating. We recorded pulling if the nail holding the capsule was pulled out of the ground and carrying if it was removed from the station. Digging, generally with the forepaws, was evident by depressions which were usually near the base of the capsule. Scratching appeared as elongated "scratch marks" made with feet and nails by pulling the forelegs or kicking the hindlegs along the ground. Rolling behavior was indicated by soil indentations on the station which usually included hair imprints. For more details see Bullard et al. (1978b) and Turkowski et al. (1979).

Placement of attractant stations along roads was randomized within each of 20 consecutive sample groups (one sample of each test candidate). Behavioral responses to each candidate lure were assessed by dividing the number of stations having the respective behavioral responses by the number of intact (i.e., not destroyed by livestock) stations. Each attractant in the test was ranked against others in the same test according to its ability to induce each of the specific responses (visits, pulling, digging, etc.). All tests were conducted on ranches near Zapata, Texas, and data from all test phases were analyzed at the Texas A and M University Institute of Statistics. An arc-sine-root transformation was performed on all visitational and behavioral rate data. We conducted a least-square analysis of variance on 18 variables and then placed visit and behavioral means into homogeneous groups, using the Scott-Knott multiple comparisons procedure (Turkowski et al., 1979).

Odor Enhancement of SFE. SFE consists of four major odor classes: (1) fruity—primarily esters, (2) sulfurous—organosulfurs, (3) sweaty—volatile fatty acids, and (4) fishy—amines (Bullard et al., 1978b). Other odor characteristics used for enhancement were: aldehyde—1 : 1 : 1 : 1 mixture of C_6–C_9 *n*-aldehydes, and fecal—an indole 1 : 1 mixture of skatole and indole (Hwang and Mulla, 1971). Each of these six mixtures was added to SFE at a concentration equivalent to their human odor difference-recognition threshold (Table 1). This threshold was the lowest concentration at which an odorant, which was mixed with another odorant, could be specifically recognized by humans.

The same 18-member human odor panel used to develop SFE (Bullard et al., 1978b) was used to determine this odor difference-recognition threshold. All panel members conformed to selection criteria outlined by ASTM (Wittes and Turk, 1967). Subjects were presented with 10 bottles (two replications) in

TABLE 1. ENHANCEMENT LEVELS OF ODOR COMPONENTS IN SFE FORMULATIONS[a]

Odor component	Mixture source	Concentration (%)[b]
Fruity	Wire loop[c]	20.0
Sulfurous	Organosulfuc[c]	25.0
Sweaty	Volatile fatty acids[c]	40.0
Fishy	Amines[c]	31.5
Aldehyde	1:1:1:1 C_6–C_9 *n*-aldehydes	20.0
Fecal	1:1.4 indole–skatole	50.0

[a]See tests A (Table 2) and B (Table 3).
[b]Percent concentration of odor component mixture added in v/v except SFE–indoles which is given in w/v.
[c]See Table I in Bullard et al. (1978b) for formulations.

which the additives increased in 0.5-μl increments starting at 0 on the left and going to 4.5 μl on the right. The base odorant was 6 μl SFE in 0.5 ml of reagent-grade propylene glycol. Subjects (Ss) were instructed to briefly sniff each bottle and record the one having the lowest detectable change in odor quality. A mean concentration value was then taken for all six fractions over two replications by adding the concentration selected by each person and dividing by the total number of people. These values represented the odor difference thresholds.

The odor difference threshold was used as a starting point in a triangle test (Byer and Abrams, 1953) designed to determine the recognition threshold of the added odorant. Ss were told to briefly sniff a 5 μl/0.5 ml propylene glycol formulation of each of the six additives and describe their qualities. They were then given six sets (one for each additive) of these bottles—two with SFE only and one with SFE plus the additive at the difference threshold. (They rested at least 2 min between each set to prevent olfactory receptor fatigue.) Concentrations were increased or decreased on subsequent days until 75% recognition accuracy was obtained in two replications of each respective test. After odor levels were determined, four groups (A, B, C, and D) of tests were conducted.

Test A—Comparison of SFE Resins vs. FEP. These propylene glycol solutions were then incorporated at 4% (v/w) polyamide resin powders by a process used in earlier SFE experiments (Bullard et al., 1978b). The process produced seven candidate formulations that were evaluated by scent station visits in the coyote attractancy tests in February 1976.

Test B—Comparisons of Concentrated Modifications of SFE. Attractant properties of the concentrated SFE formulations (liquids) were superior to the powders (Bullard et al., 1978b), therefore test A was repeated with fully concentrated liquids. The SFE, SFE–fruity, SFE–sulfurous, SFE–sweaty, SFE–fishy, SFE–aldehydes, and SFE–indoles all retained the same

component ratios. An abbreviated seven-component SFE modification (Turkowski and Bullard, in review) was also tested. This concentrated liquid mixture, divised through human odor panel techniques, consisted of the following chemicals by volume: 41.8% caproic acid, 35.1% butyric acid, 7.1% hexylamine, 7.2% trimethylamine (25% aqueous), 0.6% dimethyl disulfide, 0.2% 2-mercaptoethanol, and 8% ethyl caproate. The nine liquids were evaluated at scent stations in coyote attractancy tests in May 1977.

Test C—Comparison of Individual Odor Components. To determine influences of individual odor characters, we compared each fraction with SFE. A volume equivalent to the other three components in an SFE formulation was replaced with propylene glycol. These four treatments were compared (without blank capsules) with SFE for coyote attractancy during February 1977.

Test D—Determining Influence of Individual Component Deletion. In the final test (February 1978) we replaced one of the odor components with propylene glycol in each treatment formulation. Each of the four treatments was compared with the SFE standard.

RESULTS AND DISCUSSION

Odor Intensity. Similar rates of visitation existed for widely changed formulations of the basic four-odor component (fruity, sulfurous, sweaty, and fishy) SFE lure. In either low (Table 2) or high (Table 3) concentrations, enhancement of odor components did not significantly change visitations. Abbreviated SFE which had 63 compounds missing still equaled the 70-component SFE lure. Blank capsules elicited some visitation. This indicates that coyotes are drawn to a lure for a number of reasons and rather large changes in odor quality can exist without affecting visitation. Visitations for all fully concentrated 4-component based mixtures were above 27 and had an average of 31.1. The resin formulations in test A were all below 20 in visitation and had an average of 17. This observation is strengthened by similar results in a direct comparison between SFE–resin and concentrated SFE (Bullard et al., 1978b). Apparently, odor intensity is more important than quality in luring coyotes to a site.

Odor intensity also influenced the other behavioral responses. Indexes on concentrated SFE in February 1977 (Table 4) and 1978 (Table 5) were several-fold higher for urination, scratching, rolling, pulling, and carrying than for the diluted powdered formulations (Table 2) in February 1976. Another February 1976 test near Zapata, Texas, on concentrated SFE and SFE–resin gave similar results (Bullard et al., 1978b).

Odor Quality. Deletion of one or more of the four basic odor components of SFE (except amines) affected visitation as well as most of the other behavioral responses (Tables 4 and 5). In this and an earlier test (Bullard et al.,

TABLE 2. TEST A—COYOTE RESPONSES TO POWDERED LURE FORMULATIONS AT SCENT STATIONS NEAR ZAPATA, TEXAS, IN FEBRUARY 1976[a]

Lure	Visiting	Urinating	Defecating	Scratching	Digging	Rolling	Pulling	Carrying
SFE	15.5b	2.1a	0.6b	1.3b	1.3b	0.4a	4.0b	3.2b
SFE-fruity	19.4b	3.5a	0.4b	1.3b	1.3b	0.9a	3.6b	2.6b
SFE-sulfurous	16.4b	2.5a	0.2b	0.9b	0.9b	0.2a	3.6b	3.6b
SFE-sweaty	15.6b	1.2a	0.4b	1.0b	1.0b	0.0b	3.1b	2.9b
SFE-fishy	18.3b	2.5a	0.4b	0.6b	0.6b	0.6a	5.6a	5.1a
SFE-aldehyde	17.6b	2.3a	0.0b	1.1b	1.1b	0.2a	5.8a	5.2a
SFE-fecal	16.5b	1.9a	0.4b	2.7a	2.7a	0.2a	6.0a	4.8a
FEP	24.0a	4.0a	1.4a	2.1a	2.1a	0.4a	8.0a	6.3a
Blank	6.0c	1.0a	0.0a	0.0b	0.0b	0.0b	0.4c	0.4c

[a]The respective behavioral response was obtained at an odor attractant station which is a cleared 0.91-m circle having a 7-mm-high × 29-mm-diameter round plastic capsule in the center containing the lure. Comparisons of relative behavioral responses were deteremined by No. of behavioral responses divided by total operable stations; all values followed by the same letter are not significantly different ($P > 0.05$).

TABLE 3. TEST B—COYOTE RESPONSES TO COMPONENT-ENHANCED FORMULATIONS OF SFE (CONCENTRATED) AT SCENT STATIONS NEAR ZAPATA, TEXAS, IN MAY 1977[a]

Lure	Visting	Urinating	Defecating	Scratching	Digging	Rolling	Pulling	Carrying
SFE	36.5a	1.8b	0.0b	0.7a	0.0b	4.2a	19.0a	12.3a
SFE-fruity	30.9b	3.8b	0.9a	0.7a	0.0b	4.0a	13.3a	10.8a
SFE-sulfurous	29.1b	2.9b	0.4a	0.4a	0.0b	0.7b	14.9a	12.3a
SFE-sweaty	31.0b	3.8a	0.0b	0.7a	0.7a	1.0b	13.0a	11.9a
SFE-fishy	27.2b	2.1b	1.0a	0.9a	0.0b	3.1a	13.4a	12.7a
SFE-aldehyde	31.4b	1.8b	0.0b	1.0a	0.0b	5.9a	16.3a	14.2a
SFE-fecal	32.0b	1.4b	0.4a	2.8a	0.7a	3.8a	15.8a	13.4a
Abbreviated SFE[b]	32.8b	4.2a	0.0b	0.7a	0.0b	1.4b	13.2a	12.9a
Blank	13.0c	0.7b	0.0b	0.0a	0.0b	0.0b	1.0b	1.0b

[a]The respective behavioral response was obtained at an odor attractant station which is a cleared 0.91-m circle having a 7-mm-high × 29-mm-diameter round plastic capsule in the center containing the lure. Comparison of relative behavioral responses were determined by No. of behavioral responses divided by total operable stations; all values followed by the same letter are not significantly different ($P > 0.05$).
[b]See the text for chemical composition.

TABLE 4. TEST C—COYOTE RESPONSES TO INDIVIDUAL ODOR COMPONENTS OF SFE AT SCENT STATIONS NEAR ZAPATA, TEXAS, IN FEBRUARY 1977[a]

Lure or component	Visiting	Urinating	Defecating	Scratching	Digging	Rolling	Pulling	Carrying
SFE	43.0a	16.2a	0.0a	7.8a	0.3a	1.7a	22.1a	18.1a
Fruity	18.0c	1.7b	0.0a	4.3b	0.0a	0.0b	3.0c	2.0c
Sulfurous	14.2c	4.0b	0.0a	0.7b	0.3a	0.0b	4.6c	3.5c
Sweaty	29.9b	12.2a	0.3a	2.7b	0.3a	1.7a	11.6b	10.2b
Fishy	10.3c	1.3b	0.0a	1.3b	0.0a	0.0b	3.0c	2.0c

[a]The respective behavioral response was obtained at an odor attractant station which is a cleared 0.91-m circle having a 7-mm-high × 29-mm-diameter round plastic capsule in the center containing the lure. Comparison of relative behavioral responses were determined by No. of behavioral responses divided by total operable stations; all values followed by the same letter are not significantly different ($P > 0.05$).

TABLE 5. TEST D—COYOTE RESPONSES TO SFE FORMULATIONS WITH MAJOR ODOR COMPONENT MISSING AT SCENT STATIONS NEAR ZAPATA, TEXAS, IN FEBRUARY 1978[a]

Lure/missing component	Visiting	Urinating	Defecating	Scratching	Digging	Rolling	Pulling	Carrying
SFE	37.1a	24.7a	0.7a	5.7b	6.7a	1.7a	14.0a	10.4a
(−) Fruity	31.1b	7.4b	0.0a	5.4b	2.7b	3.0a	10.8a	7.8a
(−) Sulfurous	29.5b	9.1b	0.0a	6.1b	1.7b	2.6a	16.2a	12.5a
(− (Sweaty)	25.5b	8.1b	0.4a	5.1b	0.7b	2.0a	7.1b	5.2b
(−) Fishy	33.3a	13.2b	0.7a	9.4a	1.7b	3.0a	13.6a	9.9a

[a]The respective behavioral response was obtained at an odor attractant station which is a cleared 0.91-m circle having a 7-mm-high × 29-mm-diameter round plastic capsule in the center containing the lure. Comparison of relative behavioral responses were determined by No. of behavioral responses divided by total operable stations; all values followed by the same letter are not significantly different ($P > 0.05$).

1978b), FEP outranked SFE–resin in most behavioral categories even though the two had equivalent odor intensities in human odor panel tests. FEP had 1.5-fold more visits ($P \leq 0.05$) in test A, and a similar influence was observed for defecating, scratching, digging, pulling, and carrying (Table 2). Apparently, natural FEP had a more "full bouquet" odor quality that goes beyond the importance of having all four basic odor components present in the synthetic formulations.

Behavioral Response. Since tests A, C, and D were conducted in February, we were able to evaluate the influence of odors on scent-marking (urinating, defecating, and scratching) during the breeding season. Scent-marking occurs when urine, feces, or a gland secretion is deposited on an environmental object that will be responded to by conspecifics, including the animal that deposited the mark (Henry, 1977). The same urination rate on blank and SFE capsules in tests A and B indicate the tendency of coyotes to mark strange objects, regardless of season or odor influences.

The frequency of scent marking for coyotes in captivity has been reported to increase during the breeding season (Kleiman, 1966). Likewise, a comparison of the rates of urinating and scratching for SFE in two 1978 tests (B and D), conducted in the same area but 1 month apart, showed urinating and scratching rates several times higher in the earlier (February) test (D). Defecating rates were low in all tests. Removal of any base odor component during breeding season reduced urination (Tables 4 and 5). Volatile fatty acids were the most important single odor component (Table 5), but eliminating any of the four odor components caused a decrease in urination (Table 4 and 5). Enhanced formulations were not different from SFE for the resin powders (Table 2).

Signs of scent-rubbing or rolling by coyotes were observed throughout these tests. During scent-rubbing, the coyote usually stretches its body, turns its neck, and rolls on an object while twisting back and forth with legs thrashing in the air (Lehner, 1978; Rieger, 1979). As carrion reaches an advanced stage of decomposition foxes often scent-rub rather than consume the carrion (Albone et al., 1978). In postbreeding test B, the rolling index ranked higher than either urinating and scratching indexes for SFE, whereas the reverse was true in breeding-season tests C and D. In test C volatile fatty acids was the only individual odor component that elicited scent-rubbing, but enhancing this component in May (test B) caused a decrease.

Pulling was the most prominent behavioral response and usually the capsule was carried outside the 0.91-m circle. Sometimes it appeared that there was a link between pulling, carrying, and digging. Indexes for Test B were several-fold higher than A for most candidates, indicating that odor intensity influenced both pulling and carrying Comparison of FEP and SFE–resin in test A indicated that "full-bouquet" quality was also important. Odor intensity did not appear to have as much influence on the index for

digging (tests A and B), but test D indicated that deleting any of the four primary odor components caused a significant decrease. The fecal odor of indole (discussed below) seemed to elicit a caching response involving all three behavioral categories. Although combined pulling and digging responses were low and no statistical association to caching behavior could be made, occasionally several lure capsules were found cached under a log or in deep grass.

Chemical Influences. The influence of all four odor components in scent-marking has been discussed. However, as individual components in test C, esters, organosulfurs, and amines failed to elicit a behavioral response equivalent to SFE in any category except defecating and digging. The sweaty odor of volatile fatty acids was equal to SFE in eliciting urinating and rolling responses and was superior to the other three odor classes in four of the seven behavioral categories. Deletion of this fraction in test D produced the greatest decrease in behavioral responses. Volatile fatty acids were probably the most critical odor component in SFE. Roughton (1982) found the 10-component volatile fatty acid fraction of SFE to equal SFE in visitation by coyotes in 1976 studies in New Mexico. Volatile fatty acids, major decomposition products of proteinaceous materials, are prominent in the glands of coyote prey and are part of their own chemical communication process (Albone et al., 1978; Bullard, 1982).

Aldehydes (fat decomposition products) did not have much influence in the concentrated formulation (test B) but increased pulling and carrying in the resin powder (test A). Indoles increased digging at both concentrations and pulling and carrying in the resin powder. Carrion is commonly cached, and indoles are decomposition products of flesh proteins (Frazier, 1967). Although we could not statistically link them, it is interesting to note the parallel between pulling and digging for FEP and SFE–fecal for low odor intensitites in test A.

These tests indicate the overwhelming importance of odor intensity, but at a given level, it is the odor composition or quality in conjunction with simultaneous ongoing activity (i.e., hunting, defense, breeding, etc.) that produces a given behavioral response. A better understanding of the odor intensity–quality relationship in conjunction with environmental and physiological factors will be the key to eliciting specific behavioral responses from free-ranging coyotes.

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CHANGES IN CHEMICAL COMPOSITION OF A DECOMPOSING AQUATIC MACROPHYTE, *Lemna paucicostata*[1]

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Abstract—To understand the changes in chemical composition which may occur as an individual aquatic macrophyte decays, a species of tropical duckweed (*Lemna paucicostata*) was decomposed (aerobically and anaerobically) by its associated microbial population. Under anaerobic conditions, decomposition of the plant and microbial growth were very rapid. Short-chain carboxylic acids (C_2–C_5) and ammonia were generated in considerable amounts (reaching a maximum of ca. 30% by weight of original dry weight of *Lemna* after 71 days). In contrast, free sugars decreased, and amino compounds initially increased slightly and then decreased to the original levels. There were no physical or chemical indications of decomposition occurring under aerobic conditions. The likely biochemical pathways involved in anaerobic decomposition are discussed, and the results are compared with other anaerobic systems such as the rumen, sludge digestion, and aquatic sediments.

Key Words—*Lemna paucicostata*, decomposition, short-chain carboxylic acids, ammonia, *Biomphalaria glabrata*.

INTRODUCTION

There is evidence that decaying plant material is an important component in the environment of the snail *Biomphalaria glabrata* (Say), an aquatic host of *Schistosoma mansoni* (Sambon). Thus this snail occurs predominantly in habitats where there is depositing substrate containing detritus, and although it will eat both decayed and fresh plants, it shows a preference for the former

[1]Part I of the Chemical Ecology of Freshwater Snail–Plant–Detritus Interrelationships.

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(Pimentel and White, 1959; Mason, 1974). This detritivorous habit is characteristic of other planorbid snails, such as *Planorbis contortus*, which have been studied in detail (Calow, 1973, 1974). Yet comparatively little is known about the chemical changes which occur in the organic composition of aquatic plant material during the microbial decay process, or about their significance to aquatic invertebrates such as *B. glabrata*.

An investigation was therefore undertaken to answer this question: which chemical components are released by a decomposing aquatic macrophyte? Particular attention was focused on the short-chain carboxylic acids (C_2–C_5) since these are common end-products of microbial decomposition and are known to be consistent attractants to *B. glabrata* (Thomas et al., 1980).

Lemna paucicostata (a tropical duckweed) was used as a laboratory model for studying the decomposition process, for the following reasons: (1) *Lemna* and other floating plants tend to occur in sheltered areas of both lotic and lentic habitats, with depositing sediments, favored by *B. glabrata* (Pimentel and White, 1959). (2) Aquatic plants such as *Lemna* are likely to contribute significantly to the detritus because they have a dominant position in the neustron community, a high biotic potential, and relatively short life span (Pomeroy, 1980a). (3) *Lemna* is easily cultured in the laboratory.

In these experiments, the plant was allowed to decay under aerobic and anaerobic conditions in a closed (i.e., nutrient-limited) aquatic environment, containing only those microorganisms already present on the plant phylloplane. Concentrations were measured for the following chemical components: short-chain carboxylic acids (C_2–C_5), ammonia, amino compounds, proteins, and mono- and disaccharides.

METHODS AND MATERIALS

Glassware. Glassware was first cleaned, either in an automatic washing machine or by scrubbing in detergent and rinsing in tap water, before being washed in distilled water and dried in an oven. Where sterile conditions were required, glassware was heated to 160° C for 1 hr, cooled gradually to room temperature, and used immediately.

Water. Single glass distilled water was used for making up bulk quantities of a standard solution containing known concentrations of inorganic salts. This solution (standard snail water or SSW2) has been described by Thomas et al. (1975). Double glass distilled (DD) water (the second distillation from alkaline potassium permanganate) was used for rinsing glassware, in chemical tests where required, and for making up small-scale stock solutions. SSW2 was sterilized by boiling (2 min).

Lemna paucicostata *Cultivation.* The plants, together with the micro-

organisms on the phylloplane, were cultivated in open tanks containing SSW2 (50%) and distilled water (50%). Thus the individual plants retained their associated microbial population.

Wet–Dry Weight Ratio. Fresh *Lemna* (700 mg) was dried in vacuo over self-indicating silica gel until a constant weight (53 mg) was reached (after ca. 5 hr). Fresh *Lemna* was found to contain ca. 92.4% water.

Scanning Electron Microscopy of Leaf Surfaces. The *Lemna* leaves were first washed thoroughly in deionized water before being fixed in 5% glutaraldehyde in cacodylate buffer (50 mM, pH 7.0) while under vacuum in a Thüberg tube. This was followed by several washes in buffer and finally by dehydration in 2,2-dimethoxypropane (Morton and Trimble, 1977). The leaves were viewed through a scanning electron microscope (JEOL JSM 35C) after mounting on a Cambridge stud and splutter coating with 500 Å gold (working distance 15 mm; absorbed specimen current 1×10^{-10} A, accelerated voltage 20 kV). The magnifications are given on the micrographs.

Decomposition. The method described below was chosen to fulfill the following criteria: (1) all *Lemna* plants should "die" simultaneously; (2) only microorganisms on the plant surfaces should be present during decay; (3) this microbial population should be damaged as little as possible by the "death" of the *Lemna*. Typically, washed *Lemna paucicostata* (10 g wet weight) was homogenized (1 min) with sterilized SSW2 (100 ml) and divided into ten portions of ca. 10 ml each. Experiments were carried out in sealed, gas-tight vials ("anaerobic" conditions) and in conical flasks plugged with sterile cotton wool and/or covered with aluminium foil (aerobic conditions), kept at 26° C, with equal (12 hr) light and dark periods. The various *Lemna* samples were then left to decompose for different periods of time (from 0 to 71 days). At the end of each period, individual samples were centrifuged (3000 rpm, 10 min), and the supernatant decanted into a clean vial, sealed, and stored at −20° C until required for analysis. This procedure is summarized in Figure 1.

Chemical Analysis. Concentrations of short-chain carboxylic acids (C_2–C_5), ammonia, amino compounds, proteins and sugars in the supernatants were determined by the methods described below.

Short-Chain Carboxylic Acids. These were quantified by high-performance liquid chromatography (HPLC) after extraction and derivatization (Patience and Thomas, 1982). Briefly, the supernatant (1 ml) was acidified with HCl (1 M, 0.05 ml) to give a pH $\leqslant$ 2. This was extracted with diethyl ether (2× 3 ml). The ether was pipetted into a vial which was placed in a freezer (−20° C) for 30 min, to freeze trace quantities of water onto the sides of the vial. The dry ether was decanted into a fresh vial, and aqueous $KHCO_3$ (0.1 M, $\leqslant$ 0.1 ml) was added to convert the extracted acids into their K^+ salts. The diethyl ether was removed under a N_2 line. The acid salts were converted to *p*-bromophenacyl esters by addition of *p*-bromophenacyl bromide–18-Crown-6 (10:1) in acetonitrile and then heated (80° C, 20 min) (Durst et al.,

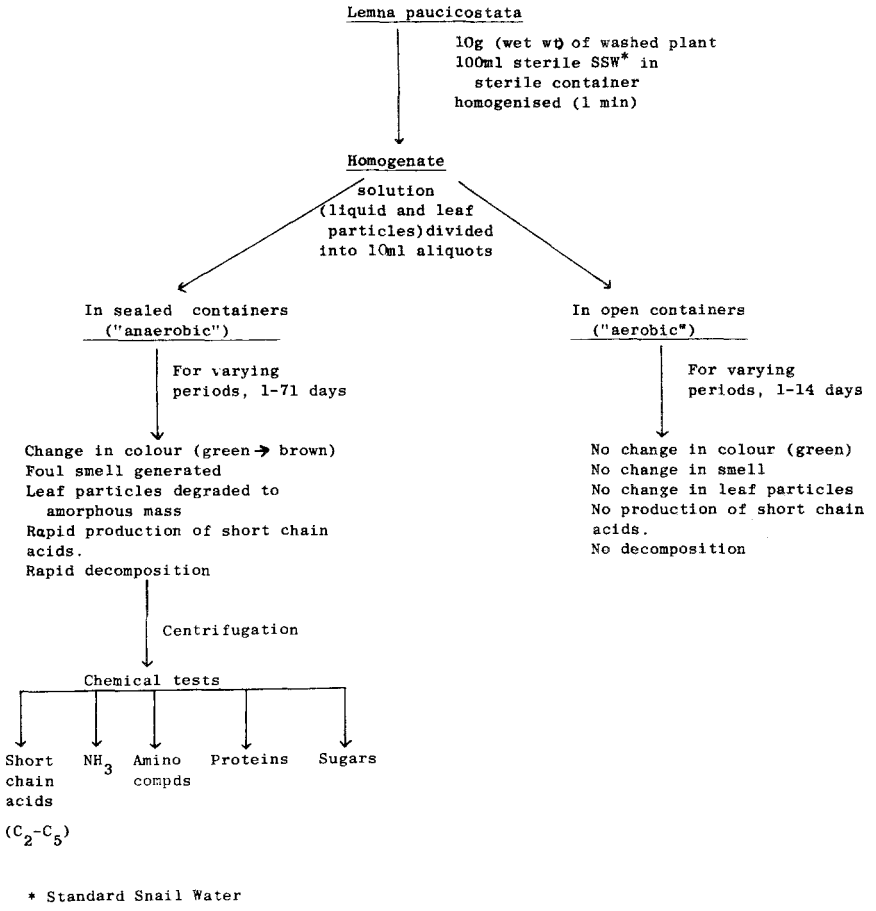

FIG. 1. Decomposition of *Lemna paucicostata*, experimental procedure.

1975). Esters were separated by HPLC on a Waters μ Bondapak C_{18} reversed-phase column eluted with acetonitrile–water (50:50) and detected at 254 nm. Figure 2 shows a typical chromatogram. Quantification was by peak area measurement (peak area = height × width at half height), and comparison with standards. Each individual sample was analyzed at least twice. Allowance was made for losses during extraction by spiking blank samples (5) with 0.5 μmol each of acetic, propanoic, and butanoic acids. Mean recoveries (% ± SD) were: acetic acid, 61 ± 9; propanoic acid, 64 ± 11; butanoic acid, 65 ± 16.

Ammonia. Ammonia (present mainly as NH_4^+) was determined by the phenate method described in Standard Methods for the Examination of Water and Wastewater (1981).

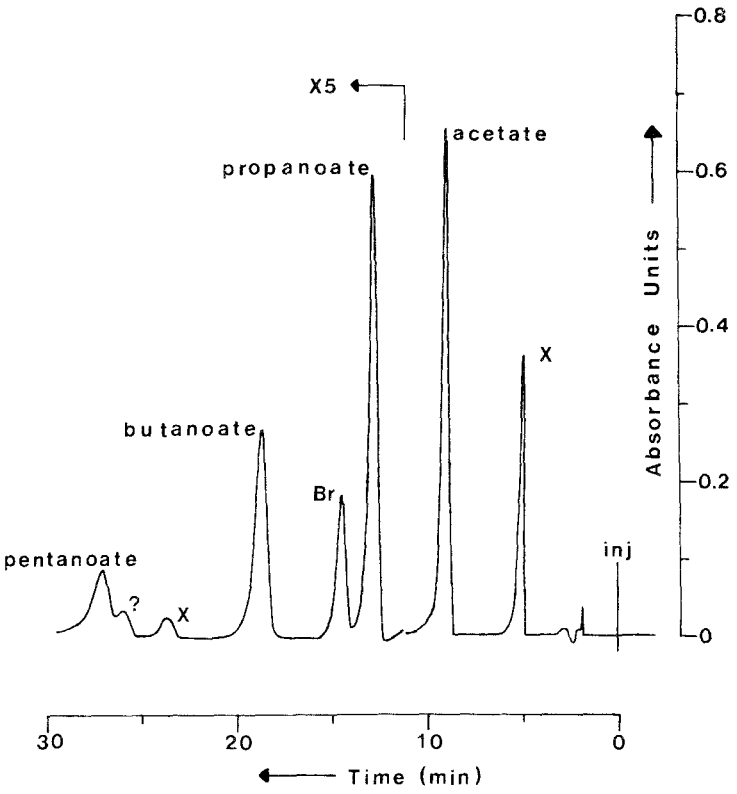

FIG. 2. HPLC separation of short chain carboxylic acids (*p*-bromophenacyl esters) from 3-day decomposing *L. paucicostata*. Conditions: 10 μl loop injection on μBondapak C_{18} column; acetonitrile–water (50:50) elution; flow rate 1 ml/min; UV detection, 254 nm. Br = *p*-bromophenacyl bromide; X = reaction by-product; ? = unknown component.

Amino Compounds. These were quantified as a class by the ninhydrin test (Rosen, 1957). However, as ammonia also responds to the test, amino compounds were determined by difference (ninhydrin test − phenate test).

Protein. Concentrations were determined by the Hartree test (Hartree, 1972). Since free aromatic amino acids also respond, protein was separated by precipitation from the aqueous solution (1 ml) with hydrochloric acid (1 M, 0.1 ml) and centrifuging (3000 rpm, 5 min). The precipitate was redissolved in NaOH (0.1M, 1 ml) before analysis.

Sugars. Mono- and disaccharides were determined qualitatively by silica gel G thin-layer chromatography (TLC) using acetonitrile–water (85:15) eluant (Gauch et al., 1979). One elution was found to be sufficient for adequate separation of individual sugars. Spots were visualized by spraying

with diphenylamine–anilinium chloride in methanol–phosphoric acid and heating, (Gauch et al., 1979; Zilic et al., 1979). Since concentrations in the supernatants were very low, each solution (500 μl) was concentrated (100×) to 5 μl before application to the plate. Identification was based on comparison of R_f values of standard solutions (xylose, fructose, glucose, sucrose, and maltose; 1 μl aqueous solution, containing 4 μg per component, applied to the plate.

RESULTS

Changes in Short-Chain Carboxylic Acids. Concentration values for the supernatant were converted to production of acids per gram (dry weight) of fresh *Lemna*, using the known ratio of fresh plant–supernatant (1 g: 10 ml) and the percentage dry weight of the plant (7.6%). Thus production of carboxylic acids with increasing decomposition is given in Table 1 and shown graphically in Figure 3. Acetate was by far the most abundant product, reaching a concentration of ca 2.5 mmol/g dry wt of *Lemna*. Propanoate, butanoate, and pentanoate were also detected, albeit at ca. 18, 16, and 8%, respectively, of acetate levels by day 71. Formate and lactate were not detected, while homologs $>C_5$ were not analyzed. It should be noted that isomers of butanoate and pentanoate were not separable by the HPLC method used.

The rate of increase in acetate concentration was nearly constant from days 0 to 3 (Figure 3). Thereafter, the rate declined gradually, and by day 28 a plateau was reached, with only small increases in concentration detected on days 42 and 71. A similar pattern was observed for the other acids.

Ammonia. Ammonia concentrations are given in Table 1 and Figure 4. Production followed the same pattern as for acetate, i.e., an initial rapid increase in concentration followed by a gradual decline in the rate of increase and a leveling of the concentration at ca. 2.9 mmol/g (dry weight) of *Lemna*. The ammonia concentration always remained close to the sum of the carboxylic acid concentrations, which probably explains why the pH stayed constant at ca. 6.5 throughout the decomposition period.

Amino Compounds. The concentrations showed some fluctuations, although they remained less than 0.7 mmol/g (dry weight) of *Lemna* throughout the 71-day period (Table 1, Figure 4). After an initial increase peaked on day 1, the concentration fell on day 2 and remained approximately constant until day 8, declined to day 14, then gradually increased up to day 71. This trend is in marked contrast to that observed for ammonia and short-chain acids.

Protein. Concentrations (Table 1) are presented as mg/g dry wt. of *Lemna* only (millimole values cannot be determined). These remained

TABLE 1. CHANGES IN CHEMICAL COMPOSITION OF DECOMPOSING *Lemna Paucicostata* OVER 71 DAYS

Component	Day									
	0	1	2	3	5	8	14	28	42	71
Acetate										
mmol/g[a]	n.d.[c]	0.29	0.62	1.00	1.38	1.68	2.01	2.18	2.25	2.53
mg/g		17.4	37.3	59.8	82.8	100.6	120.6	130.9	134.7	151.5
Propanoate										
mmol/g	n.d.	0.017	0.16	0.23	0.26	0.33	0.38	0.41	0.40	0.47
mg/g		1.3	11.5	16.7	18.8	24.2	27.8	30.3	29.7	34.4
Butanoate										
mmol/g[b]	n.d.	0.015	0.08	0.13	0.14	0.24	0.31	0.37	0.36	0.40
mg/g		1.3	6.8	11.5	12.3	20.9	27.0	32.9	32.0	35.0
Pentanoate										
mmol/g[b]	n.d.	0.019	0.05	0.07	0.08	0.13	0.18	0.20	0.19	0.21
mg/g		1.9	4.8	7.5	8.6	12.8	18.6	20.0	19.3	21.5
Ammonia										
mmol/g	0.08	0.93	1.47	1.74	1.90	2.41	2.67	2.82	2.86	2.94
mg/g	1.3	15.7	25.1	29.6	32.3	40.9	45.4	48.0	48.7	49.9
Amino compounds, mmoles/g	0.20	0.68	0.48	0.50	0.40	0.50	0.18	0.19	0.24	0.45
Protein, mg/g	1.7	2.5	2.2	2.2	2.1	2.3	2.3	2.4	2.3	4.4

[a] Per gram (dry wt) of fresh *Lemna* throughout.
[b] Straight and/or branched isomers (not separable by HPLC).
[c] n.d. = not detected (<0.005 mmol/g).

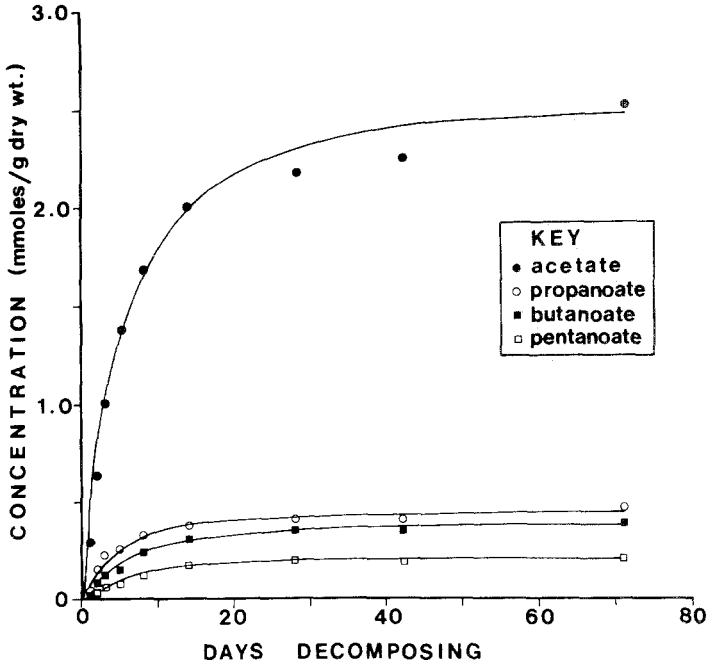

FIG. 3. Concentrations of short-chain carboxylic acids (C_2–C_5) from decomposing *L. paucicostata*.

between 1.7 and 4.4 mg/g, with no significant upward or downward trends with time.

Sugars. Figure 5 shows a diagram of the TLC plate after separation and visualization of soluble sugars in the supernatant (days 0–42). From fresh *Lemna*, the major sugars were glucose and fructose, with lesser amounts of four other sugars, including possibly maltose and/or sucrose, and xylose. Concentrations were crudely estimated by visual comparison of spot color intensity with that of a standard of known concentration. This gave values of ca. 1 mg/g dry weight *Lemna* for glucose and fructose. The six spots visible on day 0 had disappeared by day 2, and between days 2 and 14, only one (new, unidentified) spot was visible on the plate. From day 28 on, no sugars were detected.

The chemical composition of decomposed (71-day) *Lemna paucicostata* is summarized in Table 2. The left-hand column gives concentrations as a percentage value, i.e., total number of grams per component present in the supernatant after 71 days per 100 g of dry fresh *Lemna*. Thus of the original plant, on day 71 ca. 36% was in the form of water-soluble components and ca. 45% as solid material, leaving approximately 20% unaccounted for. Some of

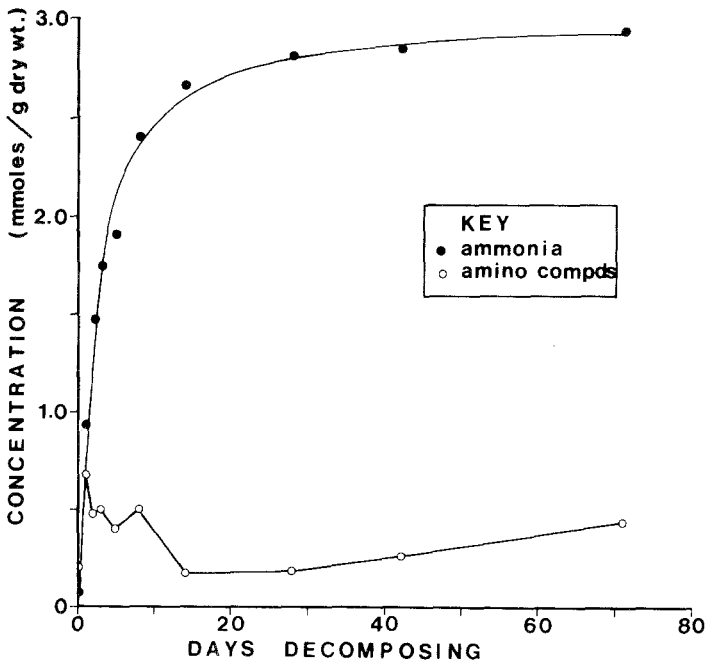

FIG. 4. Concentrations of ammonia and amino compounds from decomposing *L. paucicostata.*

this will be inorganic species, some may be (dissolved) gases, and the remainder will be organic components which we have not analyzed. However, on a mmol/g basis (Table 2, right-hand column), ammonia and acetate (i.e., ammonium acetate) almost certainly represent the major breakdown products.

Scanning Electron Microscopy of Leaf Surfaces. Figures 6 and 7, which are scanning electron micrographs of the ventral surface of *Lemna* leaf, show that the dominant microorganisms of the phylloplane community are rod-shaped and coccoid bacteria. Both of these, but particularly the former, occur at high densities. Other microorganisms present in smaller numbers are flexibacteria, caulobacteria, filamentous bacteria, and at least two species of diatoms. Similar microorganisms were found on the dorsal surface of *Lemna* leaves but in much smaller numbers.

DISCUSSION

The principal aim of this study was to quantify, as far as possible, the water-soluble end-products from decomposition of a macrophyte species,

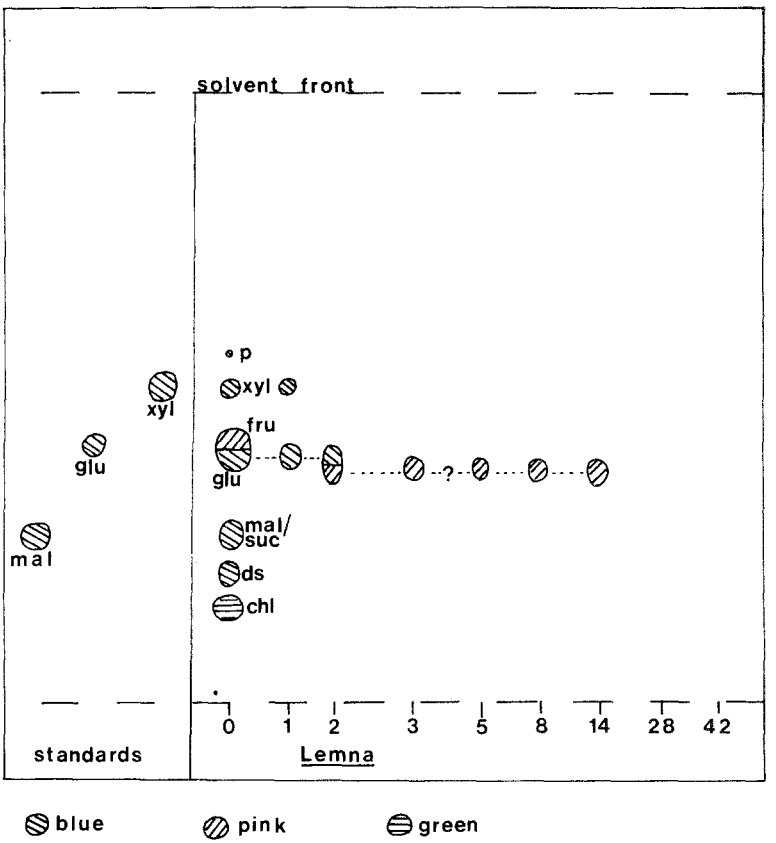

FIG. 5. Qualitative analysis of mono- and disaccharides from decomposing *L. paucicostata*, by silica gel G TLC (acetonitrile-water, 85:15, eluant). Abbreviations: mal = maltose; glu = glucose; xyl = xylose; fru = fructose; suc = sucrose; p = pentose?; ? = unknown monosaccharide?; ds = disaccharide?; chl = chlorophyll.

Lemna paucicostata. This is unusual, as previous studies of chemical changes occurring during decomposition have been concerned with material containing a mixture of plant and other substrates (e.g., sewage sludge digestion and rumen fermentation), or single or simple mixtures of synthetic chemicals. Earlier studies of decomposition of individual macrophytes have been concerned mainly with biological rather than chemical changes (Lammens and Van der Velde, 1978; Bastardo, 1979; Smock and Stoneburner, 1980).

The present experiments, whether under aerobic or anaerobic conditions, were all carried out in closed, or nutrient-limited, conditions rather than in continuous culture. This is because closed systems are more likely to simulate

TABLE 2. CHEMICAL COMPOSITION OF *Lemna* SUPERNATANT AFTER DECOMPOSITION (71 DAYS)

	%(g/100g)[a]	Millimoles/g[a]
Acetate	15.2	2.53
Propanoate	3.4	0.47
Butanoate	3.5	0.40
Pentanoate	2.1	0.21
NH_3	5.0	2.94
Amino compounds	6.7[b]	0.45
Protein	0.4	
	36.3	
"Solids"	ca. 45 (±5)	
Total	ca. 80% of original dry weight accounted for	

[a]Dry weight.
[b]Assuming mean molecular weight of 150.

conditions in nature, since the main constraint on microbial growth in the aquatic environment is the availability of inorganic and organic nutrients (Tempest and Neijssel, 1981). The conditions under which *Lemna* was decomposed resemble those found in nature in two respects: (1) Decomposition occurs within individual cells or the envelope of the leaf and would therefore cease when the resources are depleted; (2) the microdecomposers involved are those already present on the phylloplane of each plant.

It was, at first sight, surprising to find no sign of microbial decomposition under aerobic conditions, as the phylloplane microbes involved are evidently capable of aerobic existence, whereas under anaerobic conditions decomposition was extremely rapid. The following hypotheses may explain these observations. The homogenized plant tissues which have access to air may continue to metabolize and thus remain resistant to microbial decomposition. In contrast, the rapid depletion of residual dissolved oxygen in the sealed vials may cause the plant tissues to die. However, the microdecomposers are able to function as facultative anaerobes. Alternatively, the microbes may remain in a state of diapause until the plant cells die. Two observations support these hypotheses. Firstly, the homogenized tissues with access to air eventually regenerate leaflike structures. Secondly, the results for carboxylic acid production in the sealed vials were very similar to those obtained in vials where oxygen was removed by degassing and replacing with nitrogen before sealing. This indicates that anaerobic conditions were produced rapidly whether or not dissolved oxygen was present initially.

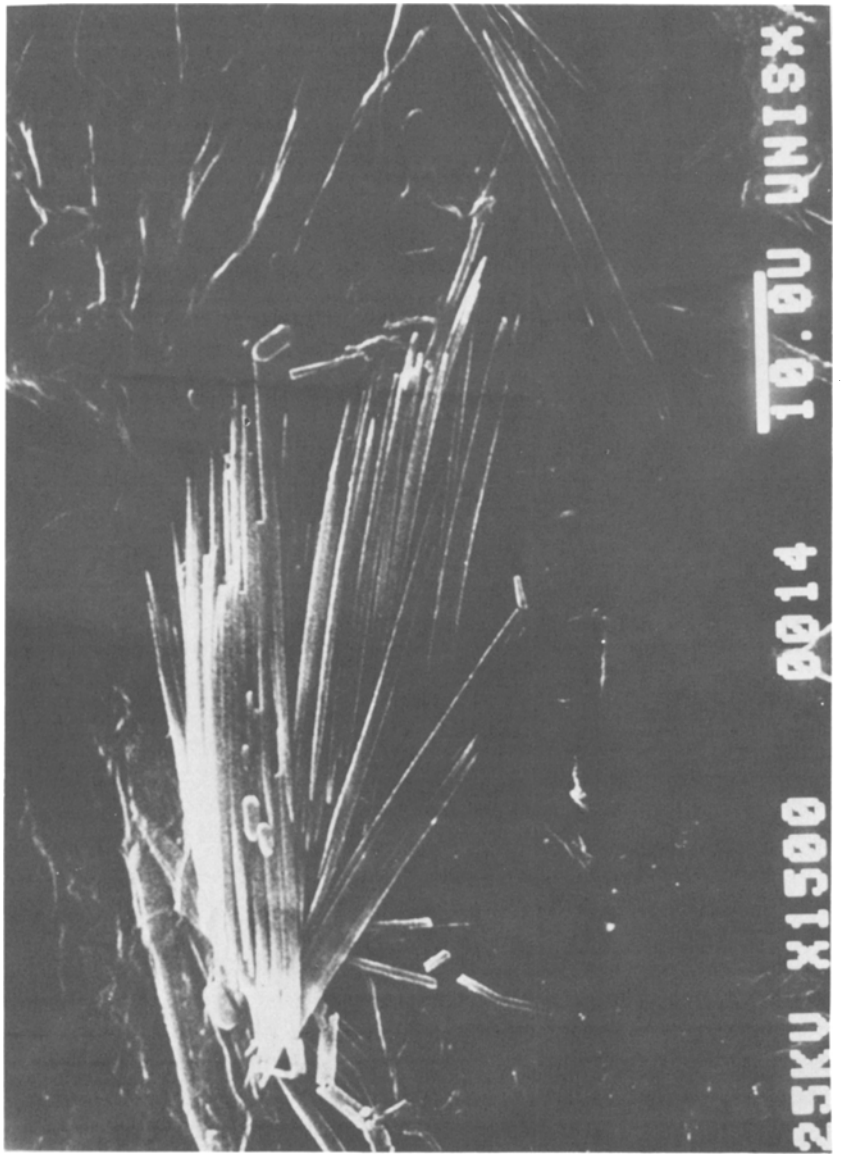

FIG. 6. Scanning electron micrograph of ventral surface of *Lemna paucicostata* leaf with coccoid and rod-shaped bacteria (×1200).

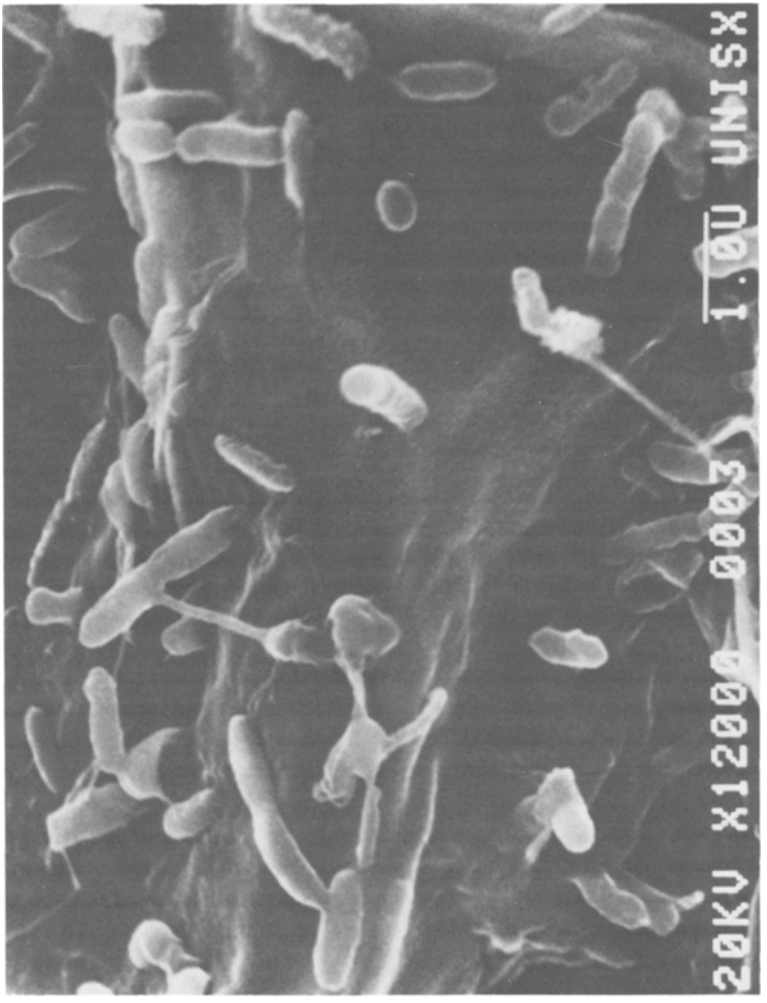

FIG 7. Scanning electron microgrpah of ventral surface of *Lemna paucicostata* leaf showing colonial diatoms with associated bacteria (×1500).

Under anaerobic conditions, there was no sign of a lag phase in the production of ammonia or short-chain carboxylic acids, and hence microbial growth. In other words, any lag period must have been minutes or hours only. The concentration profiles for the accumulation of carboxylic acids and ammonia simulate those for microbial growth in a closed environment. Mathematically (Lynch and Poole, 1979), this can be expressed as:

$$\text{Rate} = \frac{dx}{dt} = k \cdot x \left(1 - \frac{x}{x_f}\right)$$

where x = microbial population size, or end-product concentration; x_f = plateau population size, or end-product concentration; and t = time. Thus, when x is small, dx/dt is small (lag phase); when $x = \frac{1}{2}x_f$, dx/dt is a maximum; and when $x \simeq x_f$, dx/dt is small (plateau region).

Thus ammonia production rate reached a maximum on day 2 (i.e., when $x = \frac{1}{2}x_f$), acetate and propanoate on approximately day 4, and butanoate and pentanoate on approximately day 6.

Short-chain carboxylic acids are widely distributed in nature and the pathways leading to their formation are reasonably well documented. Of particular comparative relevance to this work are studies on aquatic invertebrates (to be discussed in a subsequent paper) and sediments, rumen fermentation, and anaerobic sludge digestion. The latter two processes are included because it is apparent that decomposition of *L. paucicostata* took place via nonmethanogenic fermentation (which is an anaerobic process). The presence of propanoate, butanoate, and pentanoate is particular evidence that this is the case, since these acids are not major metabolic end-products of respiration pathways. In anaerobic sludge digestion, short-chain carboxylic acids are formed as intermediates in methane generation (Jeris and McCarty, 1965; Toerien and Hattingh, 1969; Joergensen, 1978; Spoelstra, 1979; Runquist et al., 1981; Uribelarrea and Pareilleux, 1981); while in the rumen (which is basically an anaerobic plant digester), short-chain acids are produced in vast quantities as an energy source for the cow (see Russell and Hespell, 1981). The results obtained for *Lemna* decomposition and rumen fermentation bear a strong similarity when one considers the final mole ratios of carboxylic acid end-products (Table 3). Note, additionally, that lactate and formate did not accumulate in this study, and lactate especially and formate are frequently intermediates rather than end-products in the rumen (Russell and Hespell, 1981) and the nonmethanogenic phase of anaerobic sludge digestion (Toerien and Hattingh, 1969).

Biochemically there are three main organic starting materials for generation of short-chain acids—polymeric carbohydrates, proteins, and long-chain lipids such as fatty acids. The initial chemical composition of *Lemna paucicostata* was not evaluated here, except for certain water-soluble compon-

TABLE 3. COMPARISON OF *Lemna* DECOMPOSITION AND RUMEN FERMENTATION: TYPICAL MOLE RATIOS OF CARBOXYLIC ACID END-PRODUCTS

	Lemna (day 71)	Rumen[a]
Acetate	1	1
Propanoate	0.18	0.3
Butanoate	0.14	0.15
Pentanoate	0.08	0.07
Formate	n.d.[b]	often minor
Lactate	n.d.	often minor

[a]From Baldwin et al. (1977).
[b]n.d. = not detected.

ents released by homogenization. However, the bulk composition of indoor cultivated *L. paucicostata* has been reported (Chang et al., 1978) as (% dry wt): protein 46.4; total carbohydrate 15.1; fat 18.7; ash 12.4 (total 92.8). Another duckweed, *Lemna minor*, from a lake, appears to have a different composition (% dry wt) (Jabbar Muztar et al., 1978 a–c, 1979): crude protein 20.0; crude fiber 2.40; ash 16.6; inorganic 10.6 (total 65.8). Despite these differences (which may be due in part to different methods of analysis), these figures indicate the availability of suitable fermentation substrates. In each case, acetate is the major catabolic end-product—fatty acids tend to produce, via β-oxidation, little else. Carbohydrates can be degraded, via fructose-6-P and pyruvate, to give acetate, propanoate, and *n*-butanoate (Figure 8A) (Lynch and Poole, 1979) while proteins degrade to amino acids, which give, in addition to C_2–C_4 acids, ammonia plus branched C_4 and C_5 acids (Figure 8B) (Poston, 1976; Elsden and Hilton, 1978). Only certain amino acids (valine, leucine, and isoleucine) are converted largely to those branched acids. The results obtained here for protein, amino acid, and sugar concentrations in the supernatant are compatible with such pathways. Protein levels remained low and constant throughout, while amino acids increased during the first day, decreased on day 2, and then remained at an approximately constant concentration until day 8. The initial increase presumably arose from protein degradation, but a further increase was prevented by conversion to deaminated products and ammonia. The decline from days 8 to 14 coincided with the decline in microbial growth rate, and hence protein degradation. Predictably, free mono- and disaccharides in the fresh homogenate disappeared very quickly.

As *B. glabrata* is a detritivorous snail attracted to short-chain acids (Thomas et al., 1980), it is relevant to compare the data from this experiment with literature reports of such compounds (concentrations and turnover rates)

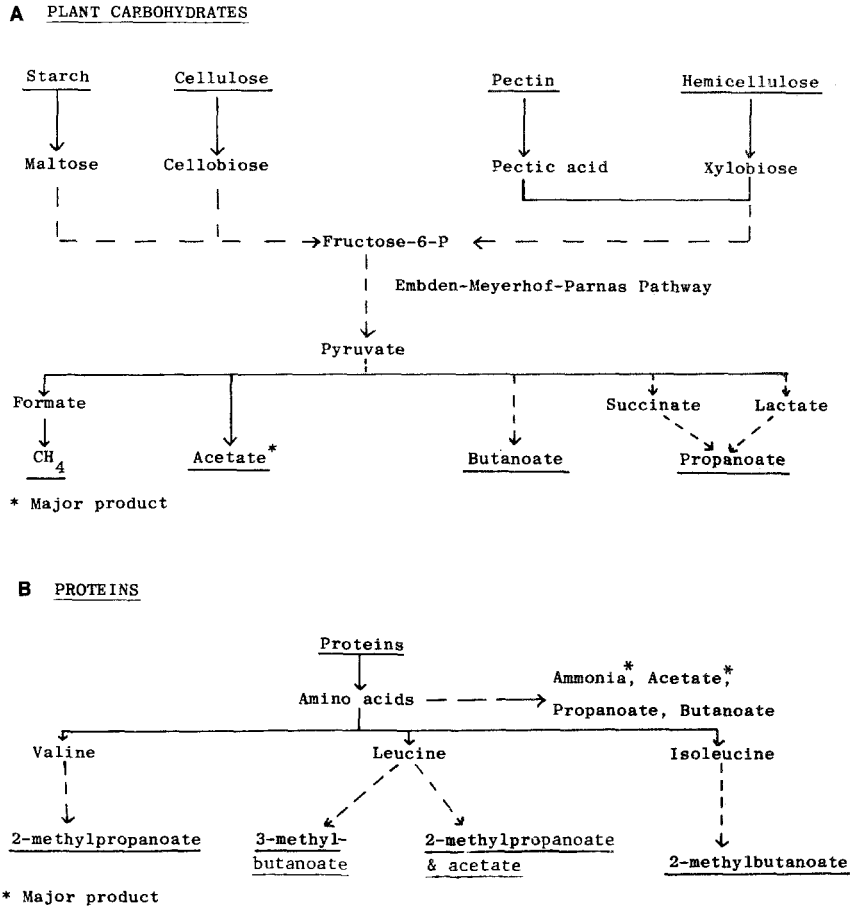

FIG. 8. Biochemical pathways (simplified) of short-chain carboxylic acid and ammonia production in fermentation. (A) from Lynch and Poole (1979); (B) from Elsden and Hilton (1978) and Poston (1976).

in aquatic surface sediments. Table 4 shows that they are present in both freshwater and marine sediments. Acetate concentrations (pool sizes) vary widely from ca. 1 to 2600 μmol/liter. However, six of the seven values are less than 250 μmol/liter, and it seems possible that this may represent a normal maximum value. Where turnover rates and concentrations were determined for the same site, the hourly turnover rates are of the same order of magnitude as concentrations, indicating that rate of production is much higher than the pool size alone would indicate. Note that at least two of propanoate, butanoate and pentanoate were also present in four of five sediments. It is, therefore, clear that short-chain carboxylic acids can be generated in large

amounts in surface sediments, and it seems probable that the biochemical processes involved in their production from decomposing plant material will be similar to those occurring in decomposing *Lemna* and rumen fermentation, for example. However, their subsequent turnover, by mineralization or methane formation, may be almost equally rapid.

There is evidence that the microorganisms involved in the decomposition process are mainly rod-shaped or coccoid bacteria. Firstly, it has been shown that these bacteria are present at high densities on the ventral surfaces of the living *Lemna* leaves. As decomposition occurred under sterile conditions, this could have been the only source of microbes. Such microorganisms also appear to be dominant components of the phylloplane communities of both aquatic and terrestrial plants (Sieburth, 1975; Lacey, 1979). Secondly there is evidence that bacteria are predominantly the primary colonizers of the remains of aquatic plant material undergoing decomposition, including *Scenedesmus* (Gocke, 1971), other phytoplankton species (Skopintsev et al., 1979a,b; Newell et al., 1981), and plant material generally (Willoughby, 1976; Fenchel and Jørgensen, 1977; Pomeroy, 1980b). In addition, Skopintsev et al. (1979b) demonstrated that short-chain carboxylic acids were produced under anaerobic conditions, although these subsequently disappeared possibly through the influence of sulfate-reducing bacteria.

It is also interesting that in both the present and other studies, the bacterial population demonstrated facultative anaerobiosis. This is perhaps not unexpected, as it is important that aquatic microorganisms can tolerate and exploit aerobic and anaerobic conditions, since both can and do occur in the same body of water or sediment (Willoughby, 1974). These micro-decomposers may well have coevolved to form a symbiotic relationship with the plants on which they live (Whatley and Sequeira, 1981). The benefits to the micro-organisms are obvious because, as leaves die, they are provided with a food source. However, the surviving plants may also benefit from the release of minerals and growth-promoting factors produced during microbial decomposition.

B. glabrata, like a number of other aquatic invertebrates, browses on partially degraded plant material and its associated community of bacteria and other microinvertebrates (Pimentel and White, 1959; Fenchel and Jørgensen, 1977; Smock and Stoneburner 1980). Under these conditions, the snail might be expected to use chemical signals to locate its food source (kairomones). Short-chain carboxylic acids generated by microbial decomposition may well be involved in this process, since propanoate and butanoate in particular are strong attractants to *B. glabrata* (Thomas et al., 1980). Although it has been shown that these acids are produced in aquatic sediments, it remains to be demonstrated that they can act as attractants under (simulated) natural conditions. Alternatively, dissolved carboxylic acids may

TABLE 4. SOME ANALYSES OF SHORT-CHAIN CARBOXYLIC ACIDS IN AQUATIC SEDIMENTS[a]

	Pool sizes					Turnover rates		
	Formate	Acetate	Propanoate	Butanoate	Pentanoate	Acetate	Propanoate	Butanoate
1. Freshwater anaerobic sediment, 12 m water column, depth of sed. 1 cm		5 ± 8 $\mu mol/l_s$				0.67 $\mu mol/l/hr$		
2. Marine anoxic sediment, 10 m water column, depth of sed: 0 cm		ca. 1	ca. 0.2 $\mu mol/l$	ca. 0.2		4 $\mu mol/l/hr$		
3. Marine reduced sediment (shallow coastal lagoon), depth of sed: not given						25	3–7 $\mu mol/kg/hr$	2–4
4a. Marine anoxic sediment, 580 m water column, depth of sed: 0–5 cm	204	97	n.d.	75	130			

4b. Marine oxic sediment, 450 m water column, depth of sed. 0–5 cm	94	111	n.d. μmol/l	48		n.d.		
5. Marine anoxic sediment, 10 m water column, depth of sed. 2 cm								
Summer		135	200	50 iso-	65 *n*-		180	7.1
Winter		210	450 μmol/l_s	20	25		330 μmol/l_s/hr	2.7
6. Marine loch sediment oxygen status: ? < 6 cm; anoxic > 6 cm 50 m water column, depth of sed. 0–3 cm		2600[b]	160 μmol/l_s	70		50		

[a] l = liter of pore water; l_s = liter of sediment (wet); kg = kilogram of sediment (wet wt); n.d. = not detected. 1. Cappenberg *et al.* 1978; 2. Ansbaek and Blackburn, 1980; 3. Sørensen et al., 1981; 4. Barcelona, 1980; 5. Sansone and Martens, 1981; 6. Miller et al., 1979; Stanley, personal communication.
[b] Assumes 80% water in wet sediment.

benefit the snail physiologically, either directly if taken up from the external environment, or through facilitating uptake of inorganic cations. For example, it has been demonstrated that certain aquatic invertebrates are capable of taking up dissolved carboxylic acids (Southward and Southward, 1970; Testerman, 1972; Fevrier et al., 1976). It has also been shown that short-chain acids can increase the permeability of plant roots to inorganic ions (Jackson and St. John, 1980). The extent to which these processes apply to *B. glabrata* will be reported subsequently.

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RESPONSES OF GRAVID SCREWWORM FLIES, *Cochliomyia hominivorax*, TO WHOLE WOUNDS, WOUND FLUID, AND A STANDARD BLOOD ATTRACTANT IN OLFACTOMETER TESTS[1]

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Abstract—Laboratory olfactometer bioassays were used to study attractiveness of whole animal wounds, wound fluid, and reconstituted dried blood to gravid screwworm flies, *Cochliomyia hominivorax* (Coquerel). Flies were attracted to odors from whole wounds that were infested with screwworm larvae but not to those from uninfested wounds that were 0–2 days old. A maximum of 59% of females were attracted to fluid from screwworm-infested wounds, and the response changed little over fluid concentrations from 3.3 to 100%. However, blood tested over a similar concentration range yielded a significant linear relationship between log dose and response. Peak attraction was 82%. Attractancy of fluid collected from infested wounds declined when the fluid was stored at −20°C for more than four weeks but attractancy of a stored blood sample remained unchanged for three months. The blood attractant(s) was also heat stable and is suitable for use as a reference standard in bioassays.

Key Words—Screwworm fly, *Cochliomyia hominivorax*, Calliphoridae, wound attractancy, host finding, olfactometer bioassay.

INTRODUCTION

Screwworm larvae, *Cochliomyia hominivorax* (Coquerel), are obligate parasites of warm-blooded animals and can cause severe livestock losses

[1] Mention of a company name or proprietary product does not imply an endorsement by the U.S. Department of Agriculture.

(Bushland, 1960). Host seeking is done by gravid flies, which oviposit on wounds or navels of newborns (Bushland, 1960). Knipling (1979) discussed the potential of an ovipositional attractant not only for screwworm detection but also for use along with the sterile-male technique for population suppression.

Liver and swormlure-baited traps have been used to monitor screwworm populations (Jones et al., 1976; Coppedge et al., 1977), but they attracted fewer gravid females than wounds did (Guillot et al., 1977b,c). Swormlure also attracted more males (Coppedge et al., 1977) than expected if it were duplicating a wound (Parish, 1937; Guillot et al., 1977a,b). Previous field trials demonstrated the attractiveness of wounded hosts but did not determine the nature of the attractive stimuli or isolate wound effects from other host effects. DeVaney et al. (1970) implicated olfactory stimuli in that flies without antennae were unable to locate a wound. Adams et al. (1979) investigated physical and physiological factors that influence screwworm responses to attractant (fluid from spent larval diet) and developed a quantitative olfactometer bioassay suitable for laboratory use.

Our goals for this investigation were to (1) test the response of gravid screwworm flies to wound-derived odors, (2) establish dose–response curves for attractants from wounds and reconstituted dried blood, and (3) determine attractant stabilities. Blood is not always attractive to gravid flies (Holt et al., 1979), and the dried blood was examined because of its potential for use as a reference standard in bioassays.

METHODS AND MATERIALS

Insects. Screwworm larvae of the 009 strain, colonized for 60–80 generations, were reared at 40 ± 1°C on a beef-based diet similar to that described by Melvin and Bushland (1940). Pupae were held at 20 ± 1°C and 50 ± 10% relative humidity. Adults, fed water and undiluted corn syrup ad libitum from separate containers, were maintained at 25 ± 1°C and 50 ± 10% relative humidity on a 12:12 light–dark photoperiod. Anesthesia (CO_2) was used only at 6 days of age when flies were separated by sex. Bioassays were conducted with 8- to 12-day-old gravid females.

Bioassays. The bioassay apparatus was a 4-port olfactometer developed by Adams et al. (1979). Air warmed to 26 ± 2°C was drawn down each port at a rate of 1 m/sec. Light intensity was about 550 lux measured at the top of sample ports. Flies were tested for 15 min in groups of 20–30/olfactometer.

Wound-fluid and reconstituted dried-blood samples were tested in two of four olfactometer ports as described by Adams et al. (1979), except that the dental wicks used as sample holders were treated with 0.85 ml water and then 0.15 ml of the test material. Unless indicated otherwise, test concentrations

were 100% wound fluid and 50% blood (1 g dried bovine blood reconstituted with 9 ml water to give a concentration 50% that of whole blood). The dried blood was obtained in one lot from the Rath Packing Co. Wound-fluid and reconstituted blood samples that were not tested immediately were stored at −20° C and used within 10 and 96 days, respectively.

Since some wounds produced only small amounts of fluid, and since the fluid content of a wound might not account for its attractiveness, methods were developed to aerate whole wounds and deliver their odors into the olfactometer. One end of a glass canula having a 3-cm-diam opening surrounded by a 1.3-cm-wide lip was applied to the shaved skin around a wound. The opposite end was fitted with inlet and outlet tubing (0.5-cm ID Teflon) oriented perpendicularly to the wound surface. Air filtered with activated charcoal entered the canula at a rate of 8 liters/min. Wound-exposed air was then delivered via one of the four choice ports into the airstream (170 liters/min) flowing through the olfactometer. The amount ranged from 1 to 4 liters/min among tests.

Uninfested Wounds. New Zealand white rabbits were shaved and aseptically wounded on the rump. Each wound was produced by removing a circle of skin approximately 2 cm in diam and then scoring a superficial, x-shaped incision in the underlying muscle tissue. Uninfested wounds were tested for attractiveness within 2 hr (animal 2) and at 1 and 2 days (animal 1) after wounding, using the aeration method of bioassay.

Infested Wounds. Wounds produced as described above were infested with screwworms and then tested for attractiveness. Fifty to 110 larvae completed development in each wound (and in those described in the next section). For the first, 1-day-old larvae were transferred from artificial diet to a 2-day-old wound on animal 1. Larvae crawled from this wound when they were 5 and 6 days old. Separate samples of wound fluid collected when infesting screwworms were 2, 3, and 6 days old were assayed (2, 3, and 3 replicates, respectively).

A partial egg mass was used to infest a 2-day-old wound on animal 2. Wound-fluid samples collected on 6 consecutive days, beginning when larvae were 2 days old, were each tested for attractancy. The intact wound was also assayed on 7 consecutive days, starting when larvae were 2 days old. (Aerations were completed each day before wound fluid was sampled.)

Four days after wounding, animal 3 was infested with 3-day-old larvae that had been reared on artificial diet. Two wound-fluid samples collected when larvae were 4 and 5 days old were each assayed twice.

Dose-Response Relationships. To determine the relationships between attractant concentration and female response, bioassays were conducted at concentrations ranging from 3.3 to 100% wound fluid and from 1.3 to 100% reconstituted blood. Dilutions were prepared by volume with water. The wound-fluid sample was pooled from two lesions (rabbits 4 and 5) simul-

taneously infested on the day after wounding with newly hatched screwworms. It was collected over a 3-day period beginning one day before larvae started to crawl from the wounds. Larvae crawled when they were 4 and 5 days old.

Efficiency of Aeration Bioassay. Tests were done so that the dose–response data could be used to estimate the amount of attractant needed to obtain a response in aeration bioassays. A blood-treated dental wick (0.15 ml 50% blood and 0.85 ml water) placed on a watchglass and aerated in the canula at 8 liters/min was bioassayed at flow rates into the olfactometer of 1 and 4 liters/min. Tests were repeated three times per flow rate.

Attractant Stabilities. Samples of wound fluid and 50% blood were bioassayed periodically during storage at −20° C to determine whether storage affected attractiveness. The wound-fluid sample was the same one used in the dose–response test.

Heat stability of the blood attractant was also examined. Dried blood was autoclaved, reconstituted to 50%, filtered, and then assayed five times. A 1-ml aliquot of the autoclaved blood was additionally heated at 120° C for 1 hr, brought back to 1 ml with 0.5 ml of water after heating, and then sampled for bioassay (two replicates).

Calculations and Statistics. The percentage of females attracted, a value that combined the activity and attractancy measures of Adams et al. (1979), was employed as the index of fly responsiveness. When two of four olfactometer ports were treated, the equation for this percentage was $[(Tr - C)/N] \times 100$, where Tr is the number of flies in treated ports, C the number in control ports, and N the total number placed in the olfactometer. When only one of four ports was treated, the equation was $[(Tr - C/3)/N] \times 100$.

Chi-square goodness of fit tests or tables of confidence intervals for the binomial distribution were used to determine whether a sample was attractive from the proportions of flies in treated and control ports. Treatment effects were analyzed by comparing fly distributions among treated ports, control ports, and the rest of the olfactometer using χ^2 calculated from contingency tables. Dose–response data were analyzed by linear regression. These procedures were according to Steel and Torrie (1960), and a 0.05 probability level was considered statistically significant.

RESULTS

Uninfested Wounds. Flies did not show significant attraction to air that had been swept over uninfested wounds: 0-, 1-, and 2-day-old wounds attracted 2 ± 3, -1 ± 2, and 0 ± 1% of females that were tested at flow rates into the olfactometer of 2, 1, and 4 liters/min, respectively.

TABLE 1. RESPONSE OF GRAVID SCREWWORM FLIES TO FLUID FROM SCREWWORM-INFESTED WOUND ON ANIMAL 2 ASSAYED BY PLACEMENT OF 0.15 ML IN TWO OF FOUR OLFACTOMETER PORTS

Larval age (days)[a]	N	No. trapped		Attracted (mean % ± SE)[b]
		Treated ports	Control ports	
2	60	4	5	−2 ± 5
3	60	7	5	3 ± 4
4	135	20	13	5 ± 6
5	143	81	8	51 ± 6*
6	90	50	5	50 ± 7*
1 postcrawl[c]	30	15	1	47*

[a] Age of infesting screwworm larvae at the time of wound-fluid collection.
[b] Asterisks indicate significant attraction, $P \leq 0.05$.
[c] Wound fluid was collected the day after screwworms had finished crawling from the wound.

Infested Wounds. Wound fluid obtained from animal 1 when infesting larvae were 6 days old attracted 50 ± 4% of test females. The attraction was significant ($P < 0.001$ of a 1:1 fly ratio between treated and control ports). However, wound fluid from this animal did not attract a significant percentage of females when larvae were 2 (2 ± 15%) or 3 days old (4 ± 2%).

Attractive wound fluid was obtained from animal 2 when larvae were 5 and 6 days old and on the day after larvae had exited the wound (Table 1). These samples attracted 47–51% of test females. No significant response occurred to fluids collected when larvae were 2, 3, or 4 days old. Air swept over the intact wound on animal 2 was attractive when the wound contained 4-, 5-, and 6-day-old larvae and for at least two days after larvae had left the wound (Table 2). When larvae were 4 days old, the intact wound attracted 39 ± 5% of test females, but 0.3 ml of wound fluid (0.15 ml in two of four olfactometer ports) did not elicit a significant response. However, this difference between bioassay methods was not evident by the time larvae were 5 and 6 days old: the whole wound attracted 52–60% of females, and 0.3 ml of wound fluid attracted 50–51%.

Fly response did not differ significantly between wound-fluid samples collected from animal 3 when infesting larvae were crawling from the wound at 4 and 5 days of age (10 and 8% attracted, respectively), and the data were pooled. Fluids from this wound, which had been infested with 3-day-old larvae, were not attractive ($P < 0.1 > 0.05$ of a 1:1 fly ratio between treated and control ports). Thus, the presence of crawl-stage larvae was not by itself sufficient to render a wound attractive.

TABLE 2. RESPONSE OF GRAVID SCREWWORM FLIES TO AIR SWEPT OVER SCREWWORM-INFESTED WOUND ON ANIMAL 2 AND DELIVERED TO FLIES VIA ONE OF FOUR OLFACTOMETER PORTS

Larval age (days)[a]	Delivery rate (liters/min)	N	No. trapped		Attracted (mean % ± SE)[b]
			Treated ports	Control ports	
2	2	75	6	15	1± 2
3	2	50	2	7	−1 ± 1
4	2	75	32	8	39 ± 5*
5	1	75	41	7	52 ± 8*
6	1	75	47	7	60 ± 6*
1 postcrawl[c]	1	65	29	16	36 ± 6*
2 postcrawl	1	50	27	9	48 ± 19*

[a] Age of infesting screwworm larvae at wound aeration.
[b] Asterisks indicate significant attraction, $P \leq 0.05$.
[c] Aeration was done the day after screwworms had finished crawling from the wound.

Dose-Response Relationships. Wound-fluid concentrations over the range of 3.3–100% were all attractive ($P \leq 0.001$), but no significant linear relationship was demonstrated (Figure 1) between the log of the concentration (x) and the percentage of females attracted (y): $y = 10.1 \log_{10} 100x + 4.9$, $r^2 = 0.39$, 95% confidence interval about the slope = −2.6 to 22.7. Ten percent wound fluid attracted 44 ± 5% of females, but the response did not improve with increased concentration.

A highly significant linear relationship between log dose and response was obtained using reconstituted dried blood (Figure 1).

Efficiency of Aeration Bioassay. When a wick treated with reconstituted blood was aerated in the canula, 61 ± 2% of females were attracted at a flow rate into the olfactometer of 4 liters/min, and 47 ± 3% were attracted at a rate of 1 liter/min. Thus, attraction to uninfested and newly infested wounds would have been readily demonstrated if they had been as attractive as 0.15 ml of the 50% blood.

Attractant Stabilities. The attractiveness of wound fluid declined when it was stored at −20° C for 37 days (Table 3). No significant change occurred within 11 days, an interval encompassing the 10-day storage period used in other wound-fluid tests.

In contrast, the attractiveness of 50% reconstituted blood did not change during storage of the aqueous blood for 96 days at −20° C (Table 4). On the average, the aliquots attracted 76% of females.

Dried blood that was autoclaved and then reconstituted attracted 79 ± 3% of females. The sample that was additionally heated at 120° C for 1 hr

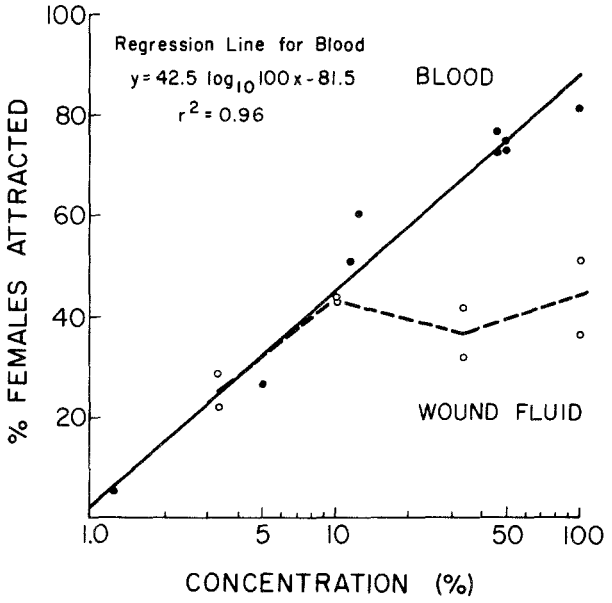

FIG. 1. Response of gravid screwworm flies to 0.3 ml/olfactometer of different concentrations of wound fluid (--○--) and reconstituted dried blood (—●—). Each data point is the mean of four replicates (25/replicate). Linear regression analysis of the eight data points obtained for wound fluid did not yield a significant relationship between $\log_{10}$ dose and attraction response.

TABLE 3. EFFECT OF STORAGE AT −20° C ON ATTRACTIVENESS OF 100% WOUND FLUID TO GRAVID SCREWWORM FLIES

Days stored	N	No. trapped[a]		Attracted (mean % ± SE)
		Treated ports	Control ports	
0–1	125	77	3	59 ± 3
4–7	175	85	9	43 ± 4
11	100	55	5	50 ± 7
18	100	54	3	51 ± 8
26	100	67	8	59 ± 7
30	100	30	6	24 ± 7
37	100	40	9	31 ± 5

[a] $\chi^2 = 47.4$ with 12 *df*, $P < 0.001$, 3×7 contingency table comparing storage treatments with respect to the proportions of flies in three olfactometer locations: treated ports, control ports, and all other portions of the olfactometer.

TABLE 4. EFFECT OF STORAGE AT −20° C ON ATTRACTIVENESS OF 50% RECONSTITUTED DRIED BLOOD TO GRAVID SCREWWORM FLIES

Days stored	N	No. trapped[a]		Attracted (mean % ± SE)
		Treated ports	Control ports	
0–2	450	367	14	79 ± 2
37	90	71	5	73 ± 3
74	150	115	4	74 ± 4
96	115	87	6	72 ± 6

[a] $\chi^2 = 5.5$ with 6 *df*, $P > 0.05$, 3×4 contingency table comparing storage treatments with respect to the proportions of flies in three olfactometer locations: treated ports, control ports, and all other portions of the olfactometer.

attracted 68 ± 2%. Both heat-treated samples were attractive, and there was no evidence for heat lability of the blood attractant(s).

DISCUSSION

Attraction of screwworm flies specifically in response to wound odors was demonstrated for the first time, confirming the existence of wound-derived olfactory cues that could facilitate host finding. Odors from screwworm-infested wounds were more attractive than those from uninfested wounds, and this result is consistent with the greater effectiveness of infested wounds in eliciting screwworm oviposition under field conditions (Bushland, 1960; Knipling, 1979). However, attractive odors were not detected in 0- to 2-day-old uninfested wounds even though uninfested wounds that were at least 2 days old yielded more egg masses than lesions less than 8 hr old (Knipling and Travis, 1937).

Aeration bioassay of an infested wound on animal 2 detected attractive odors early in the infestation when bioassay of 0.3 ml of wound fluid did not. Considering the dose–response data, the failure later in the infestation to detect a difference between bioassay procedures was probably due to assay at concentrations above that required to elicit maximum fly attraction. Given these data from wounds and the attraction demonstrated to blood-treated wicks aerated in the canula, the lack of response to odors from uninfested wounds could not be attributed to low sensitivity of the aeration method of bioassay. Thus, gravid flies locate uninfested wounds using stimuli other than chemical ones originating in the wound or uninfested wounds become olfactorily attractive under conditions different from those employed in our study.

Screwworm-infested guinea-pig wounds contained a gram-negative bacillus, nearly always in pure culture (Borgstrom, 1938; Esslinger, 1958). In addition, although sex and physiological status of responding flies were poorly defined, related bacteria produced an attractant when cultured on bovine blood (DeVaney et al., 1973; Grabbe and Turner, 1973; Eddy et al., 1975). These data suggest that wound attractancy might be of microbial origin and that uninfested wounds might become attractive if infected with specific bacteria.

Interest in the dried blood stemmed from its high attractant activity and availability in 23-kg lots. In addition, it generated a steep dose–response line when reconstituted, and it was stable, i.e., its attractiveness was not destroyed by heat treatment or affected by storage at −20° C for three months. Because of these qualities, the dried blood is suitable for use as a reference standard to monitor sensitivity of different batches of insects used for bioassay. It is also useful in experiments to determine the relative effectiveness of different bioassay methods (e.g., canula aeration vs. olfactometer-port presentation of attractant). In contrast, no linear dose–response relationship was demonstrated for wound fluid, and its attractiveness declined within 4–5 weeks when it was stored at −20° C. Attractancy of fluid from spent larval diet also declined during frozen storage (Adams et al., 1979).

The plateau in response to wound fluids at concentrations from 10 to 100% was not attributable to sample storage nor to variation among the flies used to bioassay different concentrations. Replicates of each were tested simultaneously with matched females. The plateau remains to be explained, but its occurrence at an attraction level below that obtainable with reconstituted blood suggests a suboptimal blend of attractive components or the production of compounds that interfere with the response to attractant(s). Further work is needed to determine why only some wounds become attractive and to define the conditions required for optimal attractancy.

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FUNCTION OF SECRETION OF MANDIBULAR GLAND OF MALE IN TERRITORIAL BEHAVIOR OF *Xylocopa sulcatipes* (HYMENOPTERA: ANTHOPHORIDAE)

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Abstract—Males of the carpenter bee, *Xylocopa sulcatipes*, establish and defend territories which they mark with the secretions of their mandibular glands. Chemical analysis of the glandular blend revealed that it is composed of guaiacol, *p*-cresol, and vanillin. Territorial males recognize other intruding males either by sight or by the odor emitted from their mandibular glands. They chase the intruder out of the territory immediately, but will not do so if the visitor is a female. If a female, however, is scented with the reconstituted synthetic pheromonal blend and introduced into a territory of a male, it is treated as a male and chased out of the territory. It is suggested that the blend has a dual function: Guaiacol seems to be the aggressiveness elicitor, while vanillin is the territorial marker and female attractant and therefore could be considered a sex pheromone.

Key Words—*Xylocopa sulcatipes*, Hymenoptera, Anthophoridae, territoriality, scent marking, mandibular glands, guaiacol, *p* cresol, vanillin, aggressive behavior.

INTRODUCTION

Xylocopa sulcatipes is a metasocial carpenter bee that inhabits the desert areas of Israel. It nests mostly in *Arundo donax* canes where either a single female or two cooperative (on rare occasions more than two) female construct several brood cells. After the broods have emerged, the bees enter a second reproductive cycle wherein one or several of the daughter bees may cooperate in the new brood-cell production (Gerling et al., 1982).

Reproductive activity starts early in the spring and is characterized by the

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establishment of territories by males that are seeking young females. *X. sulcatipes* males defend territories near nesting sites and/or flowering plants, thus exhibiting female and/or resource defense polygyny (Alcock, 1980). Territories that are established near nesting sites are usually a few meters long within which the male typically cruises back and forth. Territories near food resource can be of two types: in the first type males cruise around a bush or a group of flowers, while in the other type males aggregate around the flowering branches of trees (Velthius and Gerling, 1980).

In several other species of bees and wasps in which males are territorial, chemical marking of the territory has been demonstrated. In the bumble bee, *Bombus jonellus*, for example, 2,3-dihydro-6-*trans*-farnesol is the main product of the mandibular glands which are implicated in territorial marking (Bergstrom and Svensson, 1973). The mandibular glands are also the source of territorial marking chemicals in *Centris adani* (Frankie et al., 1980; Vinson et al., 1982). The secretion, which is dominated by geraniol and geranyl acetate, is deposited in a semicircular array within the male's territory.

Males of the sphecid wasp *Philanthus gibbosus* have special observation sites called perches which they perfume (Alcock, 1978), while *P. triangulum* males have been observed moving up and down grass blades and pine needles, supposedly marking them (Simon Thomas and Poorter, 1972). In the latter species, 2,5-dimethyl-3-isopentylpyrazine, accompanied by several unidentified pyrazines, was isolated from the mandibular glands and are thought to function as marking pheromones (Borg Karlson and Tengo, 1980).

Early observations on the territorial behavior of *X. sulcatipes* revealed that this species uses its mandibles for marking spots in its territory. This paper presents the chemical analysis of the mandibular gland exudates of males and experiments to determine the possible role of these exudates in territorial and aggressive behaviors.

METHODS AND MATERIALS

Chemistry. Analysis of methylene chloride extract of mandibular glands was conducted using an LKB 2091 gas chromatograph–mass spectrometer equipped with SE 30 column (2 m) or an LKB 9000 GC–MS using a 4-m SP 1000 column.

Compounds were tentatively identified by their mass spectra and further compared with spectra of authentic samples under the same conditions, as well as gas chromatographic retention time using a SE 54 capillary column.

Behavioral Observation. Observations on the territorial behavior of male *X. sulcatipes* were conducted in Hatzevah Field School in the Arava Valley, Israel. This desert settlement has an abundance of nesting sites of *X. sulcatipes* and an active population so that the areas around every group of nests or

flowering plants are dominated by one or several territorial males. Among the many sites used as territories by males, one particular site, where three males occupied three neighboring territories, was picked for observation. Each male was marked with a colored dot of enamel, nontoxic paint on its dorsum so that individual recognition was possible.

Marking behavior of two neighboring, marked males was recorded during the morning hours. Each male was observed for periods of 15 min and the marking pattern of 1 hr of observation was combined.

Bioassays were conducted in the field using tethered bees scented with different compounds and were either immobile or able to fly at the end of the thread. In order to investigate the role of the male glandular exudate in locating intruding males, females were scented with the exudates and were kept immobile and hidden within the bush in the male's territory. When, on the other hand, chasing behavior was tested, the scented female was allowed to fly at the end of the tread and as soon as the territorial male approached, the thread was pulled along inducing the male to chase the scented female. Several of the assays were conducted using the synthetic compounds identified from the glandular extracts. When a whole mixture was used, the components were mixed in the same proportions (*p*-cresol–guaiacol–vanillin 4:4:2) as found in the gland. Each bee was coated with 50 μl of solution containing a total of 0.2 μg of *p*-cresol, 0.2 μg guaiacol, and 0.1 μg vanillin. When single compounds were used, 50 μl of solvent containing 0.5 μg of the compound assayed was used. The order in which the compounds or the mixture were assayed was chosen at random. Assays were conducted during two 3-day periods during the morning hours when the activity of the bees was at its peak.

RESULTS

Territorial behavior of three neighboring marked males was observed. The territories were of the resource–defense polygyny type. The male marked with green dominated a territory of about 5 × 2 m at the center of which was a flowing bush of *Clerodendron inerme*; the male marked with blue occupied a neighboring territory about the same size with a flowering *Calotropis procera* as its center, while the third, red-marked male had a larger territory of about 10 × 3 m with both *C. procera* and *C. inerme* (Figure 1). All three territories were visited by foraging females, and its was possible to observe the behavior of territorial males toward them. Intruding males were immediately tracked by the territorial male and chased out. If, on the other hand, a female entered the territory, the male spotted her and hovered around her until she landed. He then attempted to copulate with her, if she was receptive, as soon as she started flying again. These findings are similar to those described by Velthuis and Gerling (1980).

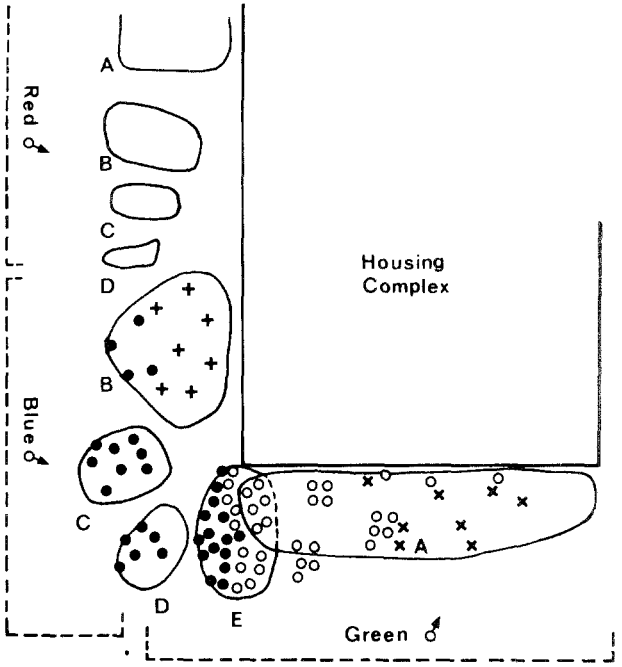

FIG 1. Scent-marking pattern of territorial males of *X. sulcatipes*. Three males were marked, of which two were observed. Marking was recorded for periods of 15 min and the marking pattern of 1 hr of observation was combined for each male. Dashed lines delineate the approximate territory of each male and the solid lines show the location of each bush in the territory. Plant names are as follows: A, *Clerodendron inerme*; B, *Caloptropis procera*; C, *Hibiscus* sp.; D, *Tamarix niloticus*; E, *Acacia* sp. ; (●) marking spot of blue male, (+) drinking spot of blue male, (○) marking spot of green male, (X) drinking spot of green male.

Males occupied their territories early in the morning, both patrolling and marking them extensively. Marking behavior was noted when the male landed on the foliage of the bush and touched it slightly with spread mandibles, a behavior that lasted for 5 ± 2 sec ($N = 30$), but at times the male remained standing on the same spot for up to 1 min. Figure 1 describes the three territories observed and the marking pattern of two of the males that occupied them. Two main points were observed: Scent marking occurred in clusters as compared to nectar drinking, which was more or less evenly distributed among the flowers. In the blue male territory, for example, *C. procera* was a nectar source but no marking on this plant was observed, while a nearby *Hibiscus sp.* bush was extensively marked. Furthermore there seemed to be a higher density of marking at the borderline between the two territories. Interestingly, only little chasing between these two males occurred, whereas

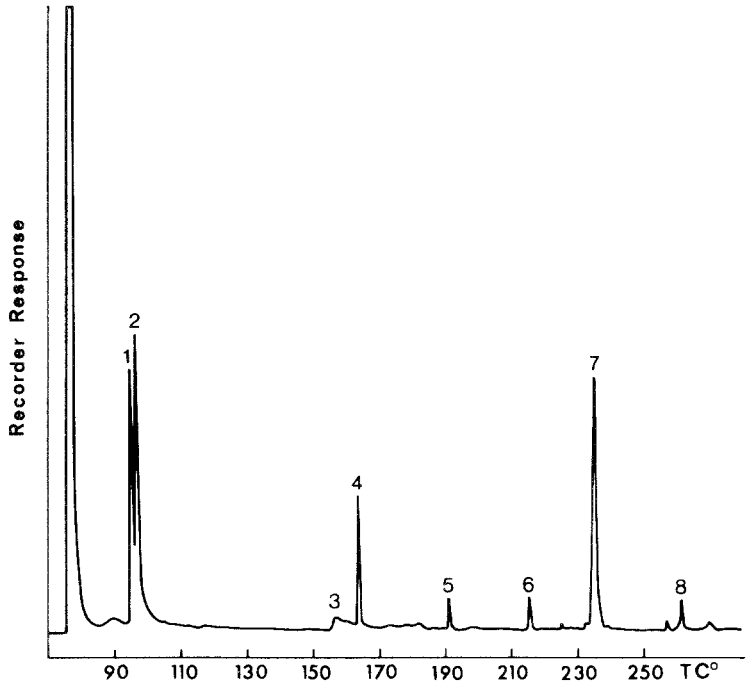

FIG. 2. Gas chromatogram of mandibular gland exudates of male *X. sulcatipes*. Numbered peaks are: 1, *p*-cresol; 2, guaiacol; 3, vanillin; 4, pentadecan; 5, heptadecan; 6, nonadecane; 7, heinicosane; 8, tricosane.

other strange males that penetrated any of the territories were evicted immediately.

Chemical analysis of the pooled mandibular gland extracts of the male revealed the presence of three major compounds: guaiacol, *p*-cresol, and vanillin, and traces of syringaldehyde, accompanied by a series of hydrocarbons ranging from pentadecane to pentacosane (Figure 2). These hydrocarbons could have originated from the mandibular cuticle, since in the extirpation of the glands the mandibles were left attached in order to avoid spillage of the exudate from the glandular reservoir. The hydrocarbons and syringaldehyde were excluded from all bioassays.

The amounts of the secretion within the gland must be rather small since gas chromatrographic analysis of exudate taken from individual glands failed to reveal any of the compounds that appeared in the pooled sample. However, the characteristic odor of cresol, guaiacol, and vanillin was emitted from the heads of roughly handled males. Efforts to establish whether the males used their mandibular gland exudates in territorial marking were also unsuccessful. Extractions were made of leaves and stems immediately after they had been

marked by territorial males, but none of the glandular components could be detected by gas chromatography. However, the acrid odor of *p*-cresol, accompanied by the more pleasant odor of vanillin, could be readily detected by the human nose.

There are two components in the defensive behavior of a territorial male. One is detection and the second is eviction of a strange male from the territory. These two components could be separated easily by using an immobilized male hidden within the bush. As soon as it was placed, the territorial male approached and inspected the experimental male although it could not have been seen beforehand. As long as the experimental male was kept immobile, the territorial male just inspected it, then continued patrolling his territory. If, however, the experimental male was pulled by a thread and as a result started flying, it was immediately pursued by the territorial male. Males that could fly at the end of a thread evoked aggressiveness in the territorial male and if, at that moment, the string attached to the tethered male was pulled, it induced chasing by the territorial male for over 20 m.

Pollen-collecting females (thus unattractive) tied to a thread were easily located by a male, but did not elicit aggressiveness. On the other hand, similar females, smeared with male mandibular gland exudates or alternatively with mixture of guaiacol, *p*-cresol, and vanillin in proportions similar to those found in the gland, induced aggressiveness by males that chased the females as if they were males (Table 1). The aggressive behavior toward each scented female lasted for a few minutes, then declined appreciably. At that time the only smell that could be detected emanating from the female was that of vanillin suggesting that the entire blend was needed for behavioral activity. In order to further elucidate this point, bioassays were conducted using the different components alone or in combinations (Table 1). A hidden female that was scented with any of the glandular secretion components was readily spotted by a territorial male. When chasing behavior was assayed, both vanillin and *p*-cresol prompted only casual inspection while with guaiacol, scented females sometimes caused a stronger reaction. In two of the assays (of the four conducted) this compound elicited chasing by the territorial male which was similar to the response to an intruding male. In the two other experiments, however, the territorial male inspected the female but did not try to evict her.

Especially interesting was the reaction toward vanillin-scented females. In three of the five assays vanillin-scented immobile females, attracted other females about 5–6 min after application. The attracted females behaved very uncharacteristically and hovered around the tethered, scented female for 1–2 min, then left. Males, as mentioned above, did not pay much attention to a vanillin-scented female except for a brief inspection. A change in their behavior, however, did occur since they stopped marking the area surrounding such females, although they continued marking other spots in the

TABLE 1. BEHAVIORAL RESPONSE OF TERRITORIAL MALE TOWARD FEMALES SCENTED WITH VARIOUS SYNTHETIC COMPONENTS OF MALE MANDIBULAR GLAND SECRETION[a]

Compound	Detection by territorial male[b]	Induction of chasing behavior[c]	Detected by females[b]
Male mandibular gland exudate	7/8	8/8	5/8
Mixture[d]	4/5	6/6	
Guaiacol	2/3	2/4	0/3
p-Cresol	1/3	0/4	0/3
Vanillin	3/5	0/4	3/5
Guaiacol + *p*-cresol	2/3	2/4	0/3

[a] Data are given as positive response by males per total number of assays.
[b] Assay conducted using an immobile hidden female for 10 min.
[c] Assay conducted using a tethered flying female.
[d] Mixture containing guaiacol, *p*-cresol, and vanillin.

area. When the female was removed, marking in the female's previous location was again noted.

DISCUSSION

Among the mate-locating behaviors exhibited by bees, the establishment and defense of a territory is most common (Alcock, 1980). In the carpenter bees investigated to date, territoriality is omnipresent and can be either near nesting sites as in *X. virginica* (Gerling and Hermann, 1978), near flowering plants as exhibited by *X. darwini* (Linsley, 1965), or in prominent places as in the case of *X. hirsutissima* (Velthuis and Camargo, 1975a,b) and *X. pubescens* (Gerling et al., 1983). *Xylocopa sulcatipes* shows flexibility in its territorial behavior, establishing territories either near the nest or around flowering bushes. In at least one area at the onset of the season, when the only flowering plant is a *Moringa peregrina* tree, male *X. sulcatipes* aggregate around it, defending only a small group of flowers (Velthuis and Gerling, 1980).

The mandibular glands of *X. sulcatipes* produce a secretion composed of a series of aromatic compounds including *p*-cresol, guaiacol, and vanillin, traces of syringaldehyde, and straight-chain hydrocarbons, the latter possibly of cuticular origin. Although not definitely proven, it appears that males use this secretion to mark spots in their territories, especially in areas that overlap with the territory of neighboring males.

The secretion produced by the mandibular glands of *X. hirsutissima* is similar to that found in *X. sulcatipes* having *p*-cresol and vanillin but lacking guaiacol (Wheeler et al., 1976). The pheromonal blend of *X. hirsutissima* also

contains *cis*-2-methyl-5-hydroxyhexanoic acid lactone and benzaldehyde as major products that are missing in the exudates of *X. sulcatipes*. *Xylocopa hirsutissima* was observed marking stems with its mandibular glands, a behavior that was first interpreted as territorial marking (Velthuis and Camargo, 1975a) but later thought to be application of sex pheromone onto the male body to lure females to its territory (Velthuis and Camargo, 1975b). Although such smearing of the ventrum was not observed in *X. sulcatipes*, the experimental results utilizing this species suggest that both functions are possibly encoded within the pheromonal blend.

Considering the small sample size utilized in the bioassays, the following conclusions regarding the role of each of the secretion components are tentative. Territorial males detect an intruder usually by sight, but can also recognize its presence by olfaction (Table 1). Females smeared with male mandibular gland exudate or reconstituted mandibular gland secretion or any of its single components were easily detected by the territorial male, even if hidden out of sight. The mixture of compounds was more active than the other preparations. The total mixture also elicited chasing behavior when assayed on a flying scented female. While a male that encountered an unscented female will hover around her but will not chase her for over a meter if moving, he will chase a scented female for over 20 m in a way similar to chasing an intruder male. Of the single components, only guaiacol, to some extent, elicited the same response, possibly constituting the aggressiveness-eliciting component of the pheromonal blend.

Vanillin, on the other hand, seems to have a different role. In both sets of experiments in which it was used, it attracted females which investigated the scented female by hovering around it, exhibiting an unusual behavior that was not otherwise observed. Since vanillin is less volatile than either guaiacol or *p*-cresol, it persists longer on the marking spot. Furthermore, marking spots are grouped together (Figure 1), forming a larger vanillin-emanating area in the territory. It is therefore possible that the mandibular gland pheromone has a dual function: eliciting aggressive behavior toward other males and attracting females to the male territory. The latter function is especially appealing since it provides another means for sexual selection by the female.

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Book Review

Insect Pheromone Technology; Chemistry and Applications. Barbara A. Leonhardt and Morton Beroza (eds.). ACS Symposium Series 190. Washington, D.C., American Chemical Society, 1982. US $35.95, 260 pp.

This book is based on the Symposium on Chemistry and Applications of Insect Pheromone Technology sponsored by the Division of Pesticide Chemistry at the 182nd Meeting of the American Chemical Society held in New York, New York, August 25–26, 1981. The editors have skillfully arranged the 14 contributions of this symposium. The chapters are written concisely and clearly by experts and are easy to read. Each chapter is preceded by a comprehensive abstract and is well documented with pertinent references. The recent publication of several new books about pheromone research and application raises the problem of repetition of material. Although some of the data has been reported recently in other volumes, this book contains mostly summaries of continuing programs and novel results.

The 14 chapters are not divided into sections, but three parts are clearly evident. The first section (chapters 1–5) contains very elegant examples of identification and synthesis of pheromones, such as the comparison of isomeric separation on various capillary columns including liquid crystal columns. Another striking example is the elucidation of the structure of periplanone A. The asymmetric syntheses of several chiral pheromones, such as the western and southern corn rootworm pheromones, grandisol and lineatin enantiomers, are novel and very interesting.

The second section of the book (chapters 6–11) describes the various formulations, their properties, methods of evaluation, and relevant examples of application. All the authors correctly emphasize the need for complete understanding of the release performance of a given formulation before it can be used in a practical pest management program. Different methods of measuring release rates of pheromones from microcapsules, hollow fibers, and laminate dispensers are described and evaluated. The possibility of constructing formulations, in particular of the laminated type, with a predictable release rate by choosing the appropriate polymer and desired thickness is very promising.

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Two chapters deal with the fate of released pheromone vapors in the field. The complexity of the sampling techniques and micrometereological problems is described. Despite the problems encountered, some conclusions drawn from work with disparlure, *Z*-9-tetradecenyl formate, and *Z*-11-tetradecanal are evaluated.

A third part of the book (chapters 12–14) describes several major efforts in management of forest pests with pheromones. An up-to-date report on the Spruce bark beetle program in Norway is presented. The report points out the difficulties both in performing a large scale mass trapping program and in evaluating scientifically its results. However, it is encouraging that a significant reduction in forest damage has been achieved. The other programs discussed in this part are those concerned with management of forest pests in North America, in particular an update on the gypsy moth project. A new convenient synthesis of chiral (+)-disparlure is described making this very active material readily available.

Pheromone researchers from various disciplines will find this book a valuable resource of current information and reference. Highlights, problems, and opportunities have been presented, encouraging further research and development. The book is strongly recommended to the growing circle of scientists who are interested both in better understanding of the chemistry of pheromones and in their practical use in pest management.

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Editors' Note

Growing evidence that chemicals released by certain plant species affect negatively the growth of receiver plants has stimulated research in allelopathy. The *Journal of Chemical Ecology* has served as a medium for publication of an increasing number of reports pertinent to this field. We are, therefore, pleased to assemble 27 papers in this issue, in cooperation with Dr. A.R. Gilmore who chaired and organized a 3-day symposium at the University of Illinois, Urbana-Champaign.[1] The symposium consisted of several sessions on allelopathy: (1) criteria of determination; (2) weed control; (3) cultivated plant communities; (4) forestry; (5) interactions of higher plants with soil organisms; (6) quantification of chemicals in plants and soil; and (7) chemistry and mechanisms of action. The same arrangement was followed in this issue.

Each paper has had the benefit of both audience exchanges at the symposium and the standard review policy of the journal. We are especially grateful to Drs. E.L. Rice and C.H. Muller, of our editorial board, A.R. Gilmore, M.A.K. Lodhi, and A.R. Putnam for having reviewed several manuscripts. We are grateful also to these reviewers: N.E. Balke, C.-H. Chou, J.O. Dawson, F.A. Einhellig, L. Fowden, P.A. Hedin, S.B. Horsley, M.L. Lacy, T.J. Mabry, Z.A. Patrick, J.A. Rasmussen, D.J. Raynal, W.J. Rietveld, and R.E. Stewart. We acknowledge the cooperation of Plenum Publishing Corporation in producing extra copies of this issue to accommodate registrants of the symposium and others interested in the "proceedings" under this single cover.

R.M. Silverstein
J.B. Simeone

[1]The symposium was sponsored by the Department of Forestry and the Department of Agronomy, University of Illinois; the USDA North Central Forest Experiment Station and the Office of Continuing Education and Public Service, Division of Conferences & Institutes, University of Illinois. Members of the Organizing Committee included Dr. A.R. Gilmore, Chair, Drs. J.O. Dawson, W.L. Banwart, D.A. Miller, W.J. Rietveld, and J.B. Simeone.

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SEPARATING THE COMPETITIVE AND ALLELOPATHIC COMPONENTS OF INTERFERENCE: Theoretical Principles[1]

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Abstract—The terms "competition" and "allelopathy" should be used in ways consistent with some set of criteria, perhaps those proposed in this discussion. Proposed proof of competitive interference includes: (1) identification of the symptoms of interference; (2) demonstration that the presence of the agent is correlated with reduced utilization of resources by the suscept; (3) demonstration of which resource(s) depleted by the agent are limiting resources; and (4) simulation of that interference (in the absence of the agent) by reduction of the supply of resources to levels that occur during interference. Proposed proof of allelopathic interference includes: (1) identification of the symptoms of interference; (2) isolation, assay, characterization, and synthesis of the toxin; (3) simulation of the interference by supplying the toxin as it is supplied in nature; and (4) quantification of the release, movement, and uptake of the toxin. It would be desirable but not essential to show that the selectivity of the toxin to various species corresponds to the range of species affected by the allelopathic agent. We propose that fulfillment of the above criteria would constitute proof of competitive or allelopathic interference. According to these criteria, it is possible that neither competitive nor allelopathic interference has been unequivocally proven at the present time. These criteria are proposed as a basis for evaluation of experimental evidence and as an indicator of deficiencies in our technology.

Key Words—Interference, competition, allelopathy, marasmin, koline, phytoinhibitins, saproinhibitins.

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INTRODUCTION

Interference is the adverse effect that neighboring higher plants can exert on each other's growth (Muller, 1969; Harper, 1977). Potential causes of this interference include: (1) competitive interference: the depletion of one or more limiting resources such as light, nutrients, or water; (2) allelopathic interference: the production of chemicals by living or decaying plant tissue which interferes with the growth of a neighboring plant; (3) indirect sources of interference: effects on the physical or biological environment which interfere with the growth of a neighboring plant, e.g., the harboring of herbivores (Halligan, 1976).

Few, if any, investigations have definitively separated components of interference because of the complexity of this ecological phenomenon. Harper (1977) indicated, "There is a need for a more rigorous philosophy in the search for cause and effect, perhaps like that adopted by pathologists and defined in Koch's postulates." The indirect sources of interference will not be considered in this discussion, although the investigator must acknowledge this possibility and test for it where appropriate.

Koch's postulates (Agrios, 1969) are a classical example of the use of criteria for scientific proof of a complex phenomenon. Once the symptoms of a plant disease have been identified, determination of the causal agent for a given plant disease requires that: (1) the organism should be isolated from the diseased plant; (2) the organism should then be grown in pure culture, and its characteristics established; (3) a healthy plant should then be inoculated with the organism and the same disease produced; and (4) the organism should be isolated again and its identity confirmed. The criteria we propose below are similarly oriented toward unambiguous identification of a causal agent; in this case the causal agent of interference is to be determined. This discussion is not intended to be a review, but rather a proposal of criteria. Therefore, selected pertinent literature will be cited only to illustrate the criteria being discussed.

The term "agent" will be used with reference to the plant or other organism causing interference, and the term "suscept" will be used with reference to the plant being adversely affected. These terms are adapted from the plant pathology literature (Agrios, 1969).

DISCUSSION

There must be evidence showing that interference occurs before any attempt is made to determine the cause(s) of interference. The additive and substitutive experiments reviewed by Harper (1977) provide an excellent basis for investigation of interference. Symptoms of interference range from the more obvious germination and mortality responses to the more subtle plastic

responses such as a reduction in size, weight, or number of organs. While many interference studies have revealed decreases in dry weight or yield, other characteristic symptoms may develop if a nutrient is limiting or if a chemical is toxic to a plant. Such characteristic symptoms would be extremely helpful in determining the cause of interference. Symptoms from nutrient deficiencies have been described in many instances (Wallace, 1961) as have symptoms from a microbial phytotoxin (McCalla and Norstadt, 1974) and from black walnut interference (MacDaniels and Pinnow, 1976). However, reports of characteristic symptoms of interference are relatively rare. It is possible that such symptoms are less conspicuous when the plant develops under conditions of limited resources or a low level of chemical toxicity than when the plant is suddenly subjected to a severe stress. It is a sound survival strategy to produce fewer, smaller, more efficient organs rather than more, larger, inefficient organs when under stress.

Preliminary tests for the cause of interference should be directed at ruling out alternative explanations as suggested by Platt (1964). Competition can be selectively eliminated by adding back-limiting resources or by using an artificial system (e.g., Bell and Koeppe, 1972, Putnam and Duke, 1974). Allelopathic interference can be selectively eliminated by using adsorbents [activated charcoal (Lockerman and Putnam, 1981a)], anion and cation exchange resins, or solvents to remove toxins from the soil, from a residue, or from an artificial system, or by using biotypes of the agent which lack the proposed toxin (Lockerman and Putnam, 1981b). Either of these approaches provides evidence which is circumstantial. The approach in which competition is eliminated may be used in combination with the approach in which allelopathy is eliminated. The combination would provide strong evidence of the relative contributions of allelopathy and competition to interference. It is important to consider the possible indirect sources of interference (e.g., Halligan, 1976; Kaminsky, 1981) as well as the possible artifacts which may be introduced in artificial systems.

Proof of Competitive Interference. Although numerous "competition" studies appear in the literature (Zimdahl, 1980), few determine which resources are limiting. Harper (1977) has indicated the difficulties in studying competition: "It is quite as difficult to identify the effects of nutrients as a resource in populations of plants as it is to disentangle the effects of light and water." We propose that the following observations are essential for proof of competitive interference:

1. Demonstrate the existence of interference by identifying the symptoms of interference and, if possible, quantitate the degree of interference.

2. Demonstrate that the presence of the agent is correlated with reduced utilization of the resources by the suscept. This indicates competition for the resource although it does not yet show this to be the cause of interference.

Experimental methods might include foliar analysis of mineral nutrients and/or monitoring the partitioning of isotopically labeled nutrients (e.g., [^{15}N]nitrate, [^{32}P]phosphate, etc.) or nutrient analogs (e.g., ^{85}Sr for Ca, or ^{86}Rb for K) between the agent and suscept. The utilization of the more dynamic resources—water, carbon dioxide, and light—must be monitored if they are potentially limiting. Competition for water or carbon dioxide might be monitored with infrared gas analysis or by use of isotopes (e.g., $^{3}H_2O$ or $^{14}CO_2$). Harper (1977) has reviewed the possibility that CO_2 is a limiting resource, although Oliver and Schreiber (1974) did not confirm this. Finally, competition for light should be studied by measuring the amount of light absorbed by the suscept alone compared with light absorbed by the suscept and agent when grown together. Such experiments are very difficult (Harper, 1977).

3. Demonstrate which of the resource(s) depleted by the agent are limiting resources. This might be done by relieving the interference through restoration of resources. [Harper (1977) has indicated that supplying one resource (such as water) might affect the availability of a second resource (such as nitrate). We have no proposal to determine potential resource interactions.] Such studies should be conducted with the agent and suscept grown together and restoring resource utilization to levels that occur when the agent is absent. For example, supplemental CO_2 (Egli et al., 1970), light (Johnston et al., 1969), nutrients, or water (Elakkad and Behrens, 1978) may be added to the competing plant, so that the suscept now utilizes as much of the particular resource as when the agent is absent. Resources should be tested individually in this manner to indicate which of the particular resources is primarily responsible for the interference. Finally, all resources which appear to be responsible for competitive interference should be restored to levels present when the agent is absent, and all symptoms of interference should be relieved.

4. Simulate interference from the agent by reducing the levels of the resources considered responsible for interference. Utilization of these resources by the suscept should be reduced to levels that occur when the agent is present, and this should cause the expected symptoms of interference in the suscept. Resources should be tested individually in this fashion in order to prove which resources are indeed causing the observed interference.

Criteria 3 and 4 may be very difficult to meet, except possibly in artificial systems. It would be difficult to monitor resource levels so precisely, even in the absence of the potential resource interactions. Such experiments remain important even if they cannot fully meet the criteria.

Proof of Allelopathic Interference. This hypothesis states that the agent interferes with the suscept by releasing a chemical that injures the suscept. This chemical may originate from living tissues and be released from leaves,

roots, or other organs; the chemical may also originate from decaying tissue. It may be desirable to distinguish toxic chemicals of plant origin by the term "phytoinhibitins" and toxic chemicals of saprophytic origin by the term "saproinhibitins." These terms are preferable to the terms "koline" and "marasmin" defined by Grummer (Rice, 1974) since the new terms describe the phenomena more clearly. Two distinct ecological phenomena are involved: chemicals of plant origin may offer a direct survival benefit; the chemicals produced by saprophytes are not controlled by the plant which produced the residues and therefore may be either beneficial or detrimental. However, the principles of proof of allelopathic interference are similar whether caused by phytoinhibitins or saproinhibitins. We propose that the following observations are essential for proof of allelopathic interference:

1. Demonstrate the existence of interference by identifying symptoms of this interference and, if possible, quantitate the degree of interference.

2. Isolate, assay, characterize, and synthesize the toxin. Isolation of a toxic substance is the first step in obtaining direct evidence that the interference is due to a chemical. Caution should be exercised to ensure that substances that would not be released by the plant or saprophyte in nature do not contaminate this toxin preparation. For the purpose of isolating the toxin, many chromatographic techniques are currently available, including those based on molecular size, charge, pK, and polarity. Next a chemical, biological, or isotopic assay should be developed. Such an assay is essential in monitoring the movement of the toxin from the agent to the suscept (discussed in No. 4, below). Characterization of the purified toxin will allow synthesis of the compound. Numerous spectral analyses are now available to aid in identification of small quantities of chemicals. Synthesis of the compound can be accomplished in two ways. First, chemical synthesis will confirm the identity of the compound. Such laboratory synthesis may also allow synthesis of the toxin in a radioactively labeled form for covenient isotopic assays. Second, the radioactively labeled compound may also be biosynthesized by the agent. This might be accomplished by supplying the agent with a radioactively labeled substrate such as $^{14}CO_2$ or [^{14}C]sucrose or some intermediate in the biosynthesis of the toxin. This may be a more convenient way to synthesize the labeled toxin. Biosynthesis will facilitate subsequent studies of the release, movement, and uptake of the compound as discussed in No. 4, below.

3. The symptoms of interference previously diagnosed should be repeated by application of the toxin at rates present in nature at the appropriate stages of suscept growth. This will show that symptoms can indeed be reproduced (quantitatively, if possible) by the toxin alone.

4. The release, movement, and uptake of the toxin should be monitored and shown to be sufficient for the observed interference. Meeting this criterion

would allow the conclusion that the toxin is released in sufficient concentration to move across the environmental barriers, whether through soil or air, and still be taken up in quantities sufficient to cause toxicity in the suscept. The biosynthesis of the radioactively labeled toxin could greatly facilitate such studies. Such biosynthesis might be done when the agent and suscept are grown together under artificial or field conditions. Monitoring the radioactively labeled toxin would provide direct evidence for the release, movement, and uptake of the toxin. As a supplement to such studies, it would be necessary to show that this toxin is absent from the tissues of the suscept when it is grown in the absence of the agent.

It would be desirable but not essential to consider the selectivity of the toxin by comparing the range of species affected by the isolated toxin to the range of species that show allelopathic injury when grown with the agent. In such a study of selectivity, the autotoxicity of the toxin should be studied as well. Such a selectivity study has been conducted by Stowe (1977) by correlating susceptibility to crude toxin preparations with spatial relationships of species in the field.

Special note should be made of the work of McCalla and colleagues (McCalla and Norstadt, 1974; Elliott et al., 1978) and the work of Tang and Young (1982) in which many of the criteria discussed above have been met in studies of saproinhibitins and phytoinhibitins, respectively.

The criteria proposed above are not inconsistent with previous statements by Whittaker (1970): "One needs to establish first that the effect is in fact chemical and not one of competition between plants for light, water, or nutrients. The source of the chemical in the plant should be found, and the route it follows from release from one plant to effect on another traced . . . Identification of the chemical is to be sought, and the manner of inhibition of germination or growth determined . . . . One wants finally to show . . . that the quantitative relations of the chemical agents identified as they occur in the soil are adequate to produce the observed degree of inhibition of other plants . . .

Two special problems in terminology should be discussed. First, production of toxins and immobilization of nutrients by saprophytes (Kimber, 1973) can presumably adversely affect plant growth. This can be considered an indirect source of interference since the substrate for the saprophytes was supplied by a higher plant. The phrase "saprophytic allelopathy" may be appropriate for the case of toxin production, and the phrase "saprophytic competition" may be appropriate in the case of nutrient immobilization. These phenomena may be studied by the appropriate set of criteria above; the saprophyte is considered to be the agent. Second, an agent may produce chemicals that prevent uptake of nutrients by a suscept (Harper and Balke, 1981). This should be considered allelopathic interference where

the mode of action of the toxin is reduction of nutrient uptake. This should not be considered competition since the agent is not directly depleting the resource. This phenomenon may be studied using the criteria for proof of allelophatic interference discussed previously.

It is obvious that final proof of allelopathic or competitive interference requires numerous experiments. For this reason it seems likely that the most progress will be made when the interaction between two particular species is studied in great detail, or when an extreme example of interference is studied in detail or in some model system. Furthermore, there is considerable need for development of technology to study interference.

Quite often the terms "competition" and "allelopathy" have been misused. For example, so called "competition" and "allelopathy" studies in most cases would be properly called "interference" studies; also conclusions are often drawn regarding the cause of interference—either allelopathy or competition—without referring to or proposing criteria for proof. Therefore the terms "competition" and "allelopathy" must be used in senses consistent with their definitions and consistent with some criteria, perhaps those proposed in this discussion.

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EXPERIMENTAL METHODOLOGIES TO EVALUATE ALLELOPATHIC PLANT INTERACTIONS: The *Abutilon theophrasti–Glycine max* Model

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Abstract—It is difficult to clearly and unambiguously demonstrate an allelopathic mechanism of plant interference. Several types of experimental methodologies such as the additive design, substitutive designs, and several types of plant yield–plant population functions are discussed in terms of their relative merits in terms of providing quantitative and qualitative information in the development of an empirical basis to describe a plant interaction. Additionally, several types of mathematical and graphical representations are presented using data from the velvetleaf (*Abutilon theophrasti*) and soybean (*Glycine max*) interaction. The design provides agronomically relevant information about crop yield losses but confounds the separate effects of population density and species proportion in mixtures. The replacement series design separates these two variables by maintaining a constant population of plants while varying the relative proportion of each species in mixtures. The replacement series diagram, relative yield, relative replacement rate, ratio diagram, a scaling test, and the regression of individual yield on the associate yield are discussed in terms of their utility in providing insights into a plant interaction. Individual plant yield–plant population functions such as the "Y–D" and "C–D" effects, the "$\frac{3}{2}$ power law of self-thinning," and the "Sakai" test provide a basis to compare plant yield per plant versus plant population responses. Several types of interactions are characterized with this methodology. None of these experimental designs will clearly demonstrate an allelopathic plant interaction alone, but they do provide high-inference experimental methodologies to develop an empirical foundation to describe accurately a plant interaction upon which more specific hypotheses can be developed.

Key Words—Allelopathy, velvetleaf, *Abutilon theophrasti*, soybean, *Glycine max*, replacement series.

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INTRODUCTION

The proper methodology to clearly and unambiguously demonstrate an allelopathic mechanism of plant interference has not yet been developed. Many types of experimental designs and techniques have been used to demonstrate allelopathy (Putnam and Duke, 1978), but their validity has not been agreed upon. The underlying problem with most methods being used is that it is difficult first to characterize the nature of the plant interaction and second to separate competitive components of that interaction as allelopathy from other forms of interference.

Until the perfect methodology is developed, the best that can be done is to search for high-inference experimental designs that can demonstrate and characterize plant interactions both quantitatively and qualitatively. Therefore, to demonstrate allelopathy, there is a need to first define the interaction between species, preferably in a field situation. From the definition of the exact interaction between species, an empirical base of both growth responses and observed symptomology can be developed. The nature of the growth responses and symptomology can provide evidence to develop an hypothesis of the mechanism of interference between the species. It must be emphasized that any single methodology is only one of many that are required to provide strong correlative evidence of an allelopathic interaction between plants. The development and use of high inference experimental designs in conjunction with subsequent, more controlled experiments can provide this type of evidence. With these considerations in mind the objectives of this paper are: (1) to determine which experimental designs now extant can give both qualitative and quantitative information about plant interactions, especially allelopathic interactions; (2) to determine which of these designs can distinguish between the separate effects of changes in plant population density and those due to species-dependent interference; and (3) to determine what statements can and cannot be derived from these designs. The experimental designs to be discussed include the additive design, substitutive designs like the replacement series, and several types of individual plant yield–plant population functions. The interaction between velvetleaf (*Abutilon theophrasti*) and soybean (*Glycine max*) will be used to demonstrate some of the interpretations to be derived from these experimental designs (Dekker and Meggitt, 1983a,b). The purpose of this paper is to present selected data from that series to illustrate possible uses and interpretations of these experimental approaches.

Several studies have been conducted that indicate an allelopathic effect from velvetleaf on soybean. Raynal and Bazzaz (1973) first suggested the possibility that the nature of interference by velvetleaf on soybean could be due to chemical inhibition. Retig (1971) and Retig and coworkers (1971, 1972) found that diffusible materials out of germinating velvetleaf seeds caused

abnormalities to tomato and cabbage roots germinating with the weed seed. The roots of those crops were larger due to increases in the number and size of parenchyma cells in the root cortex. The authors felt that these anatomical changes to the crop roots could alter normal functions such as nutrient and water uptake.

Gressel and Holm (1964) found water-soluble extracts of velvetleaf seed to be selectively inhibitory to alfalfa, radish, and turnip seed germination. Effects of these seed extracts were found to be species- and variety-specific. Velvetleaf leaf tissue extracts were also found to contain inhibitory compounds, but not necessarily the same ones found in velvetleaf seed. Neither velvetleaf seed germination nor seedling growth was inhibited by these compounds. The inhibitors were found in the highest quantity in the seed embryo and endosperm, less so in the seed coat.

Coulton and Einhellig (1977) found velvetleaf plant residues did not inhibit soybean seedling growth for the first 28 days but did decrease soybean root nodulation. Soybean seedling growth was inhibited by water-soluble velvetleaf leaf extracts. Phenolic compounds chromatographically isolated from the velvetleaf leaf tissue water-soluble extracts reduced soybean growth and leaf turgor. These compounds also caused primary root death and an increased number of secondary roots that were shorter and thicker than untreated plants.

These studies suggest that there is a high probability of allelopathic interactions playing a significant role in the interference of velvetleaf on soybean. This chemical interaction could be due to interference with soybean plant water relations and root morphology. What is lacking is a broad empirical context within which the interpretation of the importance of these discrete studies can be made.

RESULTS AND DISCUSSION

Additive Designs

Weed researchers have traditionally studied interference with additive designs (Zimdahl, 1980). Mixture responses are produced by the addition of weed plants to a constant population crop stand. This procedure makes possible the measurement and ranking of relative weed aggressiveness. The additive design may vary both the population density and the proportion of the competing species, with resultant confounding of effects. Therefore, the interpretation of the significance of the results is limited. A schematic representation of this type of design is presented in Figure 1. Because of its relevance to actual field situations in which a weed infests the area occupied by the crop, and because it permits the assessment of effects on yield and cost to the farmer, the additive design is widely used. Velvetleaf caused severe

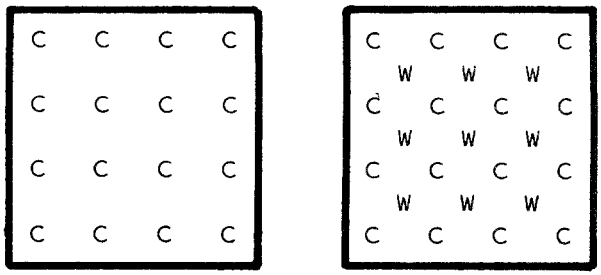

FIG. 1. Schematic representation of the additive design plot layout; each block represents 1 m^2; left: crop plants (C) at 16 plants/m^2; right: crop plants (C) at 16 plants/m^2 plus weed plants (W) at 9 plants/m^2; the overall population is 25 plants/m^2.

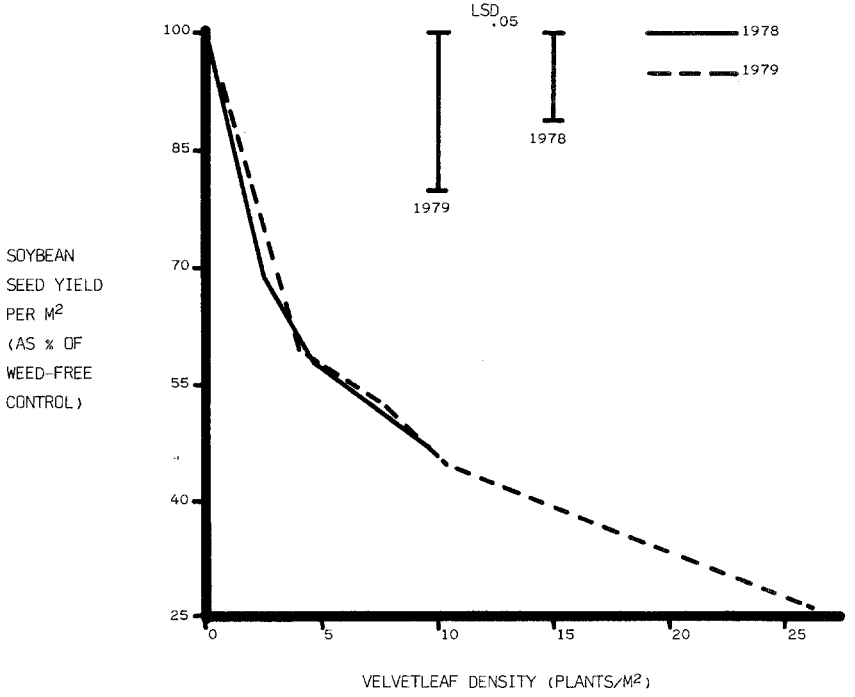

FIG. 2. Effect of various velvetleaf populations on soybean seed yield per meter^2 (expressed as a percent of the weed-free control) in a constant population soybean stand; LSD = 0.05.

soybean seed and flowering node number losses from both low and medium weed infestation levels (Figures 2 and 3). This evidence could support an allelopathic mechanism of interference, but is only suggestive. There are other competitive mechanisms that could explain these severe losses. Few inferences beyond economic yield losses can be made with data drawn from additive design experimentation.

Substitutive Designs

Many of the limitations of the additive design are eliminated by the use of substitutive designs (Durrant, 1965; Gleeson and McGilchrist, 1978; Harper, 1977; Hayman, 1954a,b; Jinks, 1954; Jinks and Hayman, 1953; McGilchrist, 1965, 1971; McGilchrist and Trenbath, 1971; Norrington-Davies, 1967, 1968; Thomas, 1970; Trenbath, 1975; Wearden, 1964; Williams, 1962). The main characteristic of a substitutive design is that the proportions of two plant species in a mix are proportionately varied while their overall density (plants

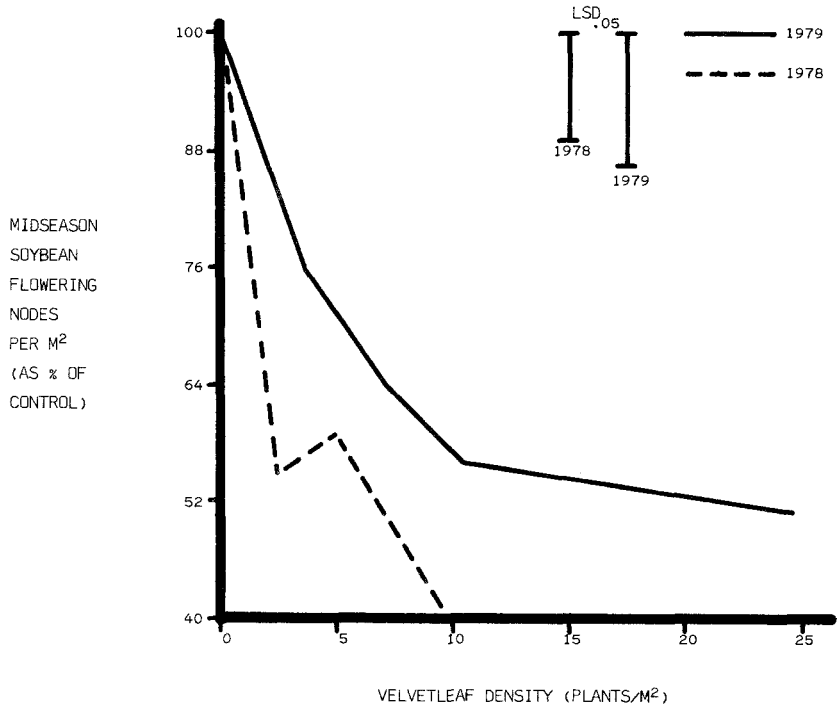

FIG. 3. Effect of various velvetleaf populations on soybean flowering nodes per meter^2 (expressed as a percent of the weed-free control) in a constant population soybean stand; LSD = 0.05.

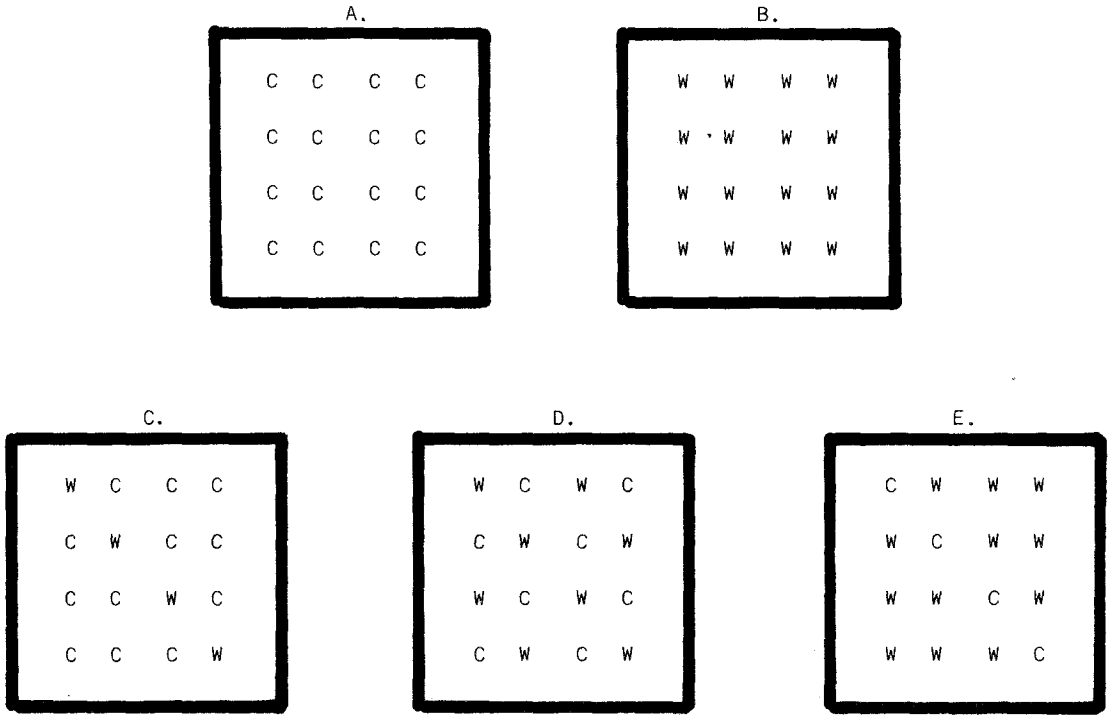

FIG. 4. Schematic representation of the replacement series plot layout; crop plants (C) and weed plants (W) at one constant population of 16 plants/m^2; each block represents 1 m^2; A: crop monoculture, B: weed monoculture; C: 75% crop–25% weed mixture; D: 50% crop–50% weed mixture; E: 25% crop–75% weed mixture.

per unit area) remains constant. Therefore, substitutive designs avoid density–proportion confounded effects and allow a precise comparison of neighbor effects at one constant population density. Perhaps the most useful substitutive design is the replacement series, as seen schematically in Figure 4.

Replacement Series Diagram. Replacement series experimental designs are usually performed with two species, e.g., a crop and a weed. This elegant analysis was first presented by de Wit (1960, 1961). Several others have utilized this methodology in their research (Baeumer and de Wit, 1968; Bakhuis and Kleter, 1965; van den Bergh and de Wit, 1960; van den Bergh and Elberse, 1962; van den Bergh, 1968; Burdon and Pryor, 1975; Dekker and Meggitt, 1983a,b; Hall, 1974a,b; Hill, 1973; Trenbath and Harper, 1972; Trenbath, 1974; Tripathi and Harper, 1973; de Wit and van den Bergh, 1965; de Wit et al., 1966). De Wit considered the competitive relations between plants and expressed those relationships mathematically. He did this by measuring how plants "crowd for the same space." Each plant possesses characteristics that allow it differentially to acquire resources, to grow, and to occupy space at a specific rate. "Space" is the exhaustible supply of growth factors. "Space"also has been defined as biological space, a composite of all growth factors and resources (Hall, 1974).

The de Wit analysis is based on the simple assumption that the yield of each species in a mixture is proportional to the share of the environmental resources it can acquire. If this sharing is unequal, then the aggressor will acquire more and the weaker species less (Trenbath, 1974).

The mathematical model is based on two species: crop C and weed W. The density, D, or number of plants per unit area is kept constant: $C + W = D$. Both species are grown in monocultures and in mixtures of varying proportions of the crop and weed. The differences between responses of plants grown in monoculture and in various mixtures reveal several qualitative relationships. The simplest level of interpretation can be discerned from the replacement series diagram (Figure 5). The actual yield of the crop or weed is represented on the vertical axis. Yield can be measured as one of several quantitative factors: plant population density; growth parameters such as seed yield, dry matter, tiller number, reproductive organ number, and leaf weight; or reproductive parameters such as propagule number.

The horizontal axis represents the relative proportions of each species' contribution to the mixture ranging from 0 to 100% and the corresponding proportions of the other species from 100 to 0%.

Perhaps the best interpretation of these diagrams is presented by Hill and Shimamoto (1973). Figure 6A represents the case in which the response of the mixture, and its individual species components, can be predicted from two monoculture responses. This relationship arises when either: (1) the crop and weed do not interfere with each other, or (2) both species' ability to interfere

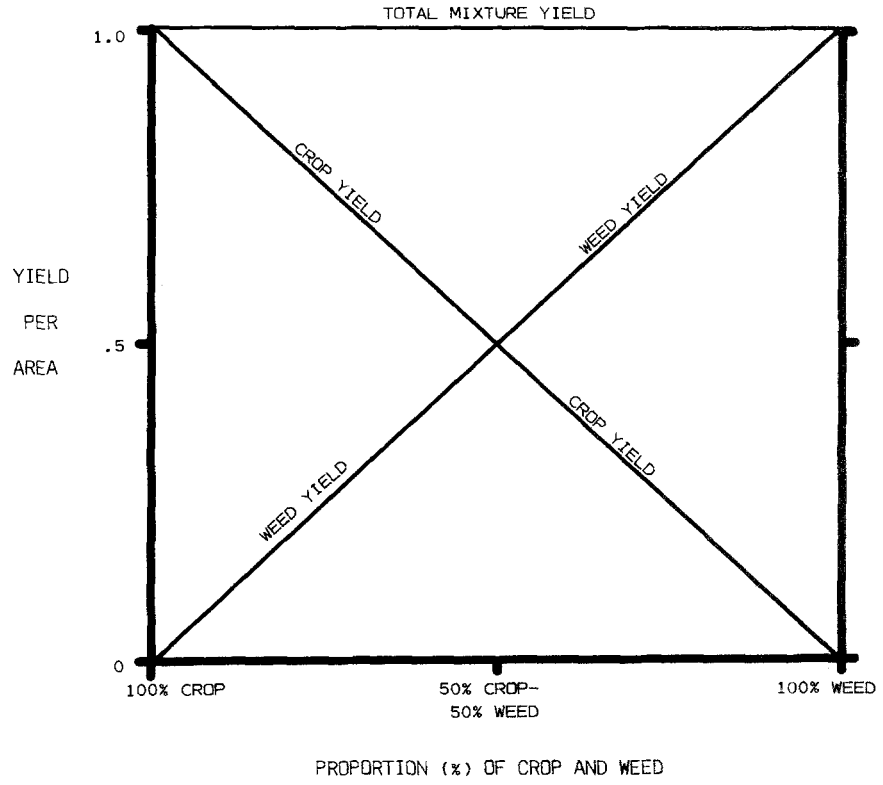

FIG. 5. The replacement series diagram of yield per unit area versus the proportion (%) of the crop or weed.

with the growth of the other is equivalent, i.e., interspecific interference is equal to intraspecific interference. The actual yeilds of the two do not have to be equal; although they make equivalent demands on the growth requisites, they may convert those requisites into biological yield with different efficiencies. This is the relationship that de Wit (1960, 1961) termed "mutually exclusive."

Figure 6B represents a compensatory interaction between crop and weed. One species gains in the mixture at the expense of the other. These gains and losses by each species counterbalance, so the responses in the total mixture cannot be predicted from the monocultural responses. The crop and weed populations make different demands on growth requisites, or they acquire these requisites with different efficiencies.

Complementary interactions occur when the gains and losses of the crop and weed do not counterbalance. Therefore, neither the total mixture yield nor the yields of the crop and weed components can be predicted from the

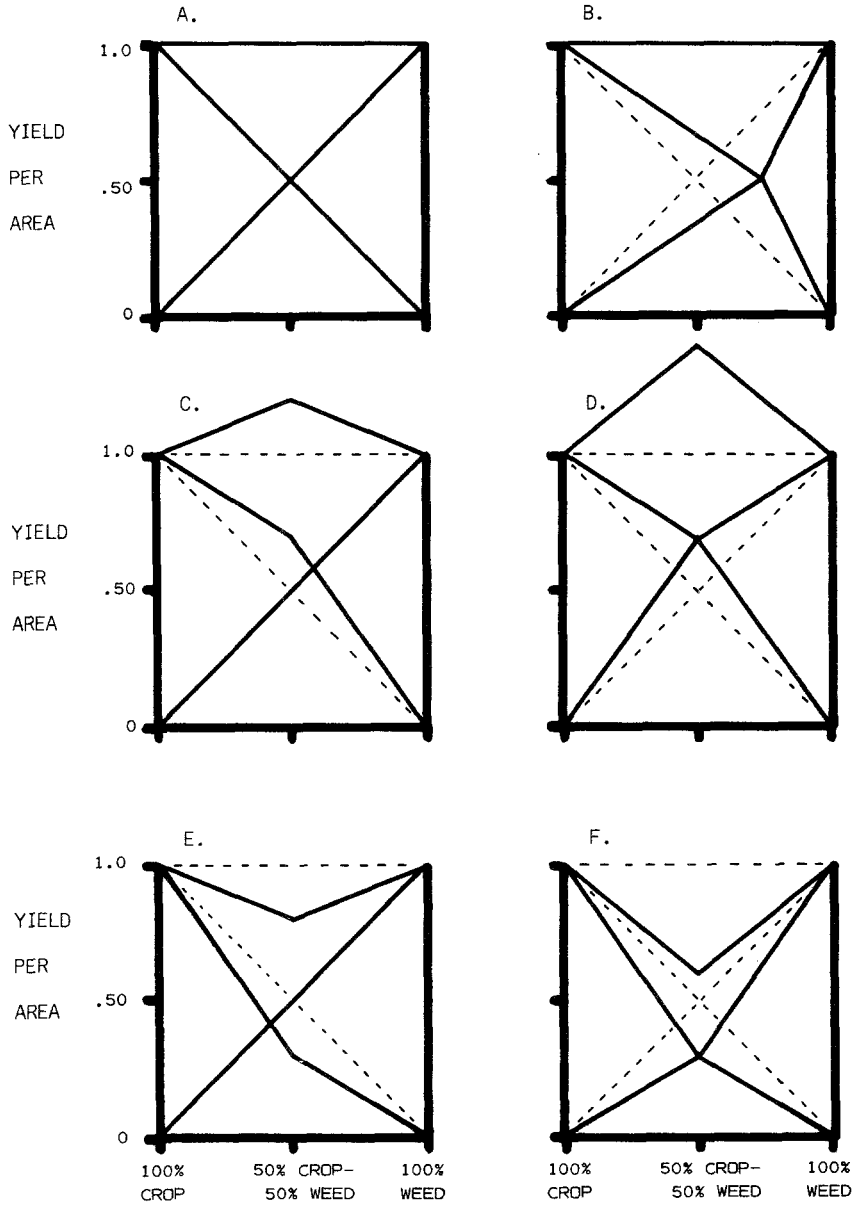

FIG. 6. Models of possible crop–weed interactions expressed in replacement series diagrams of yield per unit area versus the proportion (%) of the crop or weed; A: mutual exclusion; B: compensation; C and D: positive complementation; E and F: negative complementation.

monoculture responses. Positive complementation (Figure 6C and D) occurs when the mixture performance exceeds the average of the constituent monocultures. This occurs when the species escape, to some degree, their mutual competition. Both species fail to suffer as much as would be expected from the monoculture responses. This interaction could arise from: (1) a symbiotic relationship whereby one or both of the species aided the other (e.g., grass–legume association); or (2) a temporal displacement of the growth periods of the species (e.g., winter annual–summer annual association). Negative complementation (Figure 6E and F) occurs when mixture performance falls below that of the monoculture average. This situation occurs when one, or both, of the species does not contribute its expected share to the total yield. The interaction could arise if: (1) a species produced a toxin that reduced the growth of the other species or of both species (e.g., allelopathy); (2) a mutually stimulatory effect of individuals in pure stands is destroyed by mixing (e.g., loss of legume–*Rhizobium* spp. symbiosis). These complementary interactions can be due to the alteration in yields of only one species in an interaction (positively, Figure 6C; negatively, Figure 6E); or it can be due to the alteration in yield of both species (positively, Figure 6D; negatively, Figure 6F).

Complementation between the crop and weed can be complete or overcomplementation. Partial complementation occurs when the mixture performance is less than that of the better monoculture (positive), or more than that of the lesser monoculture (negative). Complete complementation occurs when the mixture performance is equal to that of the better monoculture (positive), or that of the lesser monoculture (negative). Overcomplementation occurs when the mixture performance is greater than that of the better monoculture (positive) or less than that of the lesser monoculture (negative). Positive overcomplementation can also be described as a cooperative interaction. Negative overcomplementation is mutual inhibition (perhaps allelopathy).

The relative degree of a species' aggressiveness can be ascertained from the component yield curves in the replacement series diagrams. Curves that are convex represent relatively more aggressive species than do the concave curves.

The velvetleaf–soybean seed yield interaction could be termed compensatory, especially for the 75% velvetleaf–25% soybean mixture (Figure 7). As such, it does not provide evidence of an allelopathic mechanism of interference. The interaction between soybean and velvetleaf reproductive organ numbers is more apparent (Figure 8) than the seed yield data. The interaction between the two species in terms of flowering nodes could be characterized as negative complementation or negative overcomplementation and is similar to Figure 6E. Velvetleaf reproductive organ production remains as we would expect from the monoculture yield. Soybean flowering node numbers were

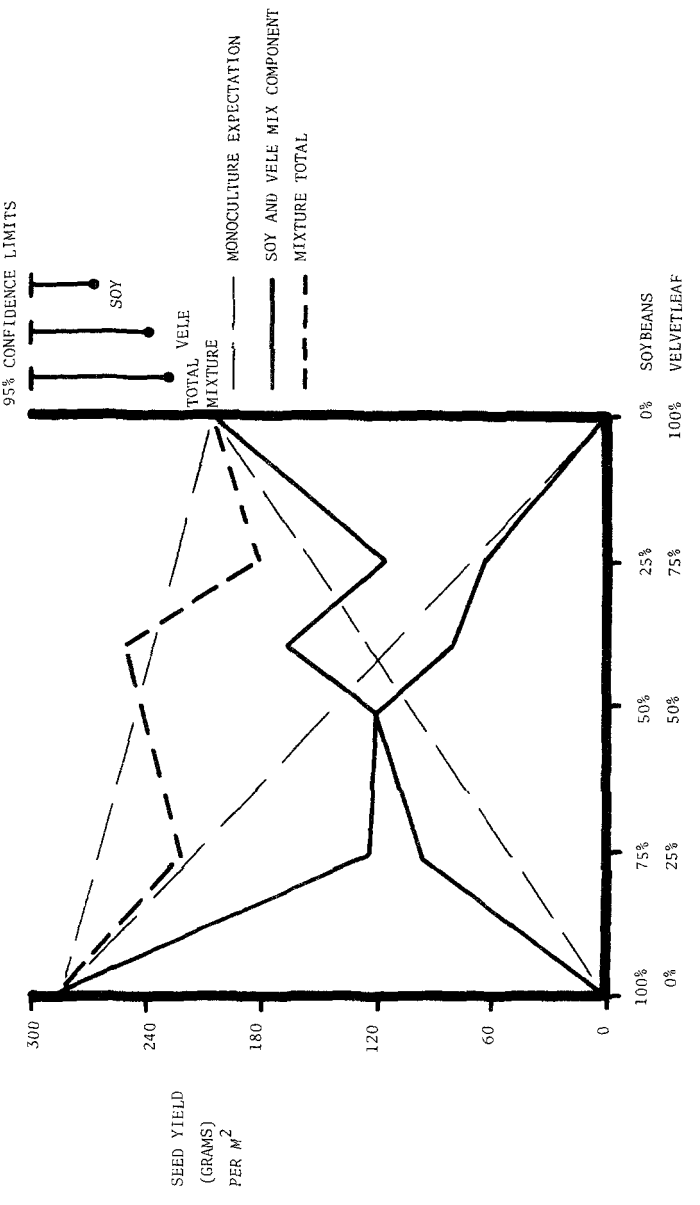

FIG. 7. Replacement series diagram of soybean and velvetleaf seed yield (grams) per meter^2 at a constant population density; confidence limits, $P = 0.05$; SOY = soybean; VELE = velvetleaf.

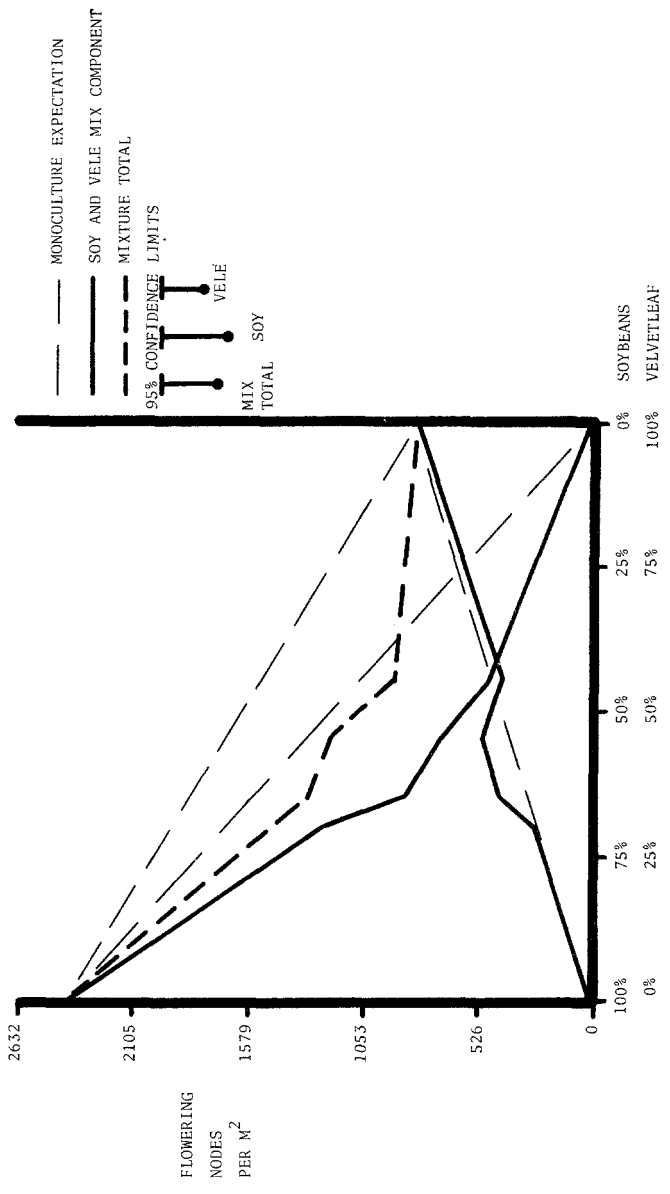

FIG. 8. Replacement series diagram of soybean and velvetleaf flowering nodes per meter^2 at a constant population density; confidence limits, $P = 0.05$; SOY = soybean, VELE = velvetleaf.

less than expected from monoculture yields. The power of the replacement series analysis allows us to discount population changes as the cause. This evidence supports the hypothesis that velvetleaf has an allelopathic effect on soybean. This effect is most direct on soybean flowering node numbers and less apparent in terms of soybean seed yield.

Relative Yield. It is difficult to compare the performance of different species in terms of absolute yields: a kilogram of velvetleaf seed is different from a kilogram of soybean seed in terms of energy content or propagule number. These difficulties can be partially overcome by the comparison of pure stand and mixture yields in terms of relative yield of each species:

$$\text{Relative yield} = \frac{\text{Yield in mixture}}{\text{Yield in pure stand}}$$

$$\text{Relative yield total} = \text{Relative yield of crop} + \text{Relative yield of weed}$$

The relative yield of both the crop and the weed can be calculated as summed to give the relative yield totals (RYT). The RYT can be used to describe the mutual interaction that occurs between the species: (1) RYT = 1: this situation implies that each species is making the same demands for "space" as the other; they are "mutually exclusive" or complementary. (2) RYT $>$ 1: this situation suggests that one or both of the species are less affected by interspecific interactions than could be predicted from their monoculture responses; it suggests that they are: (a) making different demands on the same resources; (b) occupy different niches in time or space; or (c) exhibit some sort of symbiotic relationship. (3) RYT $<$ 1: this situation occurs when one or both species are more affected by interspecific competition than would be expected from their pure stand responses and indicates mutual antagonism. Possible mechanisms that could explain this interaction are: (a) the action of phytotoxins produced by one or both species, or (b) the loss of the pure stand stimulatory effect in the mixture.

These interpretations of the RYT reveal the mutual interactions between the plant species pairs at several proportions. The yields of each species are easily comparable to each other by the use of this calculation. Also, yields differing from monoculture expectation are easily detected. The soybean relative seed yield was less than anticipated in the 75% soybean–25% velvetleaf mixture (Figure 9). This was compensated for by the higher-than-anticipated velvetleaf seed yield in that mixture. The soybean flowering node numbers were less than expected from the monoculture yield in all mixtures with velvetleaf (Figure 10).

The use of relative yields does not provide any further evidence than is provided by the replacement series diagram, but it does provide a more convenient method of detecting and communicating the interaction results obtained from this type of experimental design.

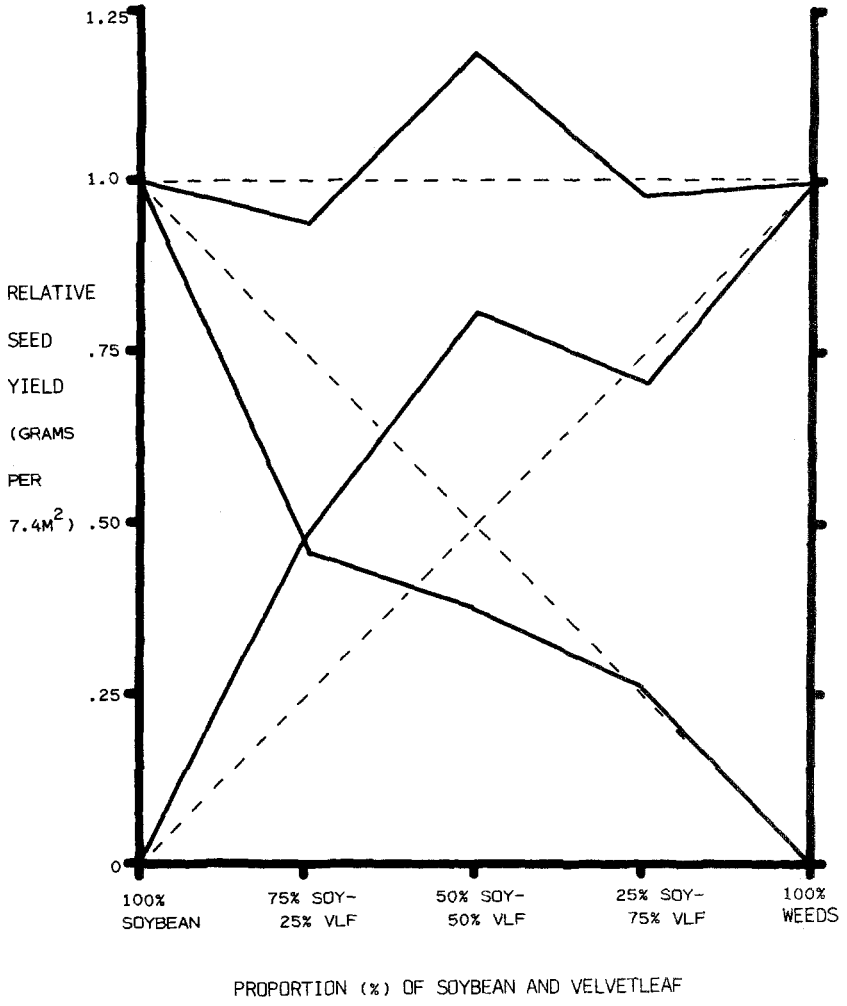

FIG. 9. Replacement series diagram of soybean (SOY) and velvetleaf (VLF) relative seed yield (grams per 7.4 meter^2).

Relative Replacement Rate (RRR). Data derived from this type of analysis can be used to represent the long-term shift favoring one species over another. This could be useful for prediction purposes in both annual and perennial cropping systems. If one species produces more yield than the other species over time, the less productive species will be excluded. The long-term dynamic equilibrium forces on annual crop and weed associations can also be determined. De Wit originally calculated the RRR from seed numbers produced by each species. The use of other yield parameters (e.g., seed dry

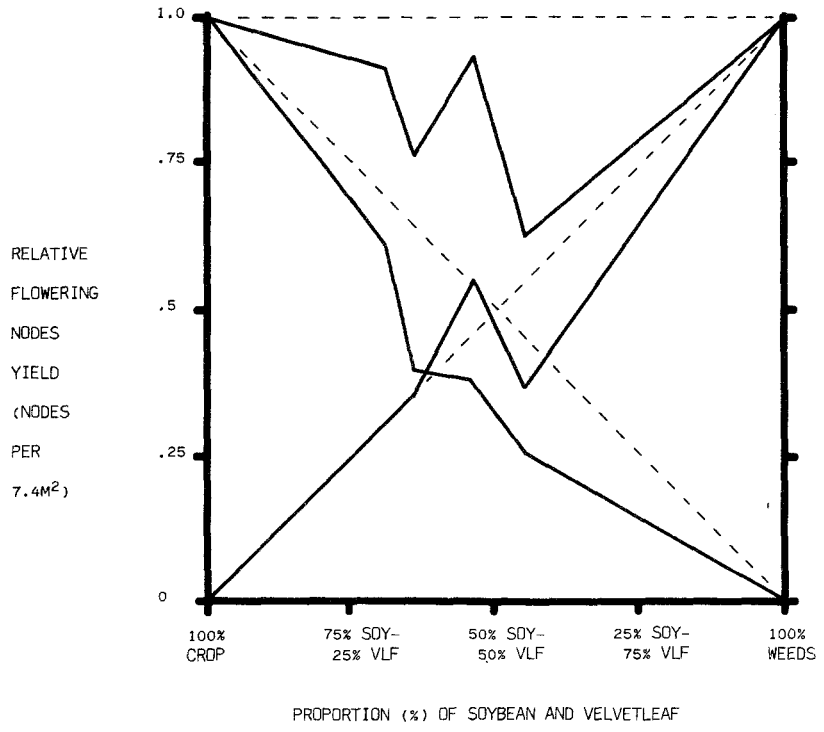

FIG. 10. Replacement series diagram of soybean (SOY) and velvetleaf (VLF) relative flowering nodes yield (nodes per 7.4 meter^2).

weight, total plant dry weight, etc.) to indicate long-term equilibria and the subsequent replacement of one species for another can reveal the intensity of interference "pressure" exerted by each yield component. The dominance of any factor—not just seed production—could be decisive in these long-term equilibria replacement shifts. It must be emphasized that there is an inherent danger in regarding this index as a predictor of what will happen between two species in a field situation in the future. It is best utilized as an indicator of the intensity of replacement pressure of one plant species on another.

The relative replacement rate (RRR) of the crop (C) species in reference to the weed (W), RRR_{CW}, can be obtained by:

$$\mathrm{RRR_{CW}} = \frac{\text{Crop yield in mix} \div \text{Crop proportion (\%) in the mix}}{\text{Weed yield in mix} \div \text{Weed proportion (\%) in the mix}}$$

The following interpretations are provided for the significance of: (1) $\mathrm{RRR_{CW}} = 1$: this implies that both species are competing for the same "space" in the same way (i.e., mutually exclusive). If $\mathrm{RRR_{CW}} = 1$ at all mixture

proportions, then the two species will tend to form a stable community. They are replacing each other on a 1-to-1 basis, and their proportionate composition will not change. (2) $RRR_{CW} >$: in this case, the crop (C) is replacing itself relative to the weed (W) at a greater rate. If $RRR_{CW} > 1$ at all mixture proportions, then the crop will replace the weed over subsequent generations. (3) $RRR_{CW} < 1$: this is the converse of (2). The weed is replacing the crop. If $RRR_{CW} < 1$ for all mixture proportions, the crop will be eliminated in subsequent generations.

The relative replacement rates of soybean to velvetleaf in terms of seed number indicates a strong direct replacement pressure by the weed against the crop component in all mixture proportions (Table 1).

Ratio Diagram. The dynamics of weed–crop interactions can be further analyzed with the use of the "ratio diagram" (Figure 11). The horizontal axis is the ratio of the area occupied by the crop to the area occupied by the weed. The vertical axis is the ratio of the actual crop yield to the weed yield. The ratio of areas occupied is plotted against the ratio of the yields obtained on a log–log function. The shape of the defined function reveals much about their interaction (Figure 11). Point C represents the crop monoculture, and point W the weed monoculture. If the calculated points fall along the 45° diagonal line for all mixture proportions, then each increment of increased (or decreased) are is met by an exact 1-to-1 increase (or decrease) in yield. In other words, the species are replacing each other on a 1-to-1 basis ($RRR_{CW} = 1$). This is represented by the diagonal line, and the interaction between the species is mutually exclusive.

Another situation can be visualized where the crop consistently yields more than the weed in all mixture proportions. This is the case where $RRR_{CW} > 1$ (Figure 12). Although this function is parallel to the diagonal ($RRR = 1$), the fact that it is consistently to the left of the diagonal implies the equilibrium will move in direction of the arrow. Each subsequent generation will let the RRR_{CW} value move toward the extinction of the weed. In the case

TABLE 1. RELATIVE REPLACEMENT RATE OF SOYBEAN IN RELATION TO VELVETLEAF (RRR_{sv}) IN TERMS OF SEED NUMBER PER m^2[a]

Mixture	RR_{sv} (SE)
66% soybean–34% velvetleaf	0.039 (0.016)
55% soybean–45% velvetleaf	0.057 (0.016)
35% soybean–65% velvetleaf	0.068 (0.050)

[a]SE = standard error, $P = 0.05$.

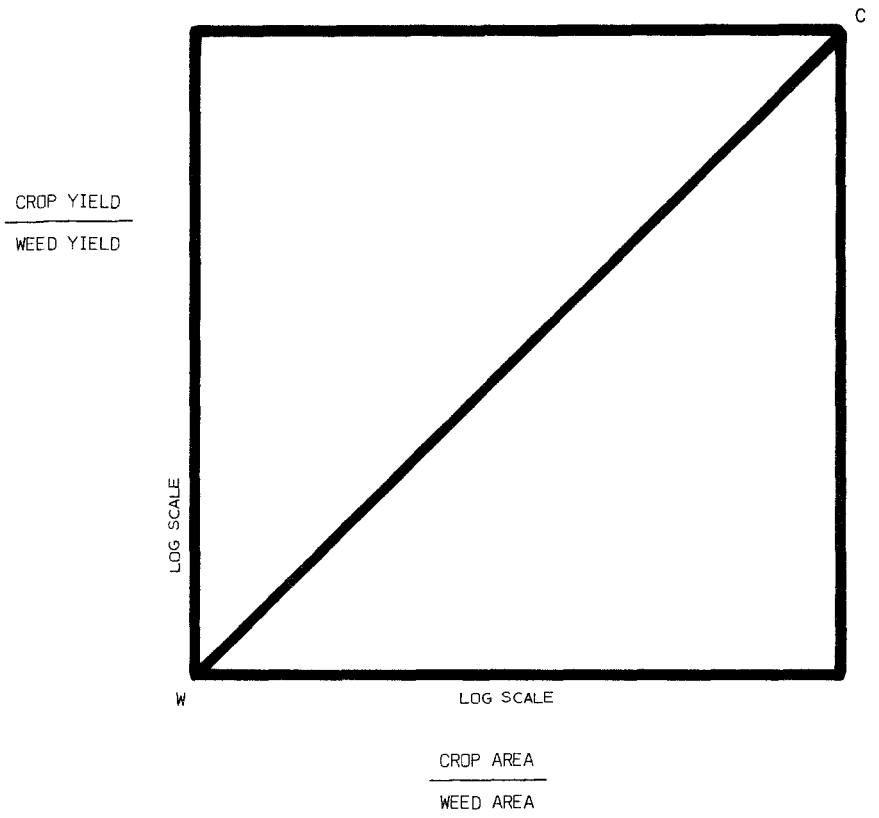

FIG. 11. The ratio diagram: the ratio of crop yield to weed yield versus the ratio of crop area to weed area; W = weed monoculture; C = crop monoculture.

represented in Figure 8, it will take three generations (three RRR_{CW} shifts along dotted line) for the weed to be eliminated (assuming no dormancy or other factor).

The converse situation can also be visualized in Figure 13. Because the weed is replacing itself at a relatively higher rate than the crop ($RRR_{CW} < 1$), the long-term shift in the population favors the weed and will lead (in this example) to the extinction of the crop in three generations. Of course this is unrealistic because most crops do not reseed themselves, but are reestablished by a farmer at a high density each year. Also, as subsequent generations move the mixture proportion in favor of the dominant species, the RRR_{CW} might also change. These frequency-dependent competitive interactions are common and will be discussed below. Despite these limitations, such analyses do reveal a dynamic equilibrium force that will enable the weed to succeed with

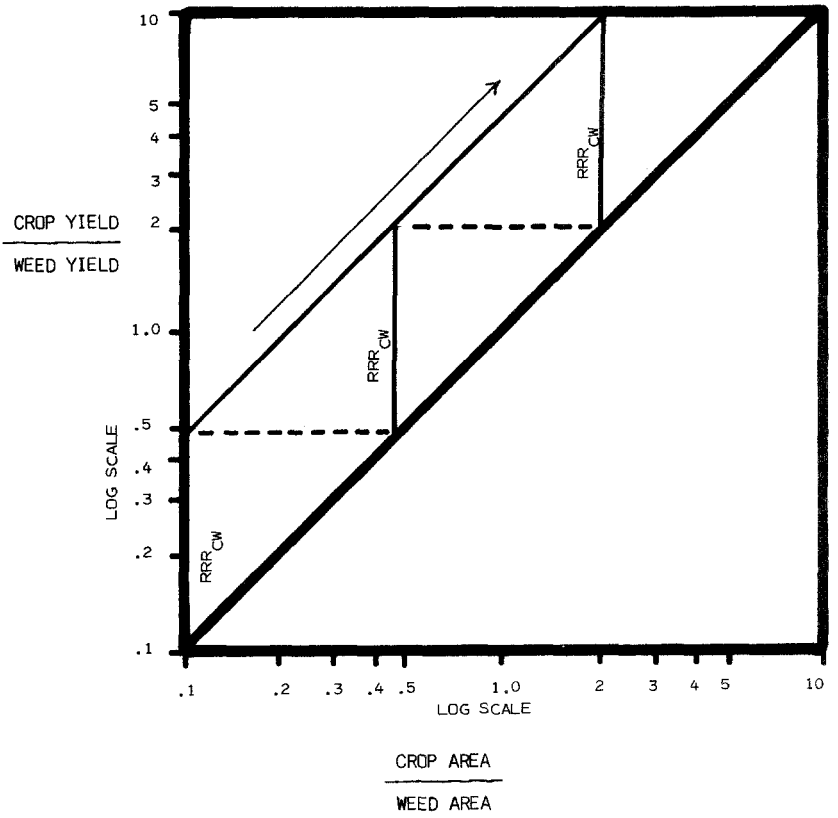

FIG. 12. The ratio diagram of the relative reproductive rate of the crop relative to the weed (RRR_{CW}) greater than unity.

continued cropping and indicate a need for a rotational crop that severely reduces weed reproductive ability. An increase in the weed seed population in the soil favors the development of adaptive strategies such as herbicide resistance.

Frequency-dependent interactions are revealed by two other possible calculated functions on the ratio diagram. These proportion-dependent interactions show species affecting each other in other ways then competing for the same space. The first is the case in which the slope of the function is not parallel to the diagonal and is less than a 45° slope (Figure 14). This type of interaction leads to an equilibrium, or stable, mixture at the intersection of the calculated function and the diagonal. For mixture proportions to the left of the diagonal ($RRR_{CW} > 1$), the increase in the replacement potential favors the crop, so it moves along the arrow by rapid replacement to the intersection point. At those ratios, the crop is the minority component, and it is favored.

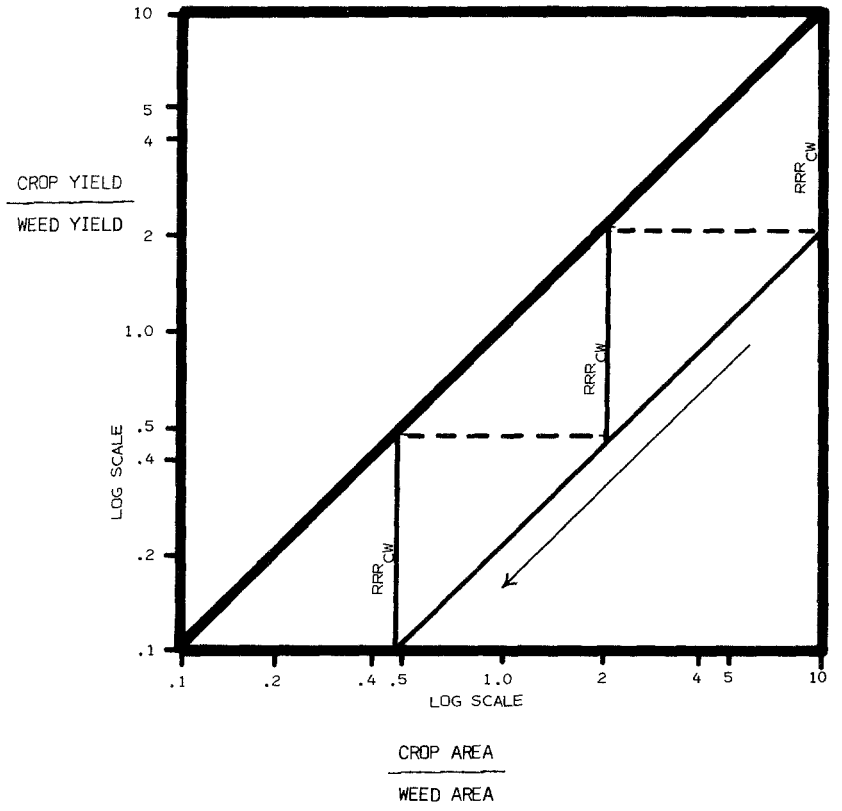

FIG. 13. The ratio diagram of the relative reproductive rate of the crop relative to the weed (RRR_{CW}) less than unity.

At points on the calculated function to the right of the diagonal, the minority component, the weed, is replacing itself relatively more rapidly than the crop. Therefore, the weed replaces the crop until it meets the intersection with the diagonal. Therefore, the equilibrium forces in this situation favor the minority component, and the mixture moves itself to a stable equilibrium mixture at the diagonal intersection point. At that point, the $RRR_{CW} = 1$, and it tends to a stable mixture there. With this interaction is the implication that both species are crowding for "space" that is not the same for both. An example would be: (1) the case where one species develops early in the season, and the other develops later; or (2) one species profits by the presence of the other (e.g., legume–grass association).

The converse situation exists when the calculated function is frequency dependent and the slope is greater than 45° (Figure 15). Then the crop and weed not only crowd for the same "space," but some other, active process is

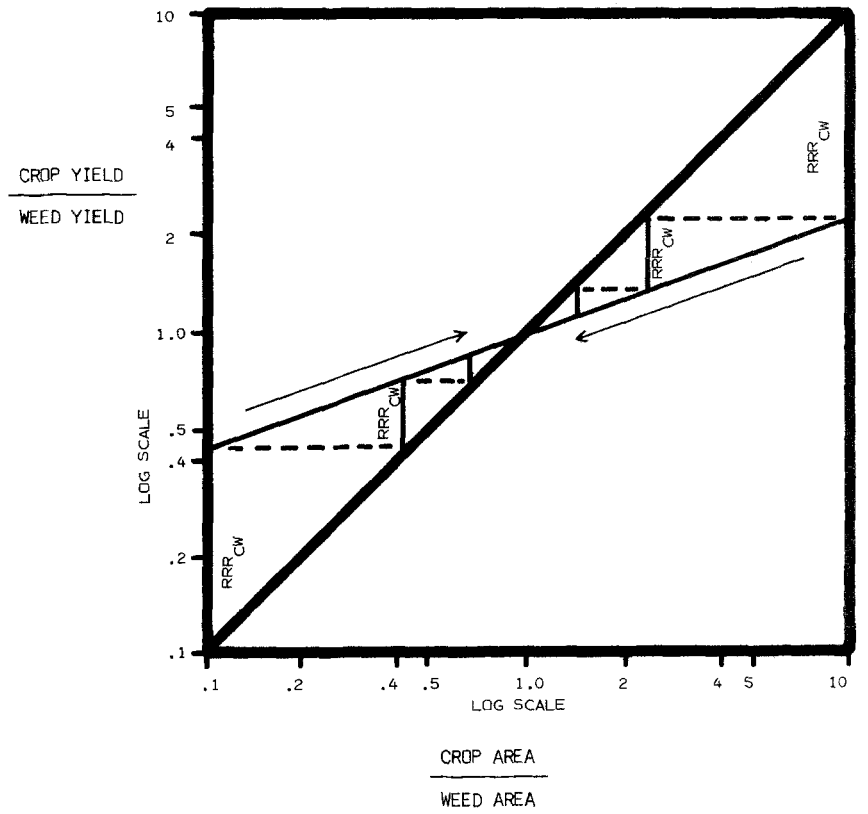

FIG. 14. The ratio diagram of the relative reproductive rate of the crop relative to the weed (RRR_{CW}) with a slope less than 45°

involved. An example would be one species producing a toxin that restricted the growth of one or both members of the mixture, allelopathy. For mixture proportions to the left of the diagonal ($RRR_{CW} > 1$), the relative replacement rate favors the increase of the crop over time with exclusion of the weed. The equilibrium goes in the direction away from the diagonal. This leads to an unstable condition. The mixture proportions to the right of the diagonal ($RRR_{CW} < 1$) tend to the exclusion of the crop over time. The mixture at the intersection in this case is not at equilibrium. The tendency favors one species to be dominant and for the replacement of the other to occur over time. The location of this intersection point will ultimately determine which species will succeed. The species in the proportional majority of the mixture at the intersection point will dominate the other. Because the direction of equilibrium forces are away from the intersection point, the most abundant species will eliminate the other over time. The ratio diagram of soybean and velvetleaf

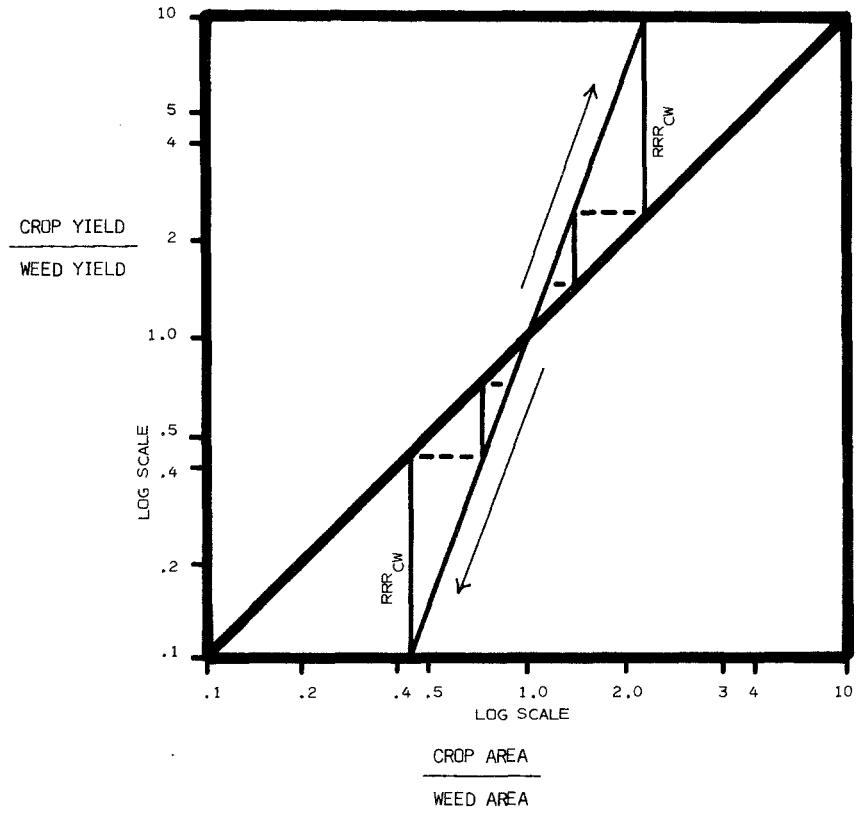

FIG. 15. The ratio diagram of the relative reproductive rate of the crop relative to the weed (RRR_{CW}) with a slope greater than 45°

seed number reinforces field observations of the weed's prolific seed production and its replacement advantage over soybean (Figure 16). There is no support for an allelopathic mechanism of interference to be derived from this diagram beyond the weed's potential to rapidly displace the crop.

Scaling Test. Hill and Shimamoto (1973) also developed a scaling test that provides tests of significance for some aspects of the replacement series diagrams. They aid in assigning responses to the appropriate interaction. Three indices, A, B, and C, must first be obtained for the weed and crop:

A = 2(mean of the 75% crop:25% weed mix) − (mean of the 50% crop:50% weed mix + mean of the crop monoculture)

B = 2(mean of the 25% crop:75% weed mix) − (mean of the 50% crop:50% weed mix + mean of the weed monoculture)

C = 2(mean of the 50% crop:50% weed mix) − (mean of the crop monoculture + mean of the weed monoculture)

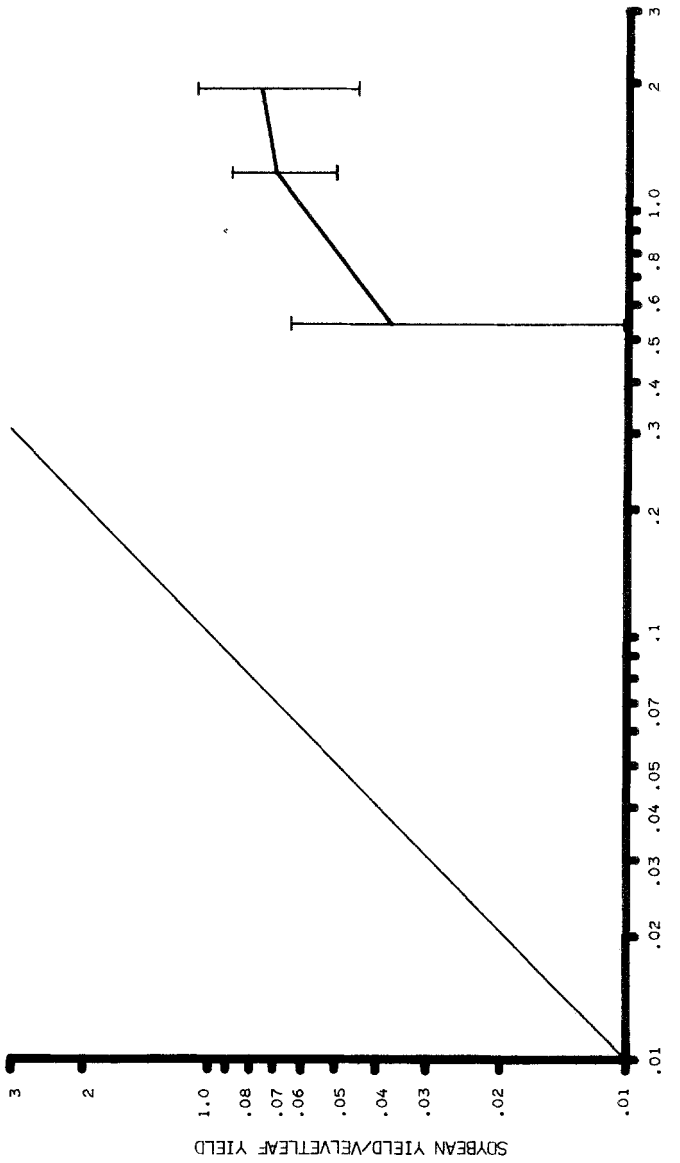

FIG. 16. The ratio diagram of soybean and velvetleaf seed number yield (seeds per meter^2); standard error, $P = 0.05$.

If all the indices (A, B, and C) are equal to zero, within the limits of experimental error, the crop–weed interaction is "mutually exclusive" or of a compensatory type. If C is significant, the interaction is complementary. The A and B indices detect those 75%:25% mixtures whose compounds interact in a way not anticipated from the response of the monoculture and the 50%:50% mixtures. The lack of significance for the soybean–velvetleaf seed yield B and C indices indicates that the interaction is compensatory or mutually exclusive, while the A index indicates the 75% crop–25% weed mixture yielded less than expected from the monocultures or 50%–50% mixture (Table 2). The scaling test has provided another method of presenting data derived from the replacement series.

Regression of Individual Mean on Associate Mean. Another revealing way to express the manner by which associated species condition the response of each other is by regressing the individual mean against the associate mean of the species in a mixture (Hill, 1973). For a given mixture proportion, the mean yield of one species is regressed against the monoculture yield times the proportion in which it appears in the mixture. For example, in the 50% crop:50% weed mixture the regression pairs are: (1) the associate mean is the yield of the 50% crop component of the mixture; and (2) the individual mean is 50% of the crop monoculture mean. When a regression function is defined by these pairs, the relative "aggressiveness" of the two species can be determined. Weak competitors will be characterized by a high individual mean performance and a low associate response (i.e., a steeper slope) (Figure 17). The implication of this is that the more aggressive species in the mixture is able to obtain a disproportionately higher share of the "space" at the expense of the weaker species. Additionally, if the regression coefficient is significant, the implication is that the species is able to compete aggressively over a wide range of mixture proportions. In other words, they have "general competitive ability." Conversely, if the regression coefficient is not significant, each species displays weaker or stronger competitive responses depending on the propor-

TABLE 2. SCALING TESTS FOR SOYBEAN–VELVETLEAF SEED WEIGHT PER AREA (1978)[a]

Scaling test indices	Values (SE)
A	−367 (244)
B	−103 (295)
C	103 (569)

[a]SE = standard error, $P = 0.05$.

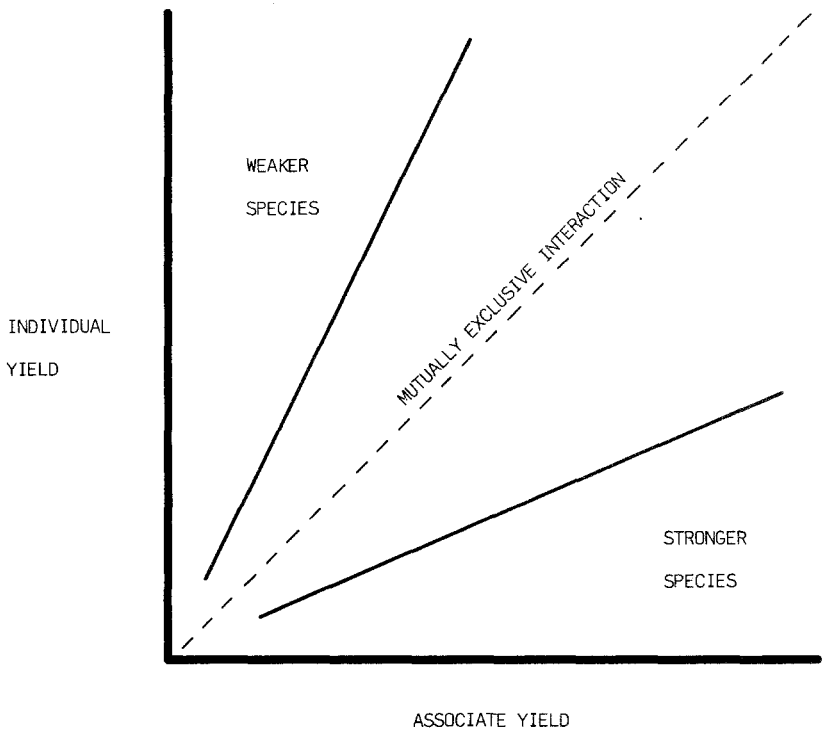

FIG. 17. Linear regression of the individual yield (monoculture) on the associate yield (mixture) to determine a species' relative interference ability.

tions of the mixture. In other words, they have "frequency-dependent competitive ability." If species respond in a mutually exclusive manner, then their regression pairs will lie along the diagonal between the two axes. Velvetleaf accumulated more seed per unit area when associated with soybean than it did when grown alone (Figure 18). Soybean responses were the converse of this situation. The lack of a significant correlation indicates velvetleaf seed yields were dependent upon the mixture proportion in which they appeared: the lower ratio velvetleaf mixtures did better relative to the comparable monoculture response than did the higher ratio velvetleaf mixture. The significant soybean correlation indicates a consistent, although poor, response in all mixtures with velvetleaf.

Individual Plant Yield-Plant Population Functions

Another methodology for experimentally evaluating plant interference is the application of mathematical models first elucidated by Mitscherlich (1919) and refined by several other researchers (Donald, 1951; Hirano and

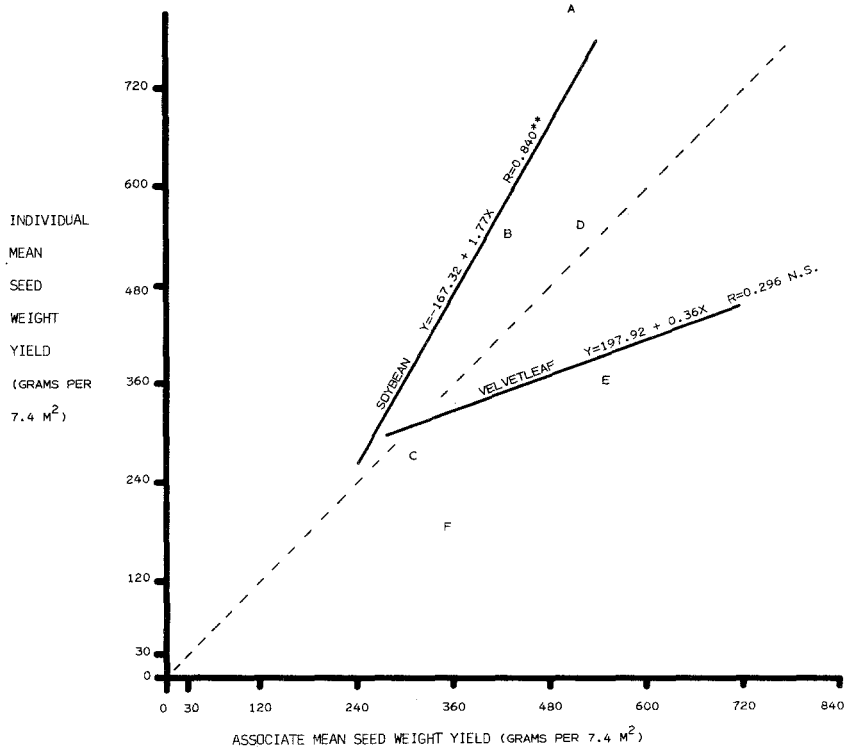

FIG. 18. Linear regression of soybean and velvetleaf seed (grams per 7.4 meter^2) individual yield on the associate yield; A = 75% soybean mixture component; B = 50% soybean mixture component; C = 25% soybean mixture component; D = 75% velvetleaf mixture component; E = 50% velvetleaf mixture component; F = 25% velvetleaf mixture component.

Kira, 1965; Hiroi and Monsi, 1966; Hozumi et al., 1956, 1968; Monsi, 1968; Yoda et al., 1957). Historically this analysis followed Mitscherlich with the statement of the "C–D effect" and the "Y–D effect" (Kira et al., 1953; Koyama and Kira, 1956; Shinozaki and Kira, 1956, 1961) and finally the "−3/2 power law" of Yoda et al. (1963). The original analysis was done in terms of intraspecific interference, but comparison of intra- and interspecific effects reveals much of the interactions of crops and weeds.

The Y–D and C–D Effects. The Y-D effect or yield-density effect indicates that, in pure stands of a species, the total yield per area becomes constant with the passage of time and is independent of density (Figure 19). Thus, $yd^{a-1} = K$ where: y = yield per unit area; d = plant density; K = constant; a = index that increases with time, $a = 0$ at time (+) zero, $a = 1$ at time (+). The Y-D effect is a corollary to the C–D effect, or

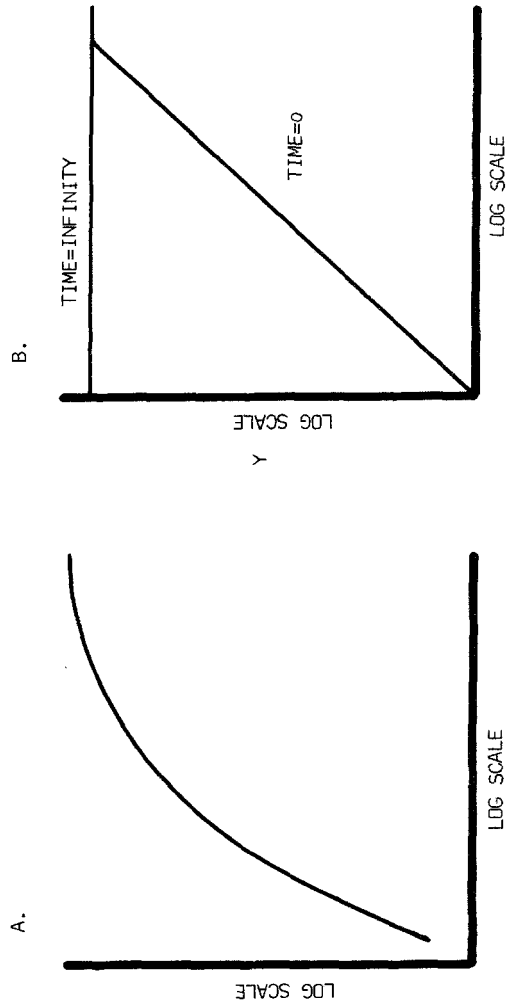

FIG. 19. Graphical representation of the Y-D effect: yield per unit area (Y) versus plant population density (D); A: response measured at maximum plant growth; B: responses measured at times zero and infinity.

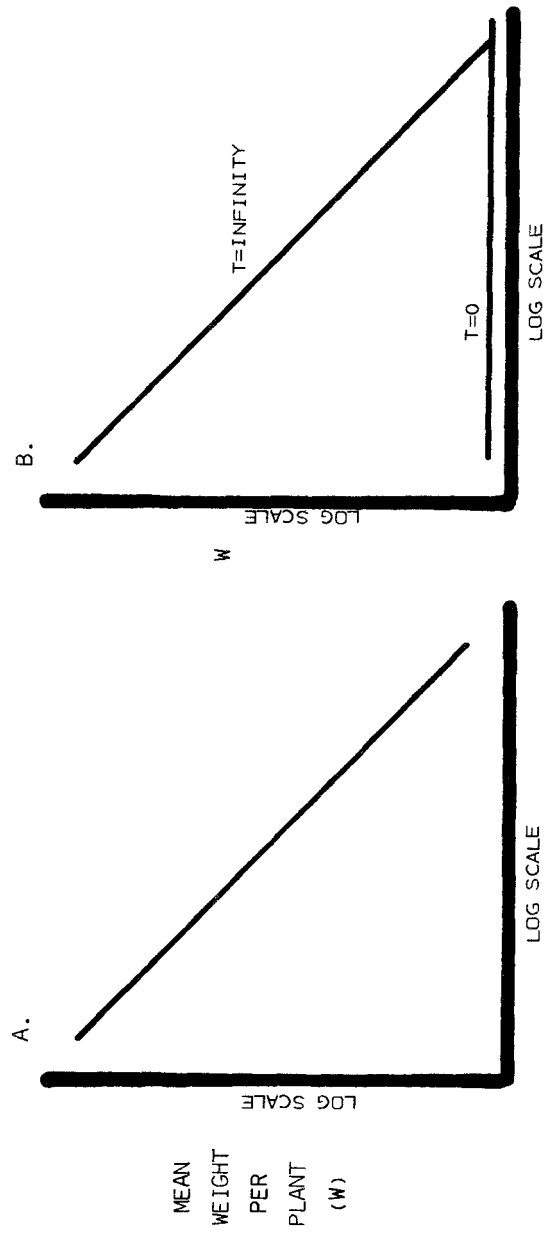

FIG. 20. Graphical representation of the C-D effect: mean weight per plant (W) versus plant population density (D); A: response measured at maximum plant growth; B: responses measured at times zero and infinity.

competition-density effect. The C-D effect states that the product of the mean weight per plant and the density are constant when sufficient time has passed (Figure 20). Thus, $wd^a = K$ where: w = mean weight per plant; d = plant population density; K = constant; and a = gradient of C-D function that increases with time, $a = 0$ at time (+) zero, $a = 1$ at time (+). As time passes, the minus slope increases to about -1, or more. These relationships hold for total plant weight, and for constituent weights: leaf, stem, root, and seed weights.

The −3/2 Power Law of Self-Thinning. Subsequent to the definition of the C-D and Y-D effects, Yoda et al. (1963) investigated overcrowded natural stands of redroot pigweed (*Amaranthus retroflexus* L.), common ragweed (*Ambrosia artemisiifolia* L.), lambsquarters (*Chenopodium album* L.), and horseweed (*Erigeron canadensis* L.) and found a slightly different relationship. What they found was: $w = cd^{-3/2}$ where: w = mean weight per plant; d = plant density; and c = a constant characteristic of the individual species. The slope of the mean plant weight versus the plant density, on log-log scales, was $-3/2$ (Figure 21). Thus the mean weight per plant is always at the

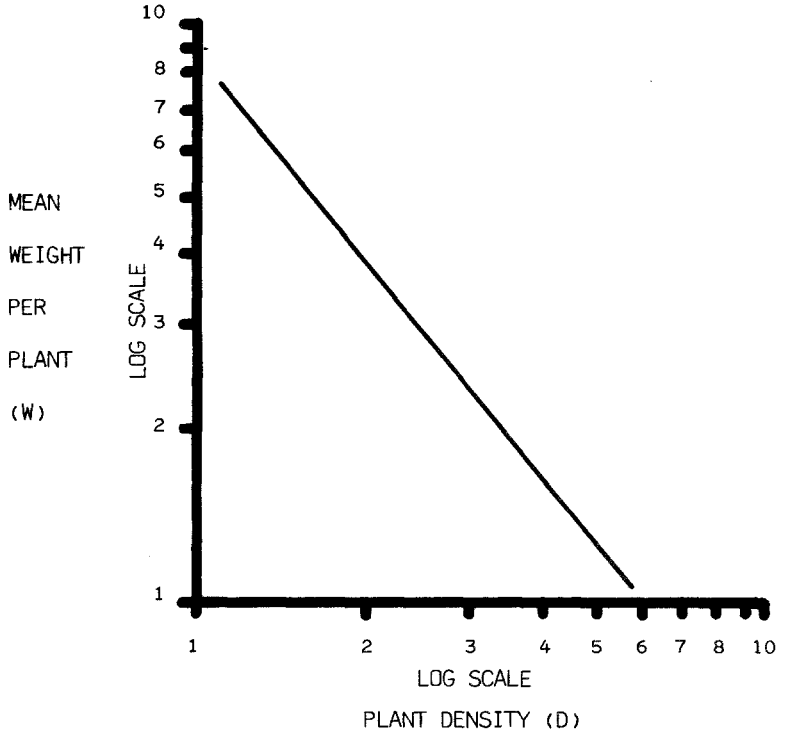

FIG. 21. Graphical representation of the $-3/2$ power law of mean weight per plant (W) versus plant population density (D).

maximum for what the land can support at a given density in overcrowded stands. The applicability of this function has been confirmed by several other researchers (Black, 1960; Bradley et al., 1966; Duff, 1966; Hiroi and Monsi, 1966; Joyce and Gallagher, 1966; Mackensie, 1962; Puckridge and Donald, 1967; White and Harper, 1970). The authors found widespread applicability of this law, but still only said its use as a model "could be accepted as a crude approximation." Its usefulness in determining interactions between crops and weeds is not diminished by this. The relationship describes the effect of both changes in plant number and individual plant size. Both of these factors are revealed in intraspecific density-dependent growth. This "normal" pattern can be compared with the growth patterns revealed in the mixture responses.

Hirano and Kira (1965) did an anlysis of a "normal" plant (persimmon, *Diospyros* spp.) and compared it to a species with known autotoxicity from root exudation [peach, *Prunus persica* (L.) Batsch]. They did not grow the species together but only compared the growth and deviations from the C-D function of the two grown in pure cultures. The basic premise of their analysis is that log–log plotting of plant population density versus the mean plant weight (or its reciprocal) can reveal two types of growth patterns (Figure 22). The assumption of this analysis is that linear functions are "normal" competitive responses, and curvilinear functions indicate allelopathic responses. The decrease in weight per plant at the higher densities indicates a growth reduction due to some factor besides the normal density-dependent growth response defined by the $-3/2$ power law. There is the possibility of other interpretations, but there is a strong inference of allelopathic growth reduction. The comparison of these intraspecific responses to those responses in mixtures could reveal selective allelopathic responses if the scope of the original analysis is extended. When velvetleaf and soybean were compared at three population densities, a decrease in flowering nodes per plant occurred at the higher density for both species in mixtures relative to their response in monocultures (Figure 23).

Sakai Test. Sakai (1955, 1957, 1961) proposed a test that can be modified slightly to allow the analysis of the replacement series data (Figure 24). The mean yield per plant of the crop and weed are plotted on the vertical axis, the relative proportion of crop and weed on the horizontal axis. Comparison of the species' response in pure stands to that in various mixture proportions will reveal the species' competitive ability. Figure 24 indicates that both species respond in pure stands as they do in mixtures. Their relative yield per plant is unchanged by being in the mixture. In Figure 24 both crop and weed yield per plant are reduced by their association in a mixture. This could be a response due to a nonselective allelopathic compound produced by either species. In Figure 24 the individual crop plant yield per plant is decreased, and the weed yield per plant increased, by their mutual association. This could be a compensatory plant interaction. In Figure 24 both crop and weed benefit by

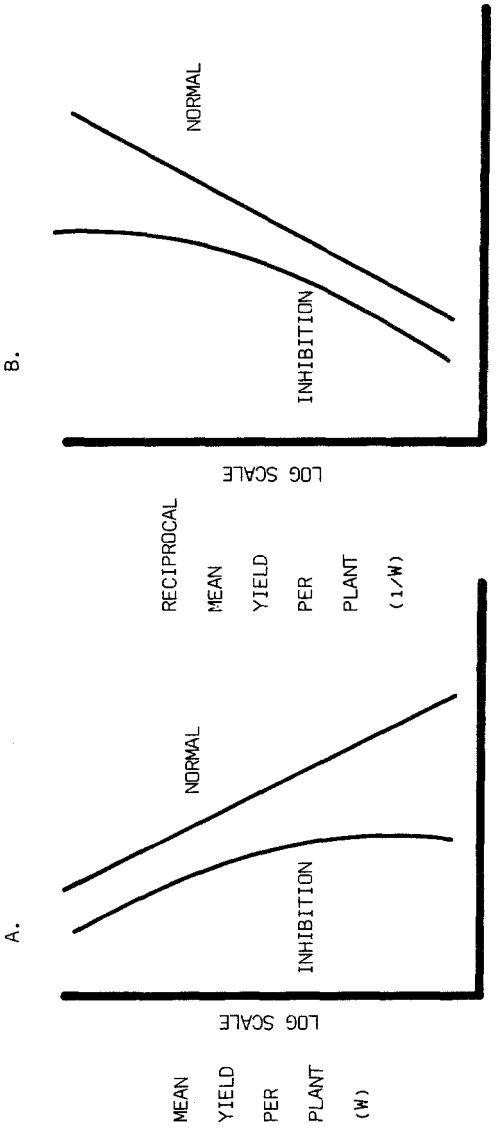

FIG. 22. Normal and inhibited growth patterns revealed by alternate graphical representations of: A: the mean yield per plant (W) versus plant population density (D); and, B: the reciprocal mean yield per plant (1/W) versus plant population density (D).

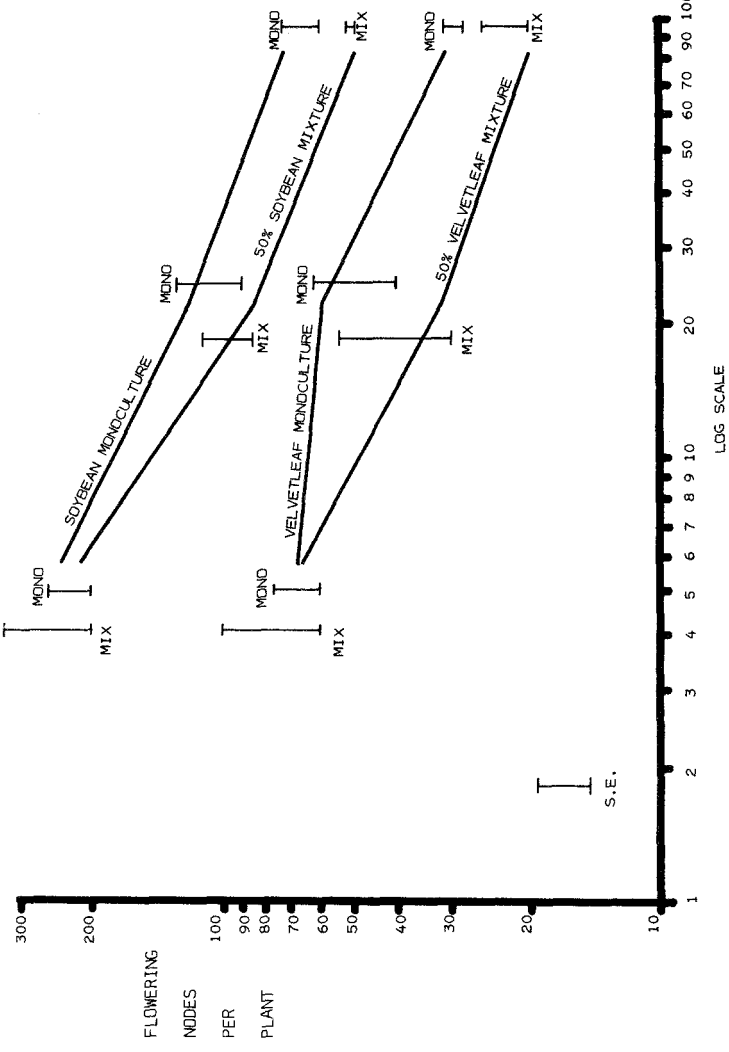

FIG. 23. Comparison of soybean and velvetleaf flowering nodes per plant versus plant population (plants per meter^2) in monoculture (MONO) and in 50% soybean–50% velvetleaf mixtures (MIX); SE, $P = 0.05$.

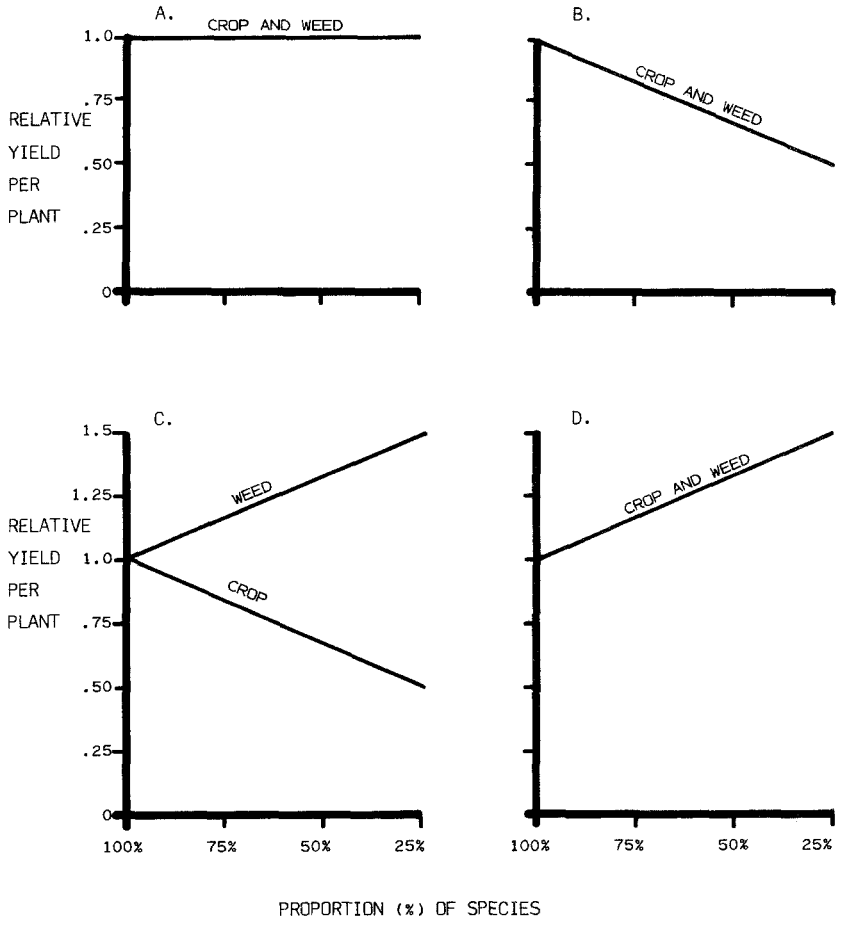

FIG. 24. Models of possible crop–weed interactions expressed by the Sakai test of relative yield per plant versus the proportion (%) of crop or weed; A: mutual exclusion; B: negative complementation; C: compensation; D: positive complementation.

their mutual association. Since they are not competing for exactly the same "space," yield per plant is increased. In all these cases, there is the possibility of a frequency-dependent yield response. In any mixture proportion, one or the other species could dominate, while at other proportions the situation could change. A comparison of flowering nodes per plant at the high population density seen previously (Figure 23) indicates a possible allelopathic mechanism is operative: both species yielded less in this mixture than when grown alone (Figure 25).

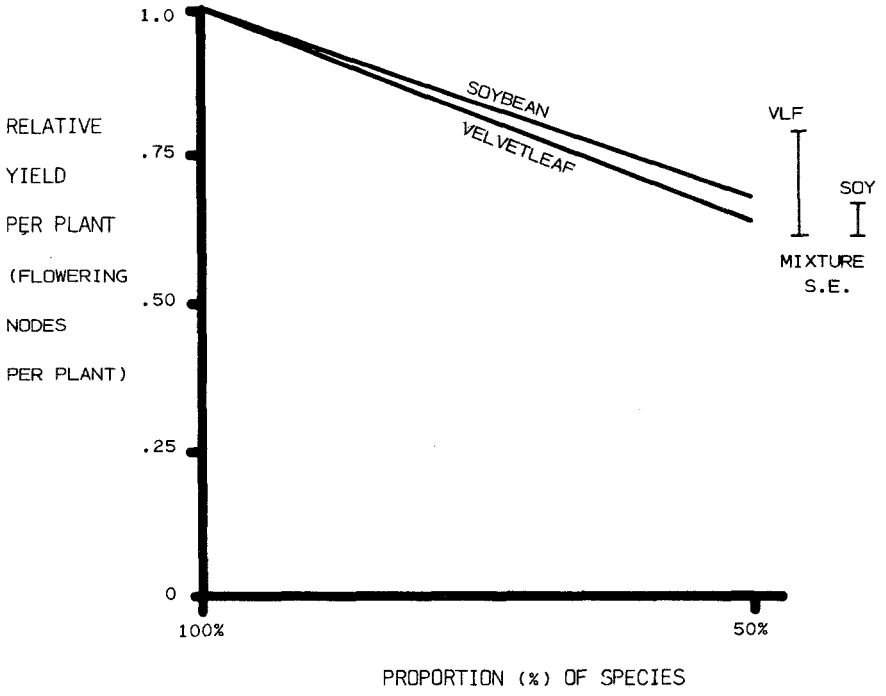

FIG. 25. The Sakai test of soybean (SOY) and velvetleaf (VLF) relative flowering nodes per plant in monoculture and in a 50% soybean–50% velvetleaf mixture when grown at a population density of 83.3 plants per meter^2; SE = standard error, $P = 0.05$.

CONCLUSIONS

The experimental designs presented here will not clearly and unambiguously detect an allelopathic mechanism of plant interference. They will provide information that is critical in the development of evidence to support an hypothesis of an allelopathic interaction. They can provide a quantitative and qualitative description of the interaction between two associated plant species. The empirical base that is established will provide a context for further, narrower, controlled experimentation. There are several statements that can be made from the data derived from these experimental designs. The additive design allowed a quantitative assessment of the effect of one plant on an associated species. The only statement that could be made was that crop losses were severe. This design is therefore unsatisfactory because very little qualitative information about the interaction between species can be inferred. The replacement series design allowed a qualitative assessment of the

interaction between two plant species without the confounding effect of changes in both population density and species proportion in mixtures. The characterization of the the interaction was made possible by the comparison of each species responses in a monoculture to those in a mixture with the other species. This design is more appealing in that all but a very few explanations are eliminated and only a few types of interactions will fit all the known facts. By the measurement of several parameters of both species, growth, population changes, and reproductivity insights can be gained into which plant components best reflect interference between species. Although several researchers have used these methodologies and provided interpretations of specific cases, much of the interpretation offered is theoretical. There exists a need for more plant associations to be characterized with these designs to give the interpretive models vitality and validity.

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WEED CONTROL USING ALLELOPATHIC CROP PLANTS

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Abstract—The concept that some crop plants may be allelopathic to common weeds of agricultural lands is receiving greater attention as an alternative weed control strategy. Several crops showing promise are: grain and forage species such as barley (*Hordeum*), oat (*Avena*), fescue (*Festuca*), and sorghum (*Sorghum*), and the agronomic species of corn (*Zea*) and sunflower (*Helianthus*). Among the problems that hinder the conclusive demonstration of allelopathic effects of crop plants are the loss of that capacity through selection and the variability among cultivars. Recent studies to evaluate the allelopathic potential of crop plants have shown that several sunflower varieties inhibit the germination and growth of associated weeds and to a greater extent than found in several biotypes of native sunflower. Aqueous extracts of dried sunflower and rape tissue inhibited or stimulated germination and growth of weeds, and the response depended upon the source of extract, the extract concentration, and the weed species tested. The validity of bioassay results was tested in a 5-year field study with sunflower and oat grown in rotation. Weed density increased in all plots but the extent of increase was significantly less in plots of sunflower than in control plots. The use of crop plants with increased allelochemical production could limit the need for conventional herbicides to early season application with late season control provided by the crop.

Key Words—Allelopathy, weed control, sunflower, *Helianthus annuus* L.

INTRODUCTION

The present U.S. crop-yield loss due to weeds is estimated to be 10%. New weed control strategies are needed to supplement current chemical, mechanical, cultural, and biological methods. Allelochemicals that inhibit the germination and growth of weed species and are produced and released by growing crop plants or their residues are receiving increased attention for

weed control. Funke (1941) suggested that the exclusive presence of certain weed species in cultivated fields may be the result of selectively inhibitory substances produced by the crop plant.

Recent studies considering the role of allelopathy in weed control have been reviewed by others (Rice, 1974; 1979; Altieri and Doll, 1978; Putnam and Duke, 1978), and all stress the difficulty of conclusively demonstrating allelopathic potential under normal cropping systems. Several crops showing promise in bioassay to inhibit the growth of certain weeds have recently been reported. Putnam and Duke (1974) evaluated 526 accessions of cucumber (*Cucumis sativus* L.) and found that some inhibited proso millet (*Panicum miliaceum* L.) growth by 75%. Lockerman and Putnam (1979, 1981) evaluated the most active cucumber accessions in field trials and found that interference caused by allelopathy and competition reduced weed densities. Massantini et al. (1977) described the effects of 141 soybean (*Glycine max.* L.) lines on *Helminthis echioides* and *Alopecurus myosuroides* and found that two lines inhibited the growth of *H. echioides* but none affected *A. myosuroides* growth. They also found that one soybean line promoted the growth of both weeds. Fay and Duke (1977) evaluated 3000 accessions of *Avena* sp. germ plasm for the production of scopoletin, a chemical identified as an allelopathic agent, and found four accessions which exuded three times the amount of a commercial oat cultivar. Leather (1983) found that several varieties of sunflower (*Helianthus annuus* L.) were more allelopathic to broadleaf weeds than the native wild sunflower described by Wilson and Rice (1968). Other research (Barnes and Putnam, 1982; Lehle and Putnam, 1982) reported the use of cover crops with high allelochemical production for weed control in orchards and no-till cropping system.

Studies are reported here that identify the allelopathic potential of crop plants and their chemical interaction with weeds and weed seeds.

METHODS AND MATERIALS

Plant Materials. Crop plants were grown under field conditions or in the greenhouse with supplemental, full-spectrum metal halide lamps. The plants were harvested at several stages of growth and broadleaf plants separated into leaf and stem tissue, and grass plants separated into leaf and culm tissue. The tissue was dried at 90°C and ground in a Wiley mill.

Seed Germination. Effects on seed germination were evaluated with extracts of dried tissue prepared by shaking 4 g of ground tissue with 100 ml of water for 24 hr at 25°C. The extracts were filtered and 10- and 100-fold dilutions prepared. Control solutions were prepared with mannitol–water adjusted to the osmotic potentials of the extracts. Germination of wild mustard [*Brassica kaber* (DC.) L.C. Wheeler var. *pinnatifida* (Stokes) L.C.

Wheeler] seeds was determined by placing 50 seeds on Whatman No. 3 filter paper wet with 3 ml of the test solution in a closed, 10-cm plastic Petri dish. The seeds were incubated for 10 days at 25° C and 97% relative humidity with 8 hr of light. Germination was scored on day 3 and day 10. Each experiment consisted of three concentrations of each tissue extract and the control treatments, each replicated six times in a completely randomized design.

Weed Seedling Growth. Seeds of velvetleaf (*Abutilon theophrasti* Medic.), jimsonweed (*Datura stramonium* L.), tall morning glory [*Ipomoea purpurea* (L.) Roth], and wild mustard were germinated in vermiculite in the greenhouse under a 14-hr photoperiod provided by supplemental metal halide lighting. When the seedlings were 5 cm tall, they were transplanted to styrofoam pots containing 670 g of quartz sand. Two grams of the dried tissue from the test plant were placed on the sand surface and leached daily by surface irrigation with one-half strength nutrient solution (Hoagland and Arnon, 1950). Shoots were harvested after 21 days by cutting at the root–shoot transition zone, dried, and weighed. Experiments consisted of a species of weed and crop plant replicated six times in a completely randomized design.

Crop Root Exudation. Crop plants were grown under greenhouse conditions as above in 4-liter ceramic pots filled with quartz sand. The sand was flushed for 1 min every 4 hr with half-strength Hoagland and Arnon's solution by recirculation from a 4-liter holding tank. The holding tank volume was maintained at 3 liters by daily addition of fresh solution. Prior to volume adjustment, 0.5 liter of the solution was removed and used to irrigate weed seedlings or as a seed germination medium.

Field Experiments. Crop varieties were evaluated over several years in plots arranged in a randomized complete block design with four replications established the first year. Control plots were open, natural weed populations without crop plants. Weed surveys were made by tabulating the density and percent cover of each species according to the method of Goldsmith and Harrison (1976) in four 0.1-m^2 quadrats on a transect at the midpoint of each plot.

RESULTS AND DISCUSSION

Seed germination with tissue extracts was variable and depended upon the extract source and concentration. Table 1 shows that wild mustard seed germination was inhibited by undiluted aqueous leaf extracts of two varieties of sunflower and native sunflower, but was stimulated by more dilute extracts from the same sources. Stem tissue extracts stimulated germination at all levels of dilution. The altered germination response over the range of concentrations suggests that the major chemical constituents extracted from

TABLE 1. TEN-DAY GERMINATION RESPONSE OF WILD MUSTARD SEED TO THREE CONCENTRATIONS OF EXTRACTS FROM LEAF AND STEM TISSUE OF TWO SUNFLOWER HYBRIDS AND NATIVE SUNFLOWER

Tissue source	Germination as % of control[a]					
	Undiluted		1/10 Dilution		1/100 Dilution	
	Leaf	Stem	Leaf	Stem	Leaf	Stem
Hybrid 201	22*	108	134*	144*	165*	173*
Hybrid 8941	27*	168*	139*	153*	77	125
Native	64*	132*	139*	157*	120	112

[a]Asterisk(*) indicates significant differences from the control at the 5% level by the Duncan's multiple-range test.

the tissue were growth regulators. Growth of the radicle was not affected after emergence.

Seedling growth of velvetleaf, jimsonweed, morning glory, and wild mustard was inhibited by leachates of Hybrid 201 sunflower leaf and stem tissue (Table 2). Root exudates inhibited velvetleaf and jimsonweed growth but stimulated the growth of morning glory as determined by the dry weight after 21 days (Table 2). Rape (*Brassica napus* L.) tissue leachates inhibited the growth of velvetleaf and morning glory but did not affect wild mustard (Table 3). As was shown in the germination tests, inhibition of seedling growth depended upon weed species and source of allelochemicals. However, no correlation was observed between stimulation or inhibition of seed germination and the growth of seedlings.

TABLE 2. GROWTH RESPONSE OF FOUR WEED SPECIES TO ROOT EXUDATES AND LEACHATES OF LEAF TISSUE AND STEM TISSUE FROM SUNFLOWER HYBRID 201[a]

Weed	Dry wt as % of control[b]		
	Leaf tissue	Stem tissue	Root exudate
Velvetleaf	63*	42*	42*
Jimsonweed	74*	63*	58*
Morning glory	78	50*	180*
Wild mustard	93	55*	

[a]Results based on dry weight after 21 days of treatment.
[b]Asterisk (*) indicates significant differences from the control at the 5% level by the Duncan's multiple-range test.

TABLE 3. GROWTH RESPONSE OF THREE WEED SPECIES TO LEACHATES OF DRIED RAPE PLANTS

Treatment	Dry weight (g)[a]		
	Velvetleaf	Morning glory	Wild mustard
Rape	1.09a	1.29a	3.19a
Control	1.37b	2.01b	2.81a

[a]Values in each column not followed by the same letter are significantly different at the 5% level by the Duncan's multiple-range test.

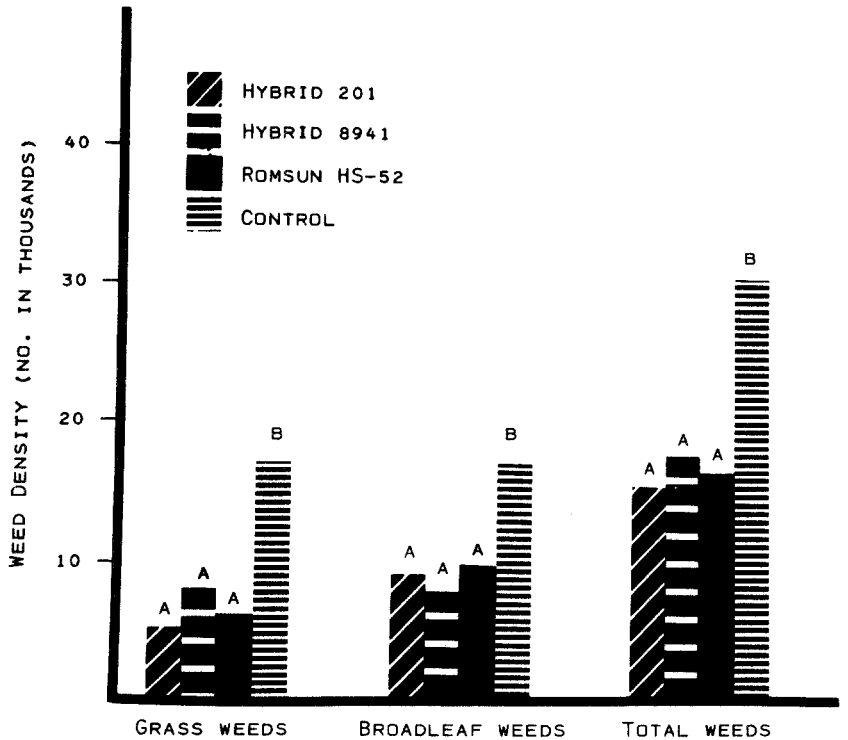

FIG. 1. Average weed density over five growing seasons in field plots of three hybrids of sunflower in rotation with oat. Vertical bars within each group having the same letter are not significantly different at the 5% level by the Duncan's multiple-range test. (Adapted from Leather, 1983.)

Average density of natural weed populations in cultivated plots increased over a 5-year sampling period. Weed density in plots with a sunflower–oat (*Avena sativa* L. cv 'Garry') rotation increased, but the rate of increase was less than in the natural populations. Figure 1 (adapted from Leather, 1983) shows the average weed density over a 5-year span in field plots with three sunflower hybrids rotated with oat. The inhibition of weed density increase in sunflower plots was probably not the result of decreased nitrogen fixation as suggested by Rice (1979) because the plots were fertilized each year according to recommendations as a result of soil analysis. Other factors, including direct allelochemical action on the germination and growth of weeds, appear to be responsible for the inhibition recorded.

The data presented indicate that variable results are obtained in bioassays for allelopathic activity; that responses are concentration dependent; and that responses to applied extracts or exudates are species specific. Field studies do not demonstrate conclusive allelopathic inhibition of weeds by crop plants but, as suggested by Putnam and Duke (1978), the results obtained show that the total crop effect is that of interfering with weed growth.

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ALLELOPATHIC INTERACTIONS IN CROP-WEED MIXTURES: Applications for Weed Management[1]

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Abstract—A very important component of the aggressive nature of weeds is allelopathic interference, the full potential of which is just being realized in the management of agroecosystems. Research results are presented which demonstrate the allelopathic interactions involved in a wide range of crop-weed combinations occurring in a great variety of habitats. This includes crops planted in weed control, crops with allelopathic potential, and noncrop plants of beneficial use for weed control as a result of allelopathic interference. Allelopathy can play a beneficial role in multiple cropping systems, crop rotations, and cover cropping. The potential role for allelopathic interactions in the design of biological weed control is proposed.

Key Words—Agroecosystems, allelopathy, weeds, biological control, multiple cropping, living mulch, interference, phytotoxins.

INTRODUCTION

Many studies have been done which demonstrate the allelopathic nature of the effects of weeds on crop growth and development (Tukey, 1969; Putnam and Duke, 1978; Rice, 1974, 1979). In such studies considerable attention has been given to the role of allelopathic interactions between different crop plants or the inhibitory effects of phytotoxins produced by weeds on crops. Relatively little research, on the other hand, has focused on the

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important role allelopathy might play in weed management. By thoroughly understanding the complexity of crop–weed interactions in different agroecosystems, allelopathy might effectively be combined with an array of weed management practices.

In order to utilize allelopathic interference successfully in weed management, it is necessary to find naturally occurring chemical compounds which inhibit seed germination, plant growth, or prevent propagule or fruit production. Such compounds can be useful in many ways, one of the best known is through the manipulation of the crops themselves in an agricultural system. It has been proposed for several years that a crop could be used to "smother" weeds (Overland, 1966). The planting of "smother crops" was often employed in the past to suppress the growth of weeds, although it was most often assumed that such crops inhibited weed growth through competition. Overland (1966) demonstrated that living plants and roots of barley (*Hordeum vulgare*) actively produced and released toxic quantities of organic compounds capable of inhibiting growth of several common crop weeds. Where crop lines have been studied that have coevolved with interference from other plant species, especially weeds, it appears that some are potentially capable of chemical inhibition. Putnam and Duke (1974) screened a large germ plasm of *Cucumis sativus* and related *Cucumis* species and found several which demonstrated allelopathic activity at least in sand culture. Under certain field conditions some cucumber accessions inhibited the growth of several weed species (Lockerman and Putnam, 1979). Fay and Duke (1977) assayed a large number of accessions of *Avena sativa* and found some very strong lines for inhibiting weeds through the active exudation of a mixture of compounds. The importance of crop inhibition of weeds has also been suggested by Bell and Koeppe (1972) and has been very actively studied by several Russian scientists with a wide array of crop–weed mixtures (reviewed in A.M. Grodzinsky, 1971–1977).

Noncrop plants, either associated with the crop species or planted in rotational sequence with them, offer possibilites for allelopathic weed control. By producing toxins effective against weeds, but not harmful for the crop, the association of such plants can be manipulated to considerable advantage. Research at Cornell University aimed at the development of "living mulches" for use with vegetable crops is a good example (Anonymous, 1981). A study of the crop–weed combination of *Linum usitatissimum* (flax) and the weedy *Camelina sativa* showed that the crop plant growth was stimulated by the presence of the weed and, on the other hand, apparently the weed also inhibitied the invasion of other weeds through a combination of allelopathic and competitive interference (Lovett and Duffield, 1981). In our studies, we have focused on different aspects of crop–weed interactions, concentrating on those cases where allelopathy might play an important role.

METHODS AND MATERIALS

In a series of experiments aimed at demonstrating the potential for allelopathic interactions in crop–weed mixtures, bioassays of aqueous extracts of fresh or air-dried plant material were employed. Crop, noncrop, and weed species were tested.

Experiment 1. The interplanting of squash (*Cucurbita pepo*) by peasants in corn–bean polycultures or in monoculture stands during the proper season in southeastern Mexico aids in weed control (Chacon and Gliessman, 1982; Gliessman et al., 1981; Yih et al. 1982). Mature leaves of interplanted squash plants were collected from a farmer's field near Cardenas, Tabasco, Mexico, in August 1979, air-dried for several days, and then 100 g of intact leaves soaked for 2 hr in distilled water. The extract was then filtered through Whatman No. 4 filter paper and the filtrate used to irrigate a seed bed composed of 50 g of quartz sand that had been screened between two sieves with 0.5- and 1.0-mm openings. The sand was washed 10 times with distilled water before being dried at 105° C for 24 hr. The sand was placed in 15 × 100-mm glass Petri dishes covered with one sheet of Whatman No. 1 filter paper (9.0 cm diameter) before being irrigated with 15 ml of the extract. Ten seeds were planted in a circular fashion around the dish at approximately 1 cm from the edge and covered with another sheet of filter paper. Seed of local varieties of corn (*Zea mays*) and beans (*Vigna sinensis*), as well as a locally available commercial variety of cabbage (*Brassica oleracea*) were used. The dish was then sealed with parafilm and stored in the dark at 26° C (±1° C) for 72 hr. After that period, percent germination and initial radicle elongation were measured. Controls were run simultaneously in distilled water.

Experiment 2. Several leguminous noncrop species are intercropped with corn in Tabasco, Mexico (Gliessman and Garcia, 1979; Gliessman et al., 1981). In a field study carried out during the wet season (June–October 1978) near Cardenas, Tabasco, two legumes were cover-cropped in rotation with corn in an area that had been used periodically for seasonal corn production for several years previously, but currently was suffering from the invasion of aggressive weed species. The 5 × 5-m plots were planted using local practices with either a velvet bean (*Stizolobium deeringianum*) or sword bean (*Canavalia ensiformis*). A control was planted with corn. After 4 months, weed species were sampled in 50 × 50-cm subplots placed in the center of the 5 × 5-m test plot, with all above-ground material separated into number of species, individuals, and dried at 70° C for 48 hr before weighing. There were five repetitions for each treatment.

To test for allelopathic potential, water extracts of fresh green leaf material were prepared in the same manner as for experiment 1 and tested against the same three indicator species.

Experiment 3. In a study of traditional agroecosystems in tropical Mexico, we found that local farmers have developed an intricate classification scheme for noncrop plants that in modern agricultural systems would probably be classified as weeds (Chacon, 1978; Chacon and Gliessman, 1982). Many of these species are considered to be beneficial for the cropping system and are thus left in the field, whereas others are harmful and removed. In an attempt to see if any correlation existed between classification and allelopathic potential, plant material was collected from farmers' fields near Cupilco, Tabasco, Mexico, during the dry season (March–May 1978), air-dried, and tested in the same manner as Experiment 1.

Experiment 4. As part of a project to assess the allelopathic potential of common weed species normally associated with local cropping systems, bioassays of a wide range of species are being performed at UC Santa Cruz. Above-ground plant material of weeds was collected at the UC Farm Project during the fall of 1981, air-dried, and then 100 g soaked in 1000 ml of distilled water for 2 hr before filtering with Whatman No. 4 filter paper. Four of the most common weeds (*Plantago lanceolata*, *Chenopodium album*, *Spergula arvensis*, and *Rhaphanus sativa*) were tested against a series of local crop types (see Table 5 for varieties). The bioassay method was the same as for experiment 1, except a commerically available quartz sand (30 mesh) was used directly in the Petri dishes, and only 12 ml of extract were used for irrigation.

RESULTS AND DISCUSSION

Crop Specifically Planted for Weed Control. Bioassay of the aqueous extracts of squash plants indicated that leaf extracts definitely possess potential for allelopathic dominance (Table 1). Although the indicator species in experiment 1 are crop species themselves, the potential for inhibition is visible in the dramatic reduction in radicle elongation displayed by cabbage

TABLE 1. ALLELOPATHIC POTENTIAL OF WATER EXTRACTS OF AIR-DRIED SQUASH LEAVES (*Cucurbita pepo*) IN LABORATORY BIOASSAYS, TABASCO, MEXICO

	Germination (%)	Radicle length (%)
Corn (*Zea mays*)	100	85.4[a]
Beans (*Vigna sinensis*)	96	79.0[b]
Cabbage (*Brassica oleracea*)	92	43.0[b]
Control	100	100.00

[a] T test significant at 5%.
[b] T test significant at 1%.

TABLE 2. EFFECT OF COVER CROPPING WITH TWO LEGUMES ON WEED DRY WEIGHT (g/m^2) AND SPECIES DIVERSITY IN A CORN PLANTING IN CARDENAS, TABASCO, MEXICO[a]

	Weeds (m^2)		
	No. individuals	No. species	Dry weight (g)
Legume species			
Canavalia ensiformis	76	11	380
Stizolobium deerengianum	57	11	285
Control	177	12	885

[a]Data from Torres Tirado, thesis in prepartion. Figures are the average of five 50 × 50-cm samples taken at random in 5 × 5-m test plots.

(only 43% of controls), whereas both corn and beans were only slightly affected. This would be expected from the two crop species with which squash is normally interplanted. Local farmers describe the use of squash for weed control; in fact, they admitted to planting it more for that purpose, and any harvest of fruit was merely an added bonus (Chacon and Gliessman, 1982). The squash can form a continuous plant cover over the low-lying weedy species, eliminating them as well as restoring productivity to the soil by adding large quantities of biomass (8–10 tons dry matter per hectare) (Amador and Gliessman, 1982).

Noncrop Plants Managed for Weed Control. Both *Stizolobium* and *Canavalia* demonstrated significant ability to reduce both weed numbers and biomass when planted as cover-crop species (Table 2). In some local communities in Tabasco, Mexico, such legumes are planted in rotation with corn and are effectively used to shorten the fallow from four to five years to only six months (Gliessman and Garcia, 1979). *Stizolobium* has been used in rotation with corn on some plots of soil continuously for 20 years, maintaining yields at close to 3.0 tons per hectare without the need for additional fertilizer applications or weedings other than the initial ground preparation with machete at the time of the corn planting. Again, using seeds of crop species as indicators due to their ease of collection and uniformity of germination, the potential for allelopathy can be seen as being at least one of the components of dominance of the legumes over associated weeds (Table 3). The fact that both legumes severely inhibited germination and growth of cabbage seeds, and not as significantly those of corn and beans, again points to the possibility of tolerance in the crop components which have coevolved through time with other plants present. Cabbage, a relatively recent introduction to new world agricultural systems, displays much less tolerance. *Canavalia*, although managed in rotation with corn also, because of its bushy

TABLE 3. BIOASSAY RESULTS OF WATER EXTRACT OF FRESH LEAF MATERIAL OF TWO LEGUMES TESTED WITH SEED OF THREE CROP SPECIES, TABASCO, MEXICO[a]

	Test species					
	Corn (*Zea mays*)		Bean (*Vigna sinesis*)		Cabbage (*Brassica oleracea*)	
Extract	Germination	Growth	Germination	Growth	Germination	*Growth*
Canavalia ensiformis	98.9	77.6**	100.0	66.3**	49.3**	35.6**
Stizolobium deerengianum	100.0	87.9*	98.7	92.9	46.7**	27.2**
Distilled water control	100.0	100.0	100.0	100.0	100.0	100.0

[a] Numbers represent the means of ten seeds repeated three times ($N = 30$). Seeds measured 72 hr following planting. Germination a simple percentage, with growth being the measured length of the main initial radicle, expressed as a percentage of control values. *T test significant at 5%; **T test significant at 1%.

rather than vining habit, can be associated as an intercropped species, thus aiding directly in weed control during the cropping season.

Weed Population Manipultaion for Weed Control. Although possible beneficial aspects of weeds in agriculture have been recognized for some time (USDA, 1938; Cocannour, 1950), only recently has research been focused on the various roles they might play in cropping systems (Cox and Atkins, 1979; Altieri, 1981). This includes soil protection and improvement, changes in populations of insects or soil-borne microorganisms, or providing some use for humans or their domesticated animals. The potential for one weed interacting with other weeds to the benefit of a crop plant, is yet another possibility.

Bioassays of a series of such noncrop species, which are classified according to a much more utilitarian "nonweed" approach, show that many of these plants possess a strong allelopathic potential (Table 4). A species such as *Lagascea mollis* is often cited by local farmers for its ability to control noxious weeds as well as to serve as a feed for animals. It displays no significant allelopathic potential and is classified as a good plant and left in cropping systems when encountered. *Guazuma ulmifolia*, a shrub encountered in early stages of secondary succession, actually shows stimulatory effects on corn. It is kept cut several centimeters above the ground, rather than eliminated, because of its use as an animal feed and the belief that it improves the soil and controls other weeds. On the other hand, a good plant like *Cleome spinosa* displays very strong allelopathic potential but is only found as widely scattered individuals in most cropping systems and has a widely accepted use

TABLE 4. PERCENT RADICLE LENGTH OF SEEDS 72 HOURS AFTER PLANTING IN WATER EXTRACTS (10%) OF COMMON WEEDS CLASSIFIED AS GOOD (G), BAD (B), OR BOTH (G/B) BY LOCAL FARMERS IN TABASCO, MEXICO[a]

Weed species	Classification	Radicle length (%)		
		Corn	Beans	Cabbage
Lagascea mollis	G	89*	93*	19
Guazuma ulmifolia	G	116	104*	19
Cleome spinosa	G	35	12	0
Sida acuta	G	64	45	3
Euphorbia sp.	G	60	61	6
Blechum sp.	B	83	72	0
Paspalum conjugatum	B	89	82	22
Melanthera aspera	B	62	58	0
Sida rhombifolia	B	27	77	48
Priva lapperlaceae	B	25	75	5
Panicum fasciculatum	B	85	90	67
Paspalum sp.	G/B	75	83	61
Leersia sp.	G/B	75	87	25
Control (distilled H_2O)		100	100	100

[a] $N = 30$. All values are significantly different from controls at least at 5% level except those with asterisks.

as a medicinal plant. It would therefore be tolerated in the cropping system despite allelopathic potential. Some of those plants classified as bad by the farmer, such as *Paspalum conjugatum*, display rather limited allelopathic potential, but reference is made to how they compact the soil and cause the yellowing of associated crops. Those plants classified as both good and bad could express allelopathic potential and thus be considered harmful for the crop but good because of some other property, such as for medicinal uses (e.g., *Leersia* sp). Among the complex set of species interactions, therefore, it is proposed that allelopathy must be considered as one possible part.

In our studies of common weed species associated with local cropping systems in Santa Cruz, California (Table 5), we are finding considerable variability in both allelopathic potential of the weeds and in susceptibility of the crop species to inhibition. Considerably more research is necessary in order to establish the basis for manipulating the weed populations themselves for better weed management. Studies comparing competitive and allelopathic components of interference between crops and weeds will allow the establishment of the tolerance levels of the different weed species for each particular

TABLE 5. PERCENT RADICLE LENGTH OF GERMINATED SEEDS 72 HOURS AFTER PLANTING IN EXTRACTS (10%) OF SEVERAL OF MORE COMMON WEEDS IN VEGETABLE CROPS IN SANTA CRUZ COUNTY, CALIFORNIA[a]

	Plantago lanceolata	*Chenopodium album*	*Spergula arvensis*	*Rhaphanus sativa*
Sweet corn (Golden Bantam)	57.5	35.3	64.1	72.4
Radish (White 252)	42.3	4.9	19.1	47.4
Lettuce (Great Lakes 118)	0	0	0	21.2
Alfalfa (Iroquois 513)	25.9	0	0	61.6
Oats (Johnny's 506)	16.8	3.1	3.0	6.8

[a] $N = 30$ for corn and oats. $N = 45$ for the others. Controls (100% response) planted in distilled water. All means significantly different from controls at 1% level.

crop type. When a crop and its accompanying weed species are considered as integral parts of the same agroecosystem, as we have observed in those agroecosystems where noncrop plants are actually classified and managed, it becomes increasingly important to understand the complexities of the relationships between the component plant parts and the environment. Studies on the mechanisms of biotic interference between the crop and noncrop components, especially through allelopathic interactions, will become more important as the economic and ecological limitations on weed control practices used currently in modern agriculture become more restrictive. Allelopathy offers a potential alternative.

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EXPLOITATION OF ALLELOPATHY FOR WEED CONTROL IN ANNUAL AND PERENNIAL CROPPING SYSTEMS[1]

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Abstract—A variety of crops, cultivars, and accessions have been evaluated over the past six years for superior capability to suppress weed growth. The most successful of these approaches has been to grow cover crops of rye (*Secale cereale*), wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*), or barley (*Hordeum vulgare*) to a height of 40–50 cm, desiccate the crops by contact herbicides or freezing, and allow their residues to remain on the soil surface. Often, up to 95% control of important agroecosystem weed species was obtained for a 30- to 60-day period following desiccation of the cover crop. The plant residues on the soil surface exhibit numerous physical and chemical attributes that contribute to weed suppression. Physical aspects include shading and reduced soil temperatures which were similarly achieved using poplar (*Populus*) excelsior as a control mulch. Chemical aspects apparently include direct release of toxins, as well as production of phytotoxic microbial products. Numerous chemicals appear to work in concert or in an additive or synergistic manner to reduce weed germination and growth.

Key Words—Rye, wheat, barley, sorghum, organic acids, no-tillage, crop residues.

INTRODUCTION

Allelopathy appears to be an important component of plant interference capability in a variety of natural ecosystems (Muller, 1966; Rice, 1974; Swain, 1977; Whittaker and Feeny, 1971). Rice (1974, 1979) indicates that allelopathy may contribute to patterning densities and the distribution of species which in specific instances can severely limit diversity of the plant community. Several

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pioneer and stage 2 weed species are also implicated in allelopathic responses against crops in a variety of agroecosystems (Kommedahl et al., 1959; Welbank, 1963; Gressel and Holm, 1964; Friedman and Horowitz, 1971; Bell and Koeppe, 1972). In the last decade, it has been hypothesized that allelopathic characteristics might be exploited for weed control purposes in a variety of agricultural settings (Putnam and Duke, 1974; 1978; Fay and Duke, 1977; Altieri and Doll, 1978).

One approach for utilization of allelopathic plants is to select and later genetically manipulate crops for their ability to suppress weeds by exudation of compounds in their immediate vicinity. This effort has been limited only to selection to date and has provided only marginal successes (Fay and Duke, 1977; Lockerman and Putnam, 1979, 1981). A second approach is to utilize a companion or rotational crop which may not be harvested in a cropping sequence, but may provide toxicity to weeds upon decay of its residues. The toxicity of plant residues has been widely studied, primarily because of adverse effects on certain rotational crops (Guenzi and McCalla, 1962; McCalla and Haskins, 1964; Guenzi et al., 1967; Patrick, 1971). These studies focused primarily on mature straw or stubble of cereal grains which release a variety of aliphatic and phenolic acids upon their breakdown.

Recent work has indicated that 30- to 40-day-old plants of sorghum (*Sorghum bicolor* (L.) Moench), rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and oats (*Avena sativa* L.) can provide excellent weed suppression after desiccation by freezing or with contact herbicides (DeFrank and Putnam, 1978; Putnam and DeFrank, 1979, 1982; Barnes and Putnam, 1981; Lehle and Putnam, 1982b). Work with sorghum indicated that organic acids are at least partially responsible for the inhibition of weed growth (Lehle and Putnam, 1982a), although other active fractions still remain to be identified. Several larger seeded crops, particularly legumes, grew vigorously in these residues, while other smaller seeded vegetable crops were severely damaged (DeFrank and Putnam, 1982). Perennial tree crops manifested no damage and sometimes showed improved growth in the presence of cereal grain residues (Putnam and DeFrank, 1979). This cultural system provides additional advantages in conservation of soil, water, and energy.

The objectives of this paper are to describe the approaches and magnitudes of success using allelopathic crops in a variety of agroecosystems. Additional papers by my colleagues will focus on specific studies, with particular emphasis on elucidation of the allelopathic mechanisms involved.

METHODS AND MATERIALS

Field Experiments. A series of field experiments were conducted over a six-year period to assess the influence of cover crop residues on emergence and

growth of annual weeds and selected annual crops. The cover crops were sown in May or September with a grain drill or Moore Uni-Drill (specialized for no-tillage) in beds 15.2 m long and 2.44 or 3.05 m wide with rows spaced 17.5 or 22.5 cm apart, respectively. The cover crops were planted in a randomized complete block design with four blocks. The cereals were sown at approximately 134 kg/hectare and sorghum or sudangrass at 84 kg/hectare. In all experiments, control plots which were not sown to cover crops were included. Two sets of controls were included in several experiments to allow assessment of conventional tillage vs. no-tillage in the absence of cover crop residues. The cover crops which did not winter kill were sprayed with paraquat (1,1-dimethyl-4,4'-bipyridinium ion) or glyphosate [N-(phosphonomethyl) glycine] at 1.1 kg/hectare just prior to seeding the subsequent crop. The spray boom was equipped with flat fan nozzles that delivered 335 liters/hectare.

Assessments of weed and crop emergence were obtained either 30 days after spraying or 30 days after sowing the indicator seeds in other experiments. Plant densities were estimated by recording emergence by species on two quadrates of 1.0 m^2 in each plot. Approximately 60 days after seeding, and coincidental with crop harvesting, weeds were also harvested from 1.0-m^2 quadrates and dried for weight determinations. The crop yield assessments were based on single destructive harvests.

In our orchard experiments, sorghum (*Sorghum bicolor*), sudangrass (*Sorghum sudanense* (Piper) Stapf.), wheat (*Triticum aestivum*), oats (*Avena sativa*), barley (Hordeum vulgare), and rye (*Secale cereale*) were evaluated. Peat moss was included as an organic mulch control. Additional orchard experiments were conducted in which straw of selected species was placed under 7-year-old Montmorency cherry (*Prunus cerasus* L.) and McIntosh apple (*Malus sylvestris* Mill.) trees at rates equivalent to 4450 kg/hectare to ascertain their influence on weed populations and tree growth. Terminal growth measurements were obtained from 10 lateral branches per tree and trunk measurements were obtained annually. Plots contained two or three trees per replicate and treatments were replicated four to six times.

Greenhouse Experiments. Two cultivars of rye, MSU-13, screened from a portion of the world's collection, and Wheeler rye, a standard cultivar in Michigan, were planted into plastic flats (25 × 25 × 7.5 cm) containing a Spinks loamy sand and grown under metal halide lighting (500 μE/cm^2/sec). Rye plants were watered with soluble fertilizer (20% N, 20% P, 20% K; 1.0 g/liter) on alternate days and were weeded prior to herbicide treatment. Thirty to 40 days after planting, rye was desiccated. Unplanted control flats were watered, weeded, fertilized, and sprayed as rye. Indicator species of weeds and crops were planted into the residues 7--10 days after herbicides were applied to rye. It was necessary to develop a system to plant indicators through the residue in a manner similar to a no-tillage situation in the field. To facilitate accurate seed placement and to ensure good seed–soil contact

necessary for germination, a planting board was designed to evaluate the response to four test species at one time. Four rows, with ten holes each, were drilled through the board. Plastic syringes (5 cc), in which the tip was cut off, were inserted through the drilled holes. The plungers were used to push seeds down through the residue to a uniform depth in the soil. In all cases, the experimental design was a randomized complete block with four replications. To assess germination, the number of plants which emerged from the 40 seeds planted was assessed 10 and 20 days after planting.

An additional series of experiments was conducted to compare the magnitude of weed suppression by rye residues with that by poplar (*Populus*) excelsior a mulching product used to stabilize highway seedings. Since this material had no adverse influence on growth of numerous crops in the greenhouse, it was utilized as a control to provide physical attributes similar to the rye. Rye was seeded and grown in Spink's loamy sand in flats as indicated previously for sorghum, and after 30 days was killed with glyphosate at 0.8 kg/hectare. The dry weight of rye residues from four replicates was determined, and equal weights of poplar excelsior were sprayed with glyphosate and applied to another set of flats. Indicator weeds were sown into the flats as before and harvested after 22 days of growth.

RESULTS AND DISCUSSION

Field Experiments. Elimination of tillage had a pronounced impact on weed density, cutting populations by more than 50% (Table 1). Biomass was also reduced at the time these data were collected, although it approached that obtained with conventional tillage by the end of the season. When tillage is eliminated, there is a shift of species from primarily dicotyledons to monocotyledons, particularly members of the Gramineae. Important agro-

TABLE 1. INFLUENCE OF TILLAGE AND CROP RESIDUES ON WEED DENSITIES AND BIOMASS IN ASSOCIATED PEA CROP

Tillage	Cover crop residues	Weeds/m^2[a]	Weed biomass[a] (kg/m^2)
Conventional	None	124.3 a	1.03 a
None	None	51.0 b	0.68 b
None	Rye[b]	4.3 c	0.47 bc
None	Wheat	3.2 c	0.34 c
None	Barley	8.2 c	0.43 c

[a] Means with different letters are significantly different at $P = 0.05$ by Duncan's multiple-range test.
[b] Average for Wheeler cultivar and MSU-13 selection.

TABLE 2. EARLY SUMMER WEED PRODUCTION AS INFLUENCED BY RESIDUES OF FALL- OR SPRING-KILLED COVER CROPS

Cover crop	Time of kill	Kill method	Weed dry weight[a] (g/m^2)
Sorghum × sudangrass hybrid	Fall	Frost	31.2 bc
Sudangrass	Fall	Frost	78.0 a
Sorghum	Fall	Frost	54.1 b
Oat	Fall	Frost	47.8 b
Rye	Spring	Desiccant	28.6 cd
Wheat	Spring	Desiccant	13.2 d
Barley	Spring	Desiccant	28.3 cd
None	Spring	Desiccant	81.5 a

[a] Means with different letters are significantly different at $P = 0.05$ by Duncan's multiple-range test.

ecosystem genera that tend to be favored in no-tillage systems are *Panicum, Echinochloa*, and *Setaria*. Among those that greatly decrease in density are *Amaranthus, Ambrosia, Chenopodium*, and *Portulaca*.

In this experiment, rye, wheat, and barley residues all similarly reduced weed densities by an average of about 90% of that where no residues were present. Biomass reductions from wheat and barley appeared slightly superior to rye. These plant residues have provided comparable weed suppression in numerous experiments conducted over a range of soils and cropping systems (Barnes and Putnam, 1981; DeFrank and Putnam, 1978; Putnam and DeFrank, 1979, 1982).

Since the sorghums and oats are susceptible to freezing, cropping systems were designed in which these crops were planted in late summer and allowed to freeze, eliminating the need for introduction of a desiccant herbicide into the system. This approach was compared to use of fall-planted rye, wheat, or barley which was killed in the spring with paraquat (Table 2). In this series of tests, the crops killed in the spring with paraquat generally provided better weed control than those killed by freezing in the fall. However, all crops except fall-killed sudangrass provided a significant reduction in weed biomass. Sorghums or oats that are fall-killed could provide some degree of weed protection on early planted crops the next spring. For the later planted, warmer season crops it may be more beneficial to overwinter the cover crop and desiccate it just prior to or at planting.

A variety of important food crops have been planted into cover crop residues to determine their tolerances (Table 3). It is difficult, and perhaps misleading, to generalize about the results, except to suggest that the larger

TABLE 3. GENERAL RESPONSE OF SEVERAL ANNUAL CROPS TO RESIDUES OF SPRING SEEDED COVER CROPS IN NO-TILLAGE SYSTEMS

	Indicator crop response[a]							
Cover crop	Cabbage	Carrot	Corn	Cucumber	Lettuce	Pea	Snapbean	Tomato
Barley (spring)	−	+	+	+	−	+	+	−
Barley (winter)	−	+	+	+	−	+	+	−
Corn	−	+	+	+	−	+	+	+
Oats	−	−	+	+	−	+	+	+
Rye	−	+	+	+	−	+	+	+
Sorghum	−	−	−	+	−	+	+	−
Sorghum × sudangrass	+	−	−	+	−	+	+	−
Wheat	−	+	+	+	−	+	+	+

[a] Plus indicates germination and growth equal to or superior to that in no-tillage controls without residues. Minus indicates significant reduction in germination, growth, or yield.

seeded crops (corn, cucumber, pea, snapbean) are consistently more tolerant than the smaller seeded species. This could be partially explained on the basis that they are sown deeper (2–2.5 cm) and may be physically separated from the residues. The smaller and more shallow seeded crops varied in their tolerance to the residues. While lettuce germination and growth were severely reduced with all residues, carrot and tomato tolerated some residues but not others. Growth and yield data from several years of research indicate that peas, beans, and cucumbers can be successfully grown in this system (Barnes and Putnam, 1981; Putnam and DeFrank, 1982). Tomato will also perform satisfactorily where rye is utilized as the cover crop.

Parallel approaches have shown excellent utility in perennial cropping systems such as orchards and vineyards. In these cropping systems, companion planting of rye or wheat in the fall does not interfere with tree growth. The companion plantings are desiccated in May, before competition begins and residues provide effective weed control for up to 60 days (Putnam and DeFrank, 1979). Long-term studies have been established in an apple orchard near Clarksville and a cherry orchard near Traverse City, Michigan. After two seasons, growth of cherry trees has been slightly enhanced by the presence of rye or sorghum residues, and all management systems with cover crops have provided growth equal or superior to that in cultivated or repeatedly sprayed controls (Table 4). In the apple study, the cover crop system has provided growth equal to repeated tillage or herbicide spraying, all of which are superior to mowed fescue sod.

Greenhouse Experiments. Numerous tests have evaluated the influence of surface residues on germination and growth of weeds and crops (Putnam and DeFrank, 1982, Barnes and Putnam, 1981). When weed seeds with known

TABLE 4. RESPONSE OF APPLE AND CHERRY TREES TO VARIOUS SOIL MANAGEMENT PRACTICES AFTER TWO SEASONS

Management method	Apple growth[a]		Cherry growth[a]	
	Trunk diameter (cm)	Av. terminal growth (cm)	Trunk diameter (cm)	Av. terminal growth (cm)
Repeated tillage	2.38 b	67.3 b	2.10 b	45.6 bc
Repeated paraquat	2.43 b	61.8 ab	2.03 b	40.4 b
Sorghum residues	2.41 b	67.2 b	2.33 c	45.9 bc
Wheat residues	2.35 b	64.2 b	2.27 bc	53.3 c
Rye residues	2.24 ab	63.6 b	2.48 c	50.6 c
Mowed fescue sod	2.02 a	55.9 a	1.77 a	24.7 a

[a]Means with different letters differ significantly at $P = 0.05$ by Duncan's multiple-range test.

TABLE 5. PERCENT EMERGENCE OF FIVE COMMON AGROECOSYSTEM WEEDS THROUGH UNDISTURBED RESIDUES OF GREENHOUSE-GROWN RYE

Weed species	No rye residue	With rye residue	Reduction (%)[a]
Ambrosia artemisiifolia L.	21	12	43
Amaranthus retroflexus L.	36	2	95
Portulaca oleracea L.	58	0	100
Setaria lutescens (Weigel) Hubb.	30	29	NS
Setaria viridis (L.) Beauv.	54	11	80

[a] All values are significantly different from their respective controls at $P = 0.01$ unless otherwise indicated.

germination characteristics were added to soils amended with rye residues, germination was greatly decreased (Table 5). Apparent explanations are release of inhibitors directly from the rye or upon its breakdown. DeFrank and Putnam (1978) obtained similar results when sorghum residues were added to the soil surface. The inhibition was lost when the residues were mixed throughout the soil. Apparently when residues lie on the surface, a zone of inhibition develops near the surface where weed seeds germinate. DeFrank (1982) has recently isolated soil microbes which produce selective toxins.

Physical impacts of mulch, i.e., shading or cooling of the soil can be achieved by utilizing poplar excelsior as a control. Poplar excelsior has little or no impact on weed germination, although it may reduce weed biomass (Table 6). In contrast, rye residues greatly reduce weed germination and growth. In addition, rye extracts and root leachates are also inhibitory (Barnes and Putnam, 1981).

Several inhibitors of seed germination have previously been isolated from cereal grains and associated soils (Guenzi et al., 1967; Patrick, 1971). Recent chemical characterization work (Lehle and Putnam, 1982a) indicates

TABLE 6. COMPARISON OF RYE RESIDUES AND POPLAR EXCELSIOR FOR WEED SUPPRESSION

Residue	Weed density[a] (No./m^2)	Weed biomass (g/m^2)
None	317 a	398 a
Poplar excelsior	243 ab	224 b
Rye	74 c	81 c

[a] Means with different letters are significantly different at $P = 0.05$ Duncan's multiple range test.

that simple organic acids (particularly aconitic acid) contribute to the inhibitory action of sorghum herbage. Several other inhibitors are present, one of which is extremely active at low concentration. We have evidence and are currently exploring the hypothesis that numerous inhibitors are present in the vicinity of these residues that may exert joint action on seed germination. The possibility of their additive, or even synergistic, action should certainly not be discounted at this time.

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ALLELOPATHIC POTENTIAL OF CORN POLLEN

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Abstract—The effects of corn pollen and aqueous leachates of pollen upon the radicle growth of *Bidens pilosa*, *Cassia jalapensis*, and *Rumex crispus* are shown. Extractions of pollen with various solvents and methods were carried out so as to assess its active principle and its effect upon *C. jalapensis*. The preliminary steps to separate and identify the allelopathic compounds of the sonicated and macerated pollen extracted with methylene chloride are described. The strongest inhibitory effect was produced by the hexane fraction. The allelopathic effect of corn pollen upon the growth of *C. jalapensis* in several substrates is shown. The possible structure of some of the active fractions is discussed as well as the possibility that the allelopathic potential of pollen might actually occur in nature.

Key Words—Corn pollen, *Zea mays chalqunoconico*, *Bidens pilosa*, *Cassia jalapensis*, *Rumex crispus*, leachates, allelopathic compounds, weeds, crop-weed interactions.

INTRODUCTION

Paradoxically the current concept of allelopathy basically coincides with the one proposed by Molisch (1937) over 40 years ago that covers not only the deleterious biochemical interactions but also the beneficial ones.

Rice (1979) recognizes that the exclusion of the beneficial effects from the definition is completely artificial, for it has been demonstrated that the stimulatory or inhibitory effects depend on the concentration of a compound in its environment (Khailov, 1974). For Rice, there is no doubt that many of the important ecological functions of allelopathy have been neglected, as the interest of a great majority of researchers is centered upon the inhibitory effect of the compounds.

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Grodzinsky (1965) conceives allelopathy as a function of the cycling of active substances within ecosystems, a process which is initiated with the production and excretion of the active compounds, or their precursors, by the donor plant to the environment. The chemical assessment of these substances is practically impossible due to their varied chemical nature and the complexity of the reactions in which they intervene. Therefore, it is necessary to evaluate them by means of specific bioassays.

Undoubtedly, the primary objective in any study of alleopathy is to know the role of the active organic compounds among the organisms in a community and the transformations and influences they might have when they are released into the environment. The knowledge of their chemical structure, specific effects, spatial dynamics, and active permanence in the substratum can give us a clear idea of their ecological significance and thus allow us to assess more accurately the value of the interference of some plants upon other plants, microorganisms, or animals.

The importance of allelopathic interactions in agricultural practice is increasingly recognized, not only with reference to the interactions between crops or between weeds and cultivated plants and their adequate management so as to improve crop production (Altieri and Doll, 1978; Einhellig and Rasmusse, 1973; Kossanel et al., 1977; Putnam and Duke, 1978; Tukey, 1969), but also with reference to the potential profit that the allelopathic agents might bring as herbicides, fungicides, antibiotics, insecticides, etc., and to their specific use to diminish crop losses due to diseases, weeds, or pests.

Putnam and Duke (1971) have hypothesized that many presently cultivated species may have possessed allelopathic substances when growing in their wild habitats. However, such a trait could have been lost through domestication with intensive breeding and selection for specific desirable characteristics. Their hypothesis was confirmed in an experiment with different accessions of *Cucumis* species. Bioassay tests confirmed that the inhibition of the growth of certain weeds was due to a toxin produced by certain of the accessions.

Many additional studies were carried out to search for cultigens with an allelopathic potential which could suppress weeds. There are many examples of cultivated plants that produce allelopathic compounds, specifically among the Gramineae, like wheat, sorghum, oat, barley, and corn. We are sure that the search will be more profitable if it is carried out in those agroecosystems in which a traditional combination of several cultivated plants is practiced, as it is very likely that the relationships between cultigens and weeds in these ecosystems have a long evolutionary history.

In fact, farmers in Mexico have acquired a great deal of empirical knowledge as a result of the management of traditional agroecosystems. These latter are found all through the country, and, as a consequence, we have a varied and abundant agricultural gene pool.

The capacity of agroecosystems to maintain their productivity through space and time is due mainly to the high species diversity achieved by the use of mixed crops and rotation practices. Moreover, diversity provides better opportunities for biological control of pests and weeds (Litsinger and Moody, 1976; Chacon, 1978).

The fact that some farmers in Mexico rarely practice grubbing in certain mixed cultivation, and that, if they do, they generally are selective, suggests a certain kind of management of the herbicide potential of these cultigens or of certain weeds against others.

Jiménez et al. (1983) studied the relationships between weeds and cultivated plants in a traditional agroecosystem (chinampas) in Mexico. They found that corn is the cultivated species with the highest allelopathic potential. This phytotoxicity was revealed by the reduced growth of weeds, especially in the second third of the crop cycle when corn is mature and flowering.

In the experiments carried out in the laboratory, corn pollen produced a strong inhibitory effect upon the growth of weeds and cultivated species.

Although the chemical structure of some of the compounds of pollen is already known, this is the first time that an allelopathic effect of pollen is reported. Hence, to confirm this allelopathic potential and to isolate and identify the phytotoxic compounds, several laboratory experiments involving chemical tests and bioassays with seeds were carried out.

METHODS AND MATERIALS

Corn pollen was collected from mature male flowers from *Zea mays chalquiñocónico* Hdez. in plots located at Mixquic, D.F., Mexico. The first bioassay carried out with pollen consisted of distributing it in five different concentrations (10, 50, 100, 150, and 200 mg) in filter paper in a Petri dish to which distilled water and 20 test seeds of *Cassia jalapensis* were added. The control contained only distilled water. Three replicates per treatment were germinated at 28° C with a 12-hr photoperiod. After 72 hr, root length was measured and statistically analyzed as a randomized complete block design.

A second bioassay was carried out in which an aqueous extract was obtained by adding 1 g of pollen to 100 ml of distilled water and shaking it during 2 hr at room temperature. The extract was passed through a Millipore filter and the final solution (C_1) as well as two dilutions of it, C_2 (50:50, C_1–H_2O) and C_3 (27:75, C_1–H_2O), were measured to assess their osmotic pressure in a freezing-point osmometer.

These three concentrations were tested upon the radicle growth of three weed species: *Cassia jalapensis*, *Rumex crispus*, and *Bidens pilosa*. Bioassays were carried out by melting the solutions (10 ml of each) with 10 ml of agar (2%) in a Petri dish where 25 test seeds were set. Radicle length was measured

after 72, 96, and 120 hr for each species, respectively, and analyzed as a randomized complete block design (with subsampling).

The water extraction was not enough to obtain all the phytotoxic material it contained, for the remaining pollen still kept most of its allelopathic activity. Therefore, extractions with several solvents of different polarity were made so as to select the one most suitable to extract the compounds.

Extraction. Hexane, ethyl acetate, methanol, and water were used as solvents. One tenth gram of pollen was placed in 10 ml of solvent, shaken for 6 hr, and filtered; 3.3 ml of each filtrate were put in a Petri dish with filter paper. The solvents were left to evaporate, and 10 ml of distilled water were added to each dish in which 20 seeds of the selected test species were set. The effect of the extracts was compared with that of the complete pollen, and the remaining pollen of the extractions were sprinkled on the seeds at a concentration of 50 mg/Petri dish (9 cm). The control had only distilled water. The seeds were germinated under the same conditions as the first bioassays with four replicates per treatment.

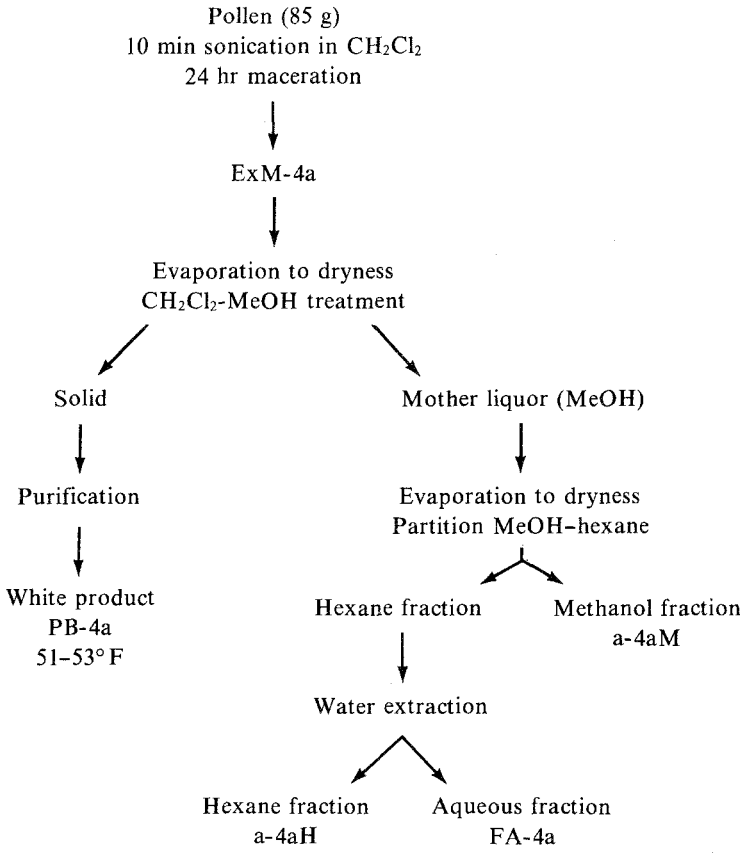
None of the solvents used in the last experiment could "adequately" extract the active principles of pollen, for the remaining pollen still proved to have a great phytotoxicity. It was subsequently discovered that pollen could be broken down by sonicating and extracting it with water. In addition, an organic solvent of medium polarity, methylene chloride, different from the ones used in the last experiment, was used. Assays with different periods of sonication in water and in methylene chloride were carried out until it was verified that 10 min was long enough to obtain a satisfactory extraction. In order to exhaust the extraction completely, part of the pollen in methylene chloride was left to soak for three days after sonication.

Immediately afterwards, bioassays were carried out as mentioned above with the following treatments: (1) complete pollen; (2) aqueous extract of sonicated pollen (10 min); (3) remaining pollen of 2; (4) extract of pollen in methylene chloride sonicated during 10 min; (5) remaining pollen of 4; (6) extract of pollen in methylene chloride sonicated during 10 min and soaked for 3 days, and (7) remaining pollen of 6. Treatments 1, 3, 5, and 7 were carried out at 50 mg/Petri dish (9 cm); treatments 2, 4, and 6 at 0.1 g/10 ml.

As the best extraction was obtained from pollen in methylene chloride sonicated for 10 min and soaked for three days, a new extraction was made by the following process:

Extraction. Eighty-five grams of pollen were sonicated during 10 min. in methylene chloride and allowed to soak for three days (Scheme 1). This procedure was repeated twice.

Isolation. The resulting extract (ExM-4a) was evaporated to dryness under reduced pressure and analyzed by TLC using silica gel chromatofolios and eluting them with BuOH–AcOH–H_2O/80:20:10.



SCHEME 1. Extraction and isolation of active fractions.

The chromatogram showed two spots with R_f of 0.81 and 0.71, respectively. The spot with R_f 0.81 was revealed with UV light (1800–3000 Å) and the one with R_f 0.71 was only visible when sprayed with sulfuric acid 5 N, and heated at 90° C for 5 min.

The total extract was treated with a mixture of methanol–methylene chloride (98:2), and the white precipitate was purified by dissolving it in a methylene chloride–methanol mixture. This corresponds to the substance with R_f 0.71 which is not revealed with UV light.

This product (PB-4a) is shown as a single spot in the last system as well as in one of petroleum–ethyl ether–acetic acid, 9:1:1.

After the white product was extracted, the remaining methanol was dried under reduced pressure, and the yellowish residue was extracted by repeated partition with hexane and methanol until the yellow-colored methanol fraction became colorless.

This latter (a-4aM) was dried under reduced pressure. TLC of this product using Siegel chromatofolios, eluting with a system of CH_2Cl_2–AcOET (60:40) and made visible with UV light (3000–4000 Å), shows six spots with the following R_f: (A) 0.44, (B) 0.31, (C) 0.14, (D) 0.13, (E) 0.06, and (F) which is found in the application point; the hexane fraction was extracted several times with water, obtaining by partition a white aqueous fraction (FA-4a) and a yellow hexane fraction (a-4aH).

The extraction was continued until the aqueous fraction turned colorless. Both fractions were analyzed by TLC in silica gel, eluting the hexane fraction with petroleum ether–ethyl ether–acetic acid 9:1:1 and the aqueous fraction with methylene chloride–ethyl acetate 60:40. The plates were examined with UV light. It was found that the hexane fraction was composed of five substances. Two of them were revealed with short-wave UV light and the remaining three with long-wave UV light. The following were obtained: (A) 0.85, (B) 0.80, (C) 0.73, (D) 0.61, and (E) 0.57.

The aqueous fraction (FA-4a) was a mixture composed of two substances which were revealed with long wave UV light showing the following R_f (A) 0.13, (B) 0.06, and a third which remained at the application point.

To detect the allelopathic activity of the fractions, bioassays with *Cassia jalapensis* were carried out under the same conditions mentioned above with the following treatments: (1) hexane fraction from the mother liquor (a-4aH); (2) methanol fraction without the white product (a-4aM); (3) aqueous fraction (FA-4a) from the hexane fraction partition with water [all at 5 mg/Petri dish (9 cm)]; and (4) white product (PB-4a) (10 mg/Petri dish, 9 cm).

In order to better understand the effect of pollen on the growth of *C. jalapensis*, a bioassay with different substrates was carried out. Seven tenths grams of pollen were mixed with 120 g of soil (sterilized and unsterilized), sand, and vermiculite in four plastic pots per treatment. Height of the seedlings was recorded every four days and, after 20 days, the percentage of inhibition and a regression analysis were calculated.

RESULTS AND DISCUSSION

The osmotic pressure of all the extracts was measured either directly, when these were aqueous, or from the aqueous solution obtained by the reconstitution of the organic solvents with distilled water. The values ranged between 0 and 20 mosm/liter and were too low to interfere with the results.

From the experiment with several concentrations of pollen sprinkled on filter paper and tested upon the radicle growth of *C. jalapensis*, a clear inhibitory effect was observed. Moreover this inhibition increased in direct proportion to the concentration (Figure 1).

Figure 2 shows the effect of the aqueous extract of pollen upon the radicle

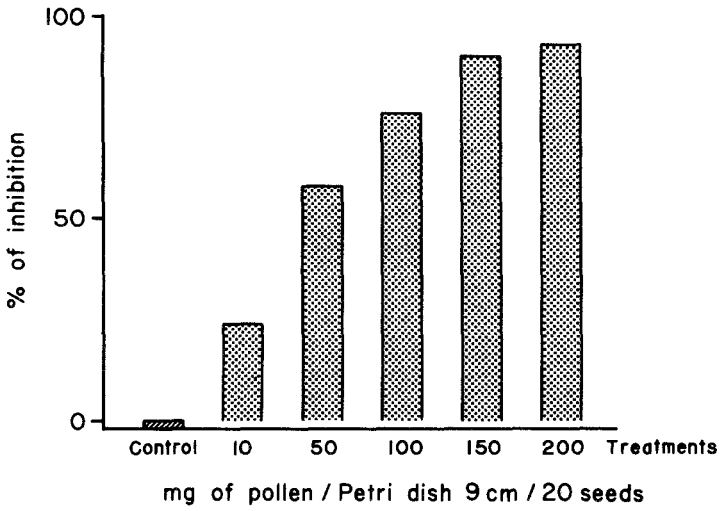


FIG. 1. Effects of five different concentrations of corn pollen sprinkled on seeds of *Casia jalapensis* on their radicle growth.

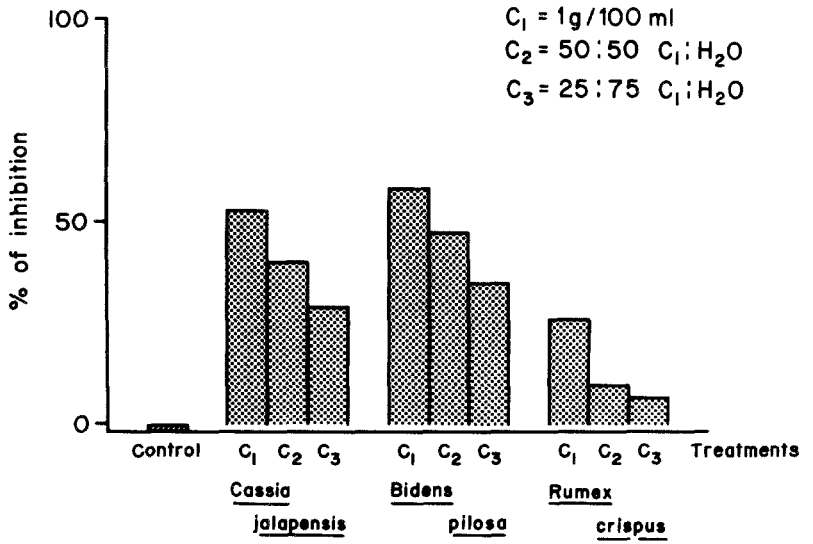


FIG. 2. Effects of the aqueous leachate of corn pollen at three different concentrations on the radicle growth of *Cassia jalapensis*, *Bidens pilosa*, and *Rumex crispus*.

growth of *C. jalapensis*, *B. pilosa*, and *R. crispus*. *B. pilosa* was the most susceptible species, while *R. crispus* was less affected. As in the last experiment, inhibition was directly proportional to the concentration, and in almost all cases it was significant to the 1% level, except C_3 upon *Rumex*.

Figure 3 shows the effects of pollen extracts from the different solvents used compared to those of complete pollen and the pollen remaining from extraction. Regarding the effect of the extracts, it can be noticed that the methanol extract produced a slight but significant inhibition upon *C. jalapensis* (5%) and *B. pilosa* (1%) and a significant stimulation (5%) upon *R. crispus*. The extract with ethyl acetate produced a significant stimulation (5%) upon *C. jalapensis*. Although the hexane extract had no significant effect upon *B. pilosa*, it stimulated significantly (5%) *R. crispus* and inhibited *C. jalapensis* (1%).

These three organic solvents extracted only a small part of the active compounds. However, the inhibitory effect of the aqueous extract was very strong upon *C. jalapensis* and *B. pilosa*. These results indicate that water is the most effective solvent to extract the allelopathic compounds of pollen. However, if we compare the effect of complete pollen with that of the pollen remaining from extraction, we notice that the inhibition produced by each (significant to the 1% level) was nearly equivalent but stronger than that

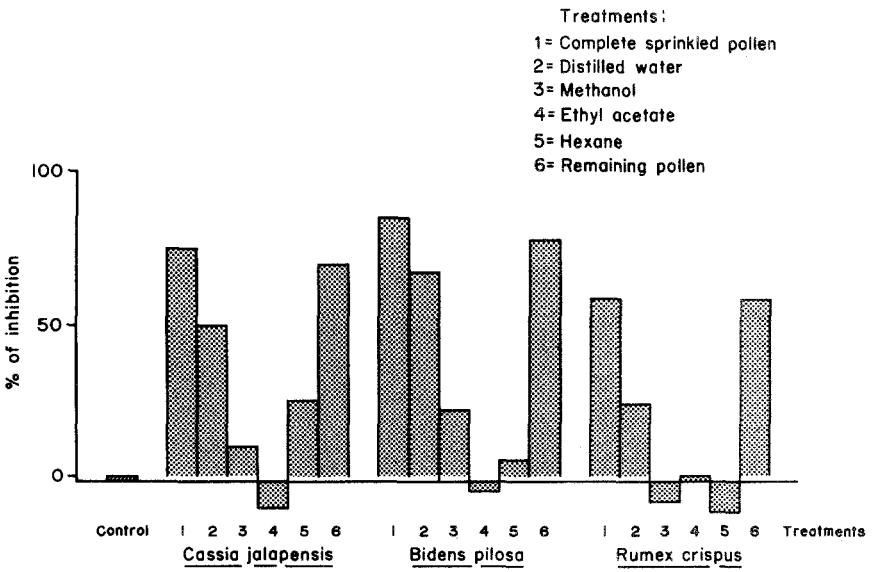


FIG. 3. Effects of some extracts of corn pollen made with water and organic solvents compared with the effects of complete pollen and the pollen remaining from the extraction on the growth of *Cassia jalapensis*, *Bidens pilosa*, and *Rumex crispus*.

produced by any extract. Therefore, a greater amount of the allelopathic compounds seemed to remain in pollen.

Sonication proved to be a good method of breaking down the pollen and extracting a greater amount of allelopathic compounds when treated with water and methylene chloride. Figure 4 shows that the most inhibitory treatment upon the growth of *C. jalapensis* was the extract with methylene chloride sonicated during 10 min and soaked for three days (6). Moreover, the pollen remaining from this extraction (7) had an inhibitory effect 30% less than that produced by complete pollen (1). The inhibition produced by all the treatments was significant to the 1% level.

The first steps to separate and identify the active compounds of the sonicated and soaked extract allowed us to observe that the methanol a-4aM and the aqueous FA-4a fraction have a similar composition; both of them seem to share three of their components located at the inferior and most polar part of their respective chromatograms (3B and 4B).

Likewise the components of the hexane fraction corresponded to the ones located at the top and less polar part of the chromatogram (2A) Figure 5.

The white product PB-4a has a Fp of 51–53° C and shows in the IR bands at 1740 S (C=O ester); 1650 W (C=C) and 720 W $(CHd)_n$. The mass spectra of this compound did not present a noticeable molecular ion, but did present peaks with m/z 18, 28, and 44 of great intensity and series of peaks with $m/2$ 57, 71, 85, 99, 113, 127, 141, etc., events which make us suspect the presence of glyceride.

From the bioassay carried out to observe the effect of the active fractions

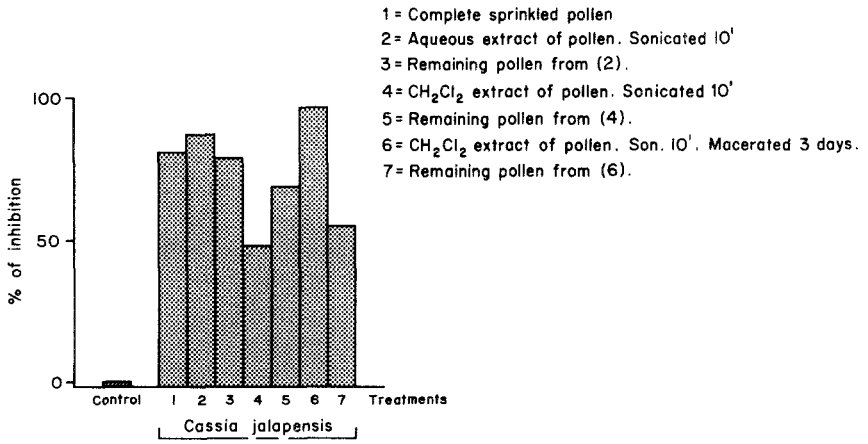


FIG. 4. Effects of sonicated aqueous and methylene chloride extracts of corn pollen, complete pollen and pollen remaining from the extractions on the radicle growth of *Cassia jalapensis*.

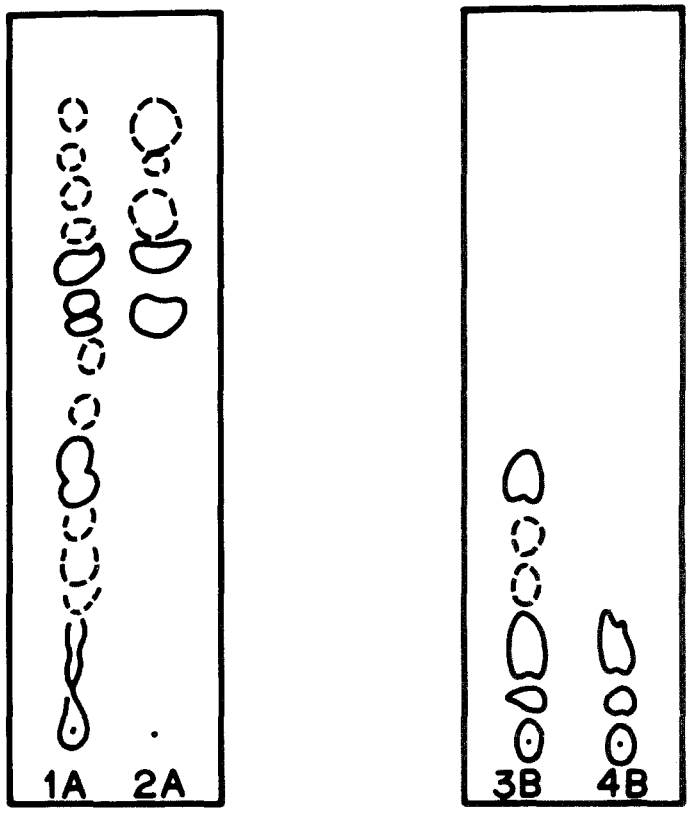


FIG. 5. Chromatograms (see scheme of extraction and isolation of active fractions). (1) Remaining methanol from the recrystallization of PB-4a (white product); (2) a-4aH (hexane fraction from the mother liquor MeOH); (3) a-4aM (methanolic fraction from the mother liquor MeOH); (4) FA-4a (aqueous fraction from the mother liquor MeOH); (A) petroleum ether–ethyl ether–acetic acid 9:1:1; (B) methylene chloride–ethyl acetate 60:40.

from the extraction of pollen upon the growth of *C. jalapensis* (Figure 6), the strongest inhibitory effect detected corresponded to the hexane fraction (a-4aH) from the mother liquor (MeOH). THe white product (PB-4a) produced 30% less inhibition and the methanolic a-4aM and aqueous FA-4a fractions had the same inhibitory effect (35% inhibition approximately). All inhibitions were significantly different from the control at the 1% level.

The white product as well as the compounds from the fraction a-4aH are being chemically characterized so as to carry out tests with pure compounds and mixtures of different concentration and to study the mode of action at the cellular and chemical level.

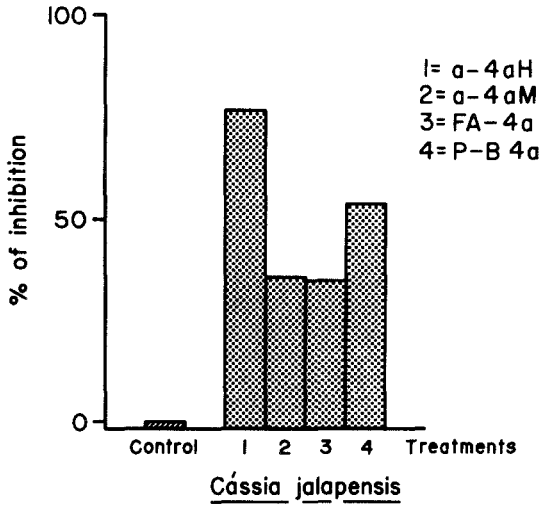


FIG. 6. Effects of some fractions of the mother liquor from the methylene chloride extract of corn pollen and the white product isolated from it on the radicle growth of *Cassia jalapensis*.

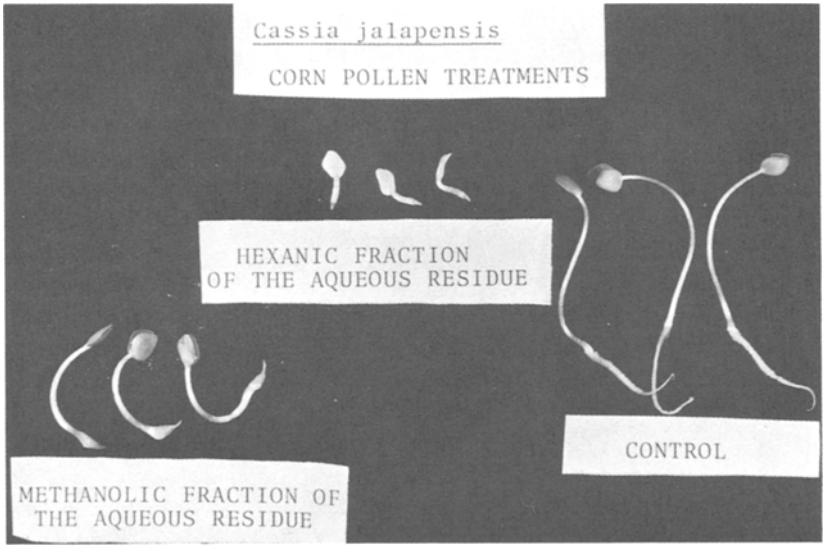


FIG. 7. Effects of the hexane fraction (a-4aH) from the aqueous residue obtained from the mother liquor and of the methanol fraction (a-4aM) from the aqueous residue obtained from the mother liquor on growth of *Cassia jalapensis*.

It is likely that some of the constituents of corn pollen are growth regulators (for example, the esters and triterpenoids, etc.) (Stanley, 1974) that may be responsible for some of the observed effects upon the growth of the seedlings. In addition to the inhibition of radicle growth, several other effects [such as thickening and deformation of the radicle as well as an absence of the pilicerous zone, inversion of tropism, and a strong chlorosis in the leaves, especially with the hexane fraction a-4aH (Figures 7 and 8)] were observed.

From the experiment with the different substrates, it was observed that the plants without pollen grew better than the ones with it (Figure 9). The variance analysis showed that inhibitions were significant at the 1% level except for the one with vermiculite. The least inhibition (28%) was observed in unsterilized soil, while the strongest ones were seen in sand (49%) and in sterilized soil (54%). Therefore it appears that microorganisms play an important role in the effect pollen might exert on the soil.

Nevertheless, an allelopathic effect was manifest in all the substrates. When all the treatments are considered together, it is apparent that the growth rate was greatly reduced. In the treatments without pollen (control) the plants grew 1.61 cm (mean value) every four days while those with pollen grew 0.61 cm (Figure 10). The growth rate of *C. jalapensis* was inhibited by 62%, so there is no doubt that plants growing in the presence of corn pollen have serious disadvantages in their establishment and development.

As mentioned before, the chemistry of pollen has been extensively

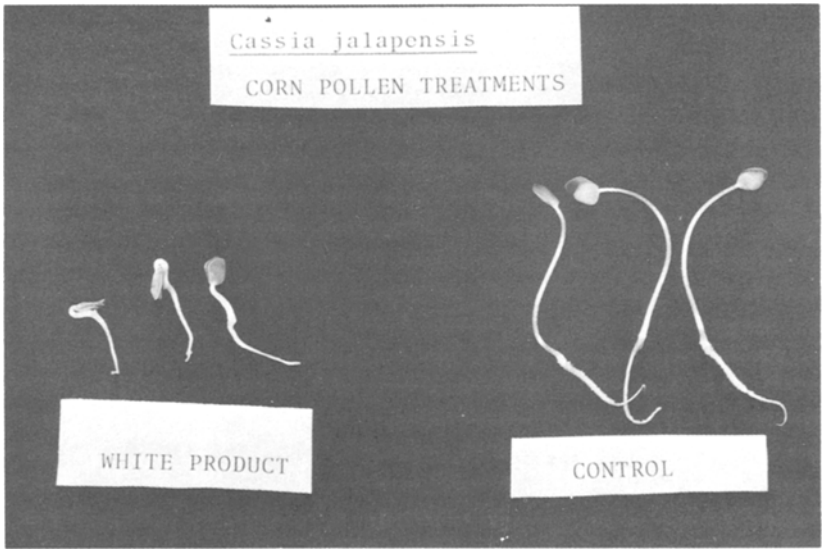


FIG. 8. Effects of the white product (PB-4a) on the growth of *Cassia jalapensis*.

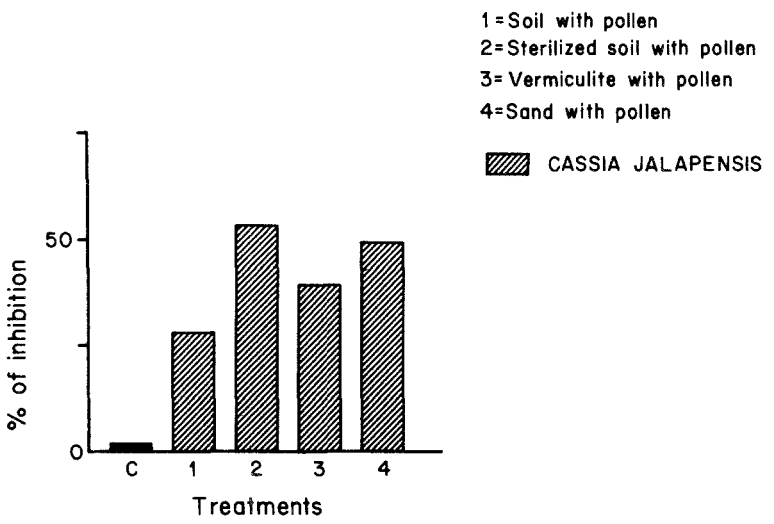


FIG. 9. Effects of complete sprinkled pollen of corn on the radicle growth of *Cassia jalapensis* growing in different substrates.

investigated (Barbier, 1970, Stanley and Linskens, 1974). Harborne (1977) states that in nutritional terms pollen is a rich source of food with 16–30% protein, 1–7% starch, 0–15% free sugar, and 3–10% fat. Trace constituents present include various vitamins and inorganic salts. There are also varying amounts of secondary substances. Pollen is often colored, especially by carotenoids and also by flavonoids, and these compounds probably act as signals to indicate availability to insect feeders (Harbornne, 1977). However, these kinds of compounds could also account for part of the phytotoxic effect of pollen upon seed germination. From the evolutionary point of view, the presence of active compounds in pollen could be determined by the selection of an adaptive mechanism which benefits the species in some way or because of chance (Rice, 1974). Harborne (1977) states that pollen has primary importance as a carrier of the male gametophytes, so that use of pollen by animals for feeding as well as the allelopathic effects of pollen upon other components of a community are only secondary. Competition between the various purposes of pollen is rarely a problem since the majority of angiosperms are overabundant in pollen production. If insects did not capitalize on the excess of pollen available to them, it could go to waste in other ways (Harborne, 1977) and affect other organisms, especially in the soil (Jiménez et al., 1983).

If we consider the amount of pollen produced by a corn plant, its density in the field (1×10^7 grains of pollen/m^2), and the inhibitory effects from the

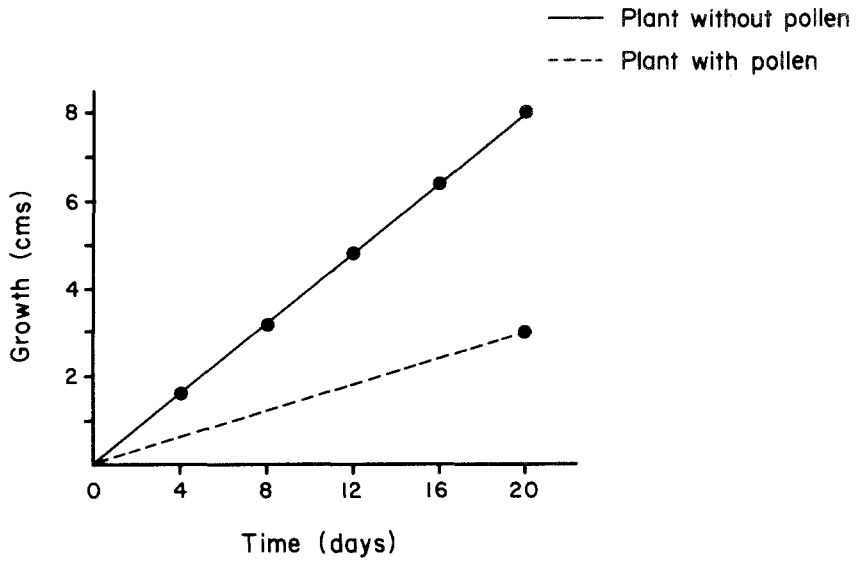


FIG. 10. Growth rate of *Cassia jalapensis* in the presence and absence of corn pollen.

plant as well as from pollen itself, it is clear that the accumulation of pollen in the soil and therefore its potential effects upon the growth of other species, particularly weeds, can be important advantages for corn over its potential competitors, especially during flowering (Jiménez et al., 1983).

Finally, it is important to consider that the pH and the weather of a region's soil are of major importance in the dynamics of the microflora and consequently in the rate of decomposition of pollen in the soil. This fact is intimately related to the release of the active principles of pollen which, under experimental conditions, was very difficult to replicate when leaching with water. However, if we consider that in a well-aereated soil with pH values of 6.5–7.5 (Stanley, 1974) pollen is rapidly oxidized, and that under these circumstances it may be attacked by fungi, yeasts, and bacteria which break the exin and release a great amount of phytotoxins (a process which could be comparable to sonication), its potential effect within the community compels us to consider that it might be an important factor in determining the productivity of corn and other cultigens and in the biological control of weeds and other kind of pests.

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INHIBITION OF PITTED MORNING GLORY (*Ipomoea lacunosa* L.) AND CERTAIN OTHER WEED SPECIES BY PHYTOTOXIC COMPONENTS OF WHEAT (*Triticum aestivum* L.) STRAW[1]

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Abstract—This study was conducted to determine if well-known phytotoxic effects of plant residues on crop growth could also be responsible for observed reductions of certain weed species in no-till cropping systems. An aqueous extract of field-grown wheat (*Triticum aestivum* L.) reduced the germination and root length of pitted morning glory (*Ipomoea lacunosa* L.) and common ragweed (*Ambrosia artemisiifolia* L.). Phytotoxicity was increased by about 70% when bioassays with the wheat extract on morning glory and ragweed were conducted in the presence of light. Phytotoxic substances were extracted from wheat with 2 N NaOH. The hydrolyzed extract was fractionated by thin-layer chromatography (TLC). The compound isolated by TLC having the greatest inhibitory effects on morning glory germination was identified using mass spectrometry and determined to be ferulic acid (4-hydroxy-3-methoxycinnamic acid). Ferulic acid at 5×10^{-3} M inhibited the germination and root length of morning glory 23 and 82%, respectively, and prickly sida (*Sida spinosa* L.) with carpels 85 and 82%, respectively. Crabgrass (*Digitaria sanguinalis* L.) germination was inhibited 100%. Ferulic acid had no effect on ragweed or prickly sida without carpels. Morning glory root and shoot biomass were reduced 52 and 26%, respectively, when morning glory was grown in sand and watered with a 5×10^{-3} M solution of ferulic acid. Ferulic acid in the presence of prickly sida seed carpels was found to undergo decarboxylation, forming a styrene derivative, 2-methoxy-4-ethenylphenol. The more phytotoxic

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styrene compound was produced by a bacterium isolated from the carpels of prickly sida seed. The study showed that ferulic acid and other compounds may indeed play a role in reducing the growth of certain weeds in no-tillage cropping systems.

Key Words—allelopathy, ferulic acid, no-tillage, prickly sida, common ragweed, weed control, 2-methoxy-4-ethenylphenol, carboxylic acid, phenol, morning glory, *Ipomoea lacunosa*, wheat, *Triticum aestivum*.

INTRODUCTION

No-tillage crop production, a cropping practice which maintains plant residues on the soil surface, is a very effective means of reducing soil erosion from wind and water, conserving soil moisture, and reducing energy consumption when compared to conventional tillage. However, crop growth and yields with this practice are sometimes reduced in comparison with yields obtained on conventionally tilled land (Duley, 1960; Fenster and McCalla, 1971). This reduction in crop growth has been attributed in part to the release or production of phytotoxic substances from plant residues (Chou and Patrick, 1976; Cochran et al., 1977; Guenzi and McCalla, 1962, 1966a, 1966b; Guenzi et al., 1967; Kimber, 1973; McCalla and Haskins, 1964; McCalla and Norstadt, 1974; Norstadt and McCalla, 1963, 1968; Patrick, 1971; Patrick and Koch, 1958; Patrick et al., 1963, 1964; Toussoun et al., 1968; Wang et al., 1967). Compounds that have been implicated as allelopathic agents include: phenolic acids, aliphatic acids, aldehydes, ketones, benzoic acids, terpenoids, coumarins, and flavonoids (Harborne, 1964; Harborne and Simmonds, 1964; Robinson, 1980; Whittaker, 1970).

Phytotoxicity is possible primarily through two mechanisms: inhibitory compounds being leached directly from plant residues or those produced by soil microorganisms using plant residues as a substrate. In an early study, Guenzi and McCalla (1962) showed that most crop residues contain water-soluble substances that can depress the growth of corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), and sorghum (*Sorghum bicolor* L.). Inhibitory effects were reduced in most cases when aqueous extracts were autoclaved, indicating that microorganisms may have been partly responsible for the phytotoxicity exhibited by the original aqueous extracts. Guenzi and McCalla (1966a) isolated and identified five phenolic acids in a number of plant residues. The phenolic acids, *p*-coumaric, syringic, vanillic, ferulic, and *p*-hydroxybenzoic acids, were all found to be inhibitory to the growth of wheat seedlings. In a subsequent study, Guenzi and McCalla (1966b) found the same five phenolic acids in NaOH extracts of soil obtained from no-till plots. The five phenolic acids were also found in soil obtained from tilled plots, but at reduced levels.

In studies conducted to determine changes in phytotoxicity during decomposition, Guenzi et al. (1967) found that aqueous extracts of weathered

corn and sorghum residues were most phytotoxic to wheat growth after 4 and 16 weeks of decomposition, respectively. The greatest phytotoxicity exhibited by aqueous extracts of wheat and oat (*Avena sativa* L.) straw occurred at or near harvest time, with essentially all toxicity gone after four weeks of decomposition. Kimber (1973) found that aqueous extracts of several grasses and legumes that had been rotting for periods of up to 21 days were inhibitory to the growth of wheat. He also showed that straws cut while still green produced a higher level of toxicity than those cut when fully mature. Toussoun et al. (1968) found that the production of water-soluble phytotoxins of barley (*Hordeum vulgare* L.) residue in soil required a soil moisture content above 30% of the soil and residue dry weight. Extracts became toxic 7–10 days after incorporation into soil with phytotoxic activity reaching a maximum in 3 weeks.

Since the early 1960s, increased emphasis has been placed on the role of soil microorganisms in the production of phytotoxic substances from plant residues. Norstadt and McCalla (1963) postulated that the inhibitory effects of crop residues might be due to a combination of toxins from plant residues and from microorganisms that are more prolific where plant residues are present. A number of fungi have been isolated from soil obtained from no-till plots. Many of the fungi produced substances toxic to higher plants (McCalla and Haskins, 1964; Norstadt and McCalla, 1968). Patrick and Koch (1958) reported that aqueous extracts of various plant residues had no effect on the respiration of tobacco (*Nicotiana tabacum* L.) seedlings. They did, however, demonstrate that substances formed during the decomposition of plant residues in the soil markedly inhibited the respiration of tobacco seedlings. In later studies, Patrick (1971) and Chou and Patrick (1976) isolated and identified a wide range of toxic compounds from decomposing rye (*Secale cereale* L.) and corn residues. In another study, Cochran et al. (1977) found that plant residues produced wheat seedling root inhibitors only after conditions became favorable for microbial growth.

The preceding represents a sampling of the extensive literature available on the detrimental effects of toxins released from surface and buried crop residues on crop plants. Little attention, however, has been given to the effect of surface crop residues on weed growth. The single factor most often cited in a nationwide survey by Worsham (1980) as a problem in no-till corn production or as a deterrent to increasing this practice was the difficulty of controlling weeds. In addition to the benefits of no-till cropping systems listed earlier, there may thus also be an added benefit of increased control of certain weeds. We have observed reduced populations of morning glory (*Ipomoea* sp.) and prickly sida (*Sida spinosa* L.) in no-till research plots and field situations in North Carolina. Although there are many significant environmental and ecological differences between conventional and no-till cropping systems, it seems possible that allelopathy may be playing a role in reducing

weed populations in no-till. In studies conducted by Barnes and Putnam (1982) the presence of a rye cover crop in a no-till vegetable production system reduced total weed biomass up to 95% when compared to controls with no residue. In addition, Lodhi (1981) reported that the number of weedy species and their dry mass remained significantly lower in a partially unharvested corn field, as compared to a clear-cut field.

We hypothesized that the allelopathic influence of a wheat mulch was partially responsible for reduced populations of morning glory and other weeds in no-till. To test this hypothesis, experiments were designed to: (1) determine if aqueous extracts of wheat straw are inhibitory to morning glory, (2) attempt to isolate and identify any inhibitory component(s) from wheat straw, and (3) determine quantitatively the phytotoxicity of any identified toxin.

METHODS AND MATERIALS

Wheat ('McNair 1813') plant material used in this study was harvested from the field in early spring after the wheat had tillered but before heading. The plant material was dried at 50° C for 48 hr.

Aqueous Extracts

Five-gram samples of wheat plant material were soaked in 150 ml of water for 10 hr at room temperature. The mixture was filtered with Whatman No. 1 filters and the filtrate used to germinate pitted morning glory (*Ipomoea lacunosa* L.) and ragweed (*Ambrosia artemisiifolia* L.) seed. The effect of the extract on germination was tested in the presence and absence of light. Twenty morning glory and ragweed seeds were placed in separate 9-cm Petri dishes fitted with filter paper and wetted with 5 ml of the extract. The seeds were germinated in a growth chamber which was set for 12-hr days and temperatures of 30° C day and 21° C night. The dishes for the dark test assays were covered with aluminum foil prior to placing in the growth chamber. After 5 days, percent germination and root length data were obtained.

Isolation of Inhibitory Compound

Extractions were done by the procedure of Guenzi and McCalla (1966a). Five-gram wheat samples were hydrolyzed with 2 N NaOH for 4 hr at room temperature. The alkaline extract was filtered and the filtrate acidified to pH 2 with concentrated HCl and extracted with diethyl ether. The ether fraction was dried over $CaSO_4$ and concentrated to a volume of 20 ml in a rotary flash evaporater. The concentrated ether fraction was used to spot thin-layer chromatography (TLC) plates. Silica gel G obtained from Sigma Chemical

Company was used to prepare 20 × 20-cm plates poured to a thickness of 1.0 mm. Seven separate spots of 200 μl each were made on each plate. The plates were developed in a benzene–methanol–acetic acid system (80:10:5 v/v/v). Compounds separated by one-dimensional TLC were detected by exposing the developed plates to long-wave (3360 Å) UV light. The isolated compounds noted under UV light were individually scraped from the TLC plates and placed in acetone. The silica gel was removed by filtration. The acetone was removed by evaporation. The individual compounds were then bioassayed for morning glory seed germination inhibition. Six presoaked morning glory seeds were germinated in 4.5-cm Petri dishes in the presence of the test compound diluted with 2 ml of water. Each test compound was prepared by combining 14 identical spots from the developed TLC plates. Controls were prepared using unspotted plates put through the same procedure. The Petri dishes were incubated for 4 days at 25° C. At the completion of the bioassay, percent germination and root length data were obtained.

Identification of Inhibitory Compound

One compound isolated by TLC was found to be very inhibitory to morning glory seed germination and was identified using mass spectrometry. A LKB 2091 GC-MS was used for GC-MS analysis. Operating conditions of the GC-MS analysis are given in Table 1. In addition to GC-MS, the sample was also analyzed by direct probe.

Bioassay for Toxicity of Inhibitory Compound(s)

Since the identity of the inhibitory compound was determined to be ferulic acid (4-hydroxy-3-methoxycinnamic acid), ferulic acid obtained from Sigma Chemical Company was used in germination and greenhouse bioassays.

TABLE 1. GC-MS CONDITIONS USED IN IDENTIFICATION OF UNKNOWN COMPOUND

Instrument	LKB 2091
Column	6 ft × $\frac{1}{8}$ in. Ultrabond
Column temperature	230°C
Carrier gas	He at 8 ml/min.
Injector temperature	240°C
Separator temperature	210°C
Source temperature	210°C
Ionizing energy	70 eV
Trap current	50 μA
Scan cycle	2.0 sec

Germination Studies. Ferulic acid solutions of 5×10^{-3}, 1×10^{-3}, and 5×10^{-4} M, buffered to pH 6.5 with sodium phosphate (final concentration 0.01 M) were used to germinate morning glory, prickly sida, ragweed, crabgrass (*Digitaria sanguinalis* L.), corn, and soybean (*Glycine max* L. Merr.), all obtained commercially. The weed and crop seed were germinated in 9-cm Petri dishes. The number of seeds per dish varied depending on species: there were 20 prickly sida, ragweed, and crabgrass seeds per dish, 15 morning glory, and 10 corn and soybean seeds each per dish. To each dish, 10 ml of test solution was added. The Petri dishes were incubated for 4 days (prickly sida and crabgrass, 5 days). At the termination of the bioassay, percent germination and root length data were taken. In addition to ferulic acid, caffeic acid, another naturally occurring phenolic acid, was tested for comparison purposes. The bioassay conducted with caffeic acid was identical to that for ferulic acid, with the exception that only morning glory and prickly sida were studied.

Greenhouse Study. Morning glory was grown from seed in 473-ml wax-covered paper pots in either sand or a clay soil. Holes were made in the bottom of pots for drainage. In each pot, three morning glory plants were established. Pots were watered daily and nutrients, prepared according to Weber (1977), were supplied as needed.

One week after planting, morning glory seedlings were watered with 5×10^{-3}, 1×10^{-3}, or 5×10^{-4} M ferulic acid solutions. The ferulic acid solutions were buffered with sodium phosphate as described earlier. Fifty milliliters of test solution were added to the pots daily. Morning glory plants were watered with the test solutions for a period of three weeks, at which time the plants were harvested, and shoot length, and shoot and root fresh weights were obtained.

RESULTS

Aqueous Extracts

The effect of the aqueous wheat extract on morning glory and ragweed germination is shown in Table 2. In the dark, the presence of the wheat extract reduced morning glory germination and root length 27 and 66%, respectively. The wheat extract did not, however, significantly reduce ragweed germination in the dark, but did reduce ragweed root length 86%. Ragweed was chosen in this test because levels of this weed have not been observed to be reduced by no-till cropping practices. Greater phytotoxicity was observed when the bioassays were conducted in the presence of light. The enhanced phytotoxicity due to light was particularly apparent on ragweed. Ragweed seed wetted with the wheat extract and germinated in the presence of light did not germinate, yet light alone had a slight stimulatory effect on ragweed germination.

TABLE 2. EFFECT OF LIGHT ON MORNING GLORY AND RAGWEED GERMINATION WITH AQUEOUS WHEAT EXTRACT

Treatment	Morning glory		Ragweed	
	Germination (%)	Root length (cm)	Germination (%)	Root length (cm)
Dark + extract	53 b[a]	1.5 c	29 b	1.1 c
Dark check	73 a	4.4 a	34 b	7.6 a
Light + extract	30 c	1.0 c	0 c	0.0 d
Light check	85 a	2.6 b	45 a	3.0 b

[a]Values within columns sharing the same letter are not significantly different according to the Waller–Duncan procedure, assuming a K ratio of 100.

Morning glory germination and root length were reduced 65 and 62%, respectively, when seed wetted with the wheat extract were germinated in the light.

Isolation of Inhibitory Compound

The hydrolyzed extract of wheat straw yielded many compounds when subjected to TLC separation. Approximately 12 spots were noted on the developed TLC plates when exposed to long-wave UV light. When individual spots were removed and bioassayed for morning glory seed germination, only two of the spots significantly inhibited germination (Table 3). Only the data for the two inhibitory spots are presented. Of the two inhibitory compounds, the compound with R_f 0.5 was the more inhibitory. This compound reduced morning glory germination and root length 94 and 89%, respectively. The few seeds which did germinate developed very hard, short compact roots. Although the substance at R_f 0.5 was found to be the most inhibitory, concentration differences of the various components in the

TABLE 3. EFFECT OF COMPOUNDS ISOLATED FROM WHEAT BY TLC ON MORNING GLORY SEEDLING GROWTH

R_f value	Germination (%)	Root length (cm)
0.5	6 c[a]	0.3 c
0.95	67 b	1.5 b
Check	94 a	2.7 a

[a]Values within columns sharing the same letter are not significantly different according to the Waller–Duncan procedure assuming a K ratio of 100.

ether extract may have been partially responsible for the greater inhibition of the test solution containing the compound at R_f 0.5.

Identification of Inhibitory Compound

Of the two compounds isolated by TLC that had activity on morning glory germination, only the one at R_f 0.5 on the TLC plate was analyzed by GC-MS. The reconstructed gas chromatogram of the material at R_f 0.5 is shown in Figure 1. A mass spectrum was obtained for each of the components in the gas chromatogram shown in Figure 1. The sample was also analyzed by direct probe. The extracted ion current profiles of several ions from the direct probe analysis are shown in Figure 2. The most abundant ion obtained by the direct probe analysis is m/e 194. Only the mass spectrum of peak 5 on the reconstructed chromatogram contained a m/e value of 194, indicating that peak 5 was the major component in the mixture. From the mass spectrum of peak 5 (Figure 3), the identity of peak 5 was determined to be ferulic acid. Although a mass spectrum of an authentic sample of ferulic acid was not obtained, the identity of the unknown was determined by comparing the mass spectrum of peak 5 to spectra of ferulic acid found in the literature (Cornu and

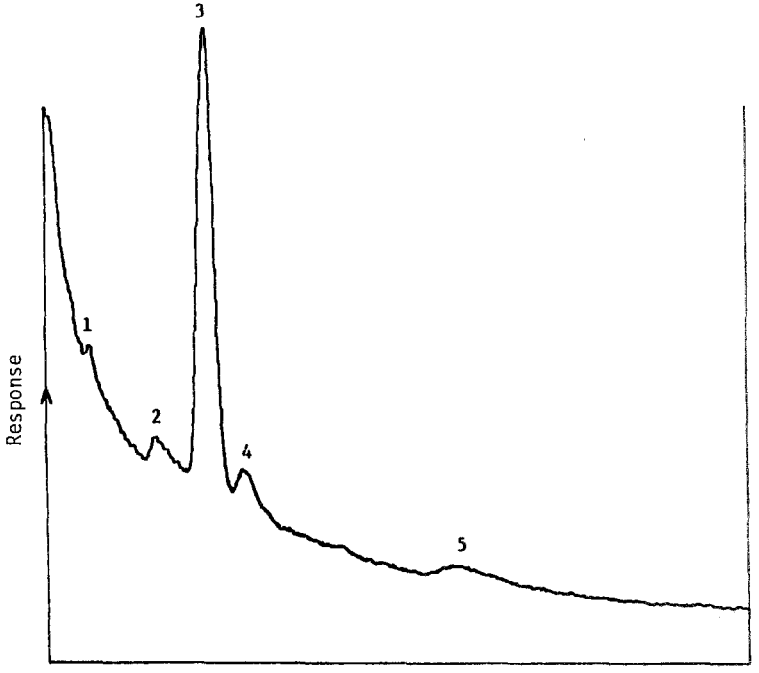


FIG. 1. Reconstructed gas chromatogram of inhibitory fraction isolated from wheat by TLC (R_f 0.5).

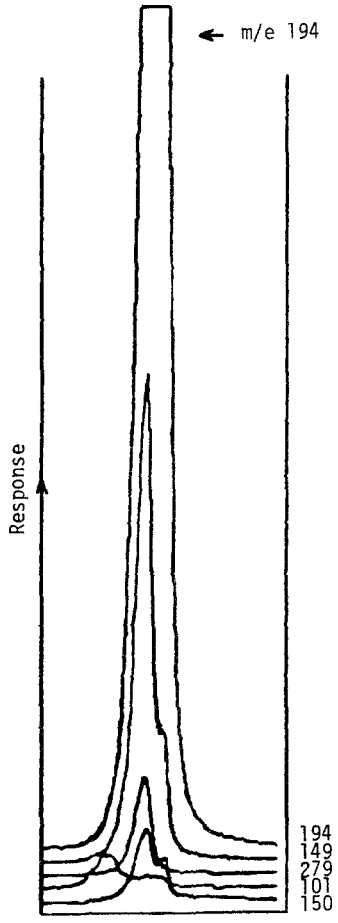


FIG. 2. Extracted ion current profiles of m/e 194, 149, 279, 101, and 150 ions from the direct probe analysis of sample from TLC plate (R_f 0.5).

Massot, 1975; Stenhagen et al., 1974). The mass spectrum of ferulic acid (Figure 3) shows a strong molecular ion peak at $m/e = 194$. The loss of CH_3 and CO_2 from the molecular ion are probably responsible for fragment peaks at m/e values of 179 and 150, respectively.

In addition to mass spectrometry, the UV absorbance spectra, the R_f values using previously described conditions, and the color reactions with diazotized *p*-nitroaniline of suspected ferulic acid (peak 5) and an authentic sample of ferulic acid were identical. The preceding not only indicates the identity of the unknown as ferulic acid, but also that the other components in the mixture, peaks 1–4 in Figure 1, were present at very low levels. Of the other components in Figure 1, only peak 3 (dioctyl phthalate) was identified.

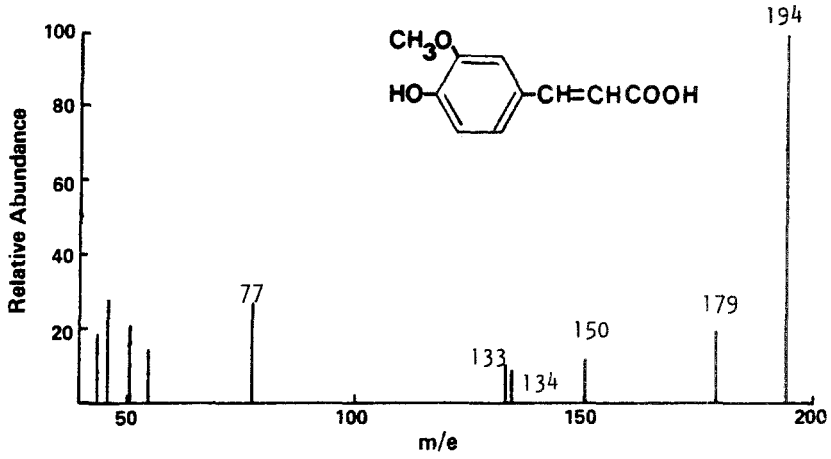


FIG. 3. Mass spectrum of ferulic acid (peak 5 of Figure 1).

Since peak 5 is smaller than peak 3 in the reconstructed gas chromatogram, adsorption or thermal decomposition of ferulic acid upon GC injection may be occurring which would be expected with a polar compound such as ferulic acid.

Toxicity of Ferulic Acid

Germination Studies. The effect of ferulic acid on the germination and root length of morning glory, ragweed, prickly sida, and crabgrass is shown in Table 4. Significant reductions in germination or root length of morning glory, crabgrass, and prickly sida seed enclosed in carpels were observed only at the highest concentration of ferulic acid. Ferulic acid, however, had no effect on either ragweed or pricly sida seed with carpels removed. Of the four weed species bioassayed with ferulic acid, crabgrass appeared to be the most sensitive. No crabgrass germination was observed at the high concentration of ferulic acid. None of the concentrations of ferulic acid used had any effect on the germination of corn or soybean (Table 5). Ferulic acid did, however, inhibit the root growth of both corn and soybean at the highest concentration.

The results of the bioassay conducted with caffeic acid, are given in Table 6. Caffeic acid was found to be less inhibitory to both morning glory and prickly sida in comparison to ferulic acid. Only prickly sida seed enclosed in carpels were inhibited by caffeic acid. Although caffeic acid appeared to be less inhibitory to prickly sida in comparison to ferulic acid, the inhibitory effects of both acids were enhanced by the presence of the pricly sida seed carpel. The carpel alone, however, had no effect on prickly sida seed germination.

TABLE 4. EFFECT OF FERULIC ACID ON MORNING GLORY, RAGWEED, CRABGRASS, AND PRICKLY SIDA SEED GERMINATION

							Prickly sida			
	Morning glory		Ragweed		Crabgrass		Without carpel		With carpel	
Ferulic acid (M)	Germ. (%)	Root length (cm)	Germ. (%)	Root length (cm)	Germ. (%)	Root length (cm)	Germ. (%)	Root length (cm)	Germ. (%)	Root length (cm)
5×10^{-3}	58	0.3	26	1.5	0	0	16	1.4	3	0.3
1×10^{-3}	68	1.4	29	1.6	29	0.9	18	1.6	13	1.7
5×10^{-4}	65	1.8	27	1.6	34	1.0	15	1.8	21	2.1
Check	75	1.7	27	1.6	30	1.1	19	1.6	20	1.7
LSD (0.05)	15	0.6	NS	NS	19	0.3	NS	NS	9	0.3

TABLE 5. INFLUENCE OF FERULIC ACID ON GERMINATION OF CORN AND SOYBEAN SEED

	Corn		Soybean	
Ferulic acid (M)	Germ. (%)	Root length (cm)	Germ. (%)	Root length (cm)
5×10^{-3}	68	0.9	63	2.7
1×10^{-3}	83	3.7	65	2.7
5×10^{-4}	85	3.3	70	3.7
Check	83	2.9	82	4.4
LSD (0.05)	NS	1.2	NS	1.2

Greenhouse Study. The effect of ferulic acid on morning glory height and root and shoot fresh weight in two soil types is shown in Table 7. Significant reductions of morning glory root and shoot fresh weights in both soil types were observed at the high (5×10^{-3}) concentration of ferulic acid. There was no reduction of morning glory shoot length due to ferulic acid in either soil system. The reduction of morning glory root biomass due to ferulic acid was greater in sand than in soil, yet shoot biomass was reduced less in sand than in soil.

DISCUSSION

Of the many types of phytotoxic compounds released from decaying plant material by microbial activity or leaching, the phenolic acids are probably the most common (Rice, 1974; Whittaker, 1970). Ferulic acid, as

TABLE 6. EFFECT OF CAFFEIC ACID ON MORNING GLORY AND PRICKLY SIDA SEED GERMINATION

			Prickly sida			
	Morning glory		Without carpel		With carpel	
Caffeic acid (M)	Germ. (%)	Root length (cm)	Germ. (%)	Root length (cm)	Germ. (%)	Root length (cm)
5×10^{-3}	55	1.6	14	1.6	4	1.0
1×10^{-3}	57	1.9	14	1.3	13	2.4
5×10^{-4}	60	2.0	14	1.8	16	2.3
Check	70	1.9	14	1.8	16	1.9
LSD (0.05)	NS	NS	NS	NS	10	0.9

TABLE 7. EFFECT OF GROWTH MEDIUM AND FERULIC ACID ON MORNING GLORY GROWTH IN GREENHOUSE

Ferulic acid (M)	Growth medium	Height (cm)	Fresh Weight (g)	
			Root	Shoot
5×10^{-3}	Sand	46.6 a[a]	0.9 b	2.2 b
1×10^{-3}	Sand	49.2 a	1.8 a	3.0 a
5×10^{-4}	Sand	52.5 a	1.8 a	3.1 a
Check	Sand	52.5 a	1.9 a	3.0 a
5×10^{-3}	Soil	6.7 b	0.6 b	0.9 c
1×10^{-3}	Soil	12.9 b	0.7 ab	1.6 b
5×10^{-4}	Soil	27.9 a	0.7 ab	2.1 a
Check	Soil	17.7 ab	0.9 a	2.0 a

[a] Values within columns within one soil type sharing the same letter are not significantly different according to the Waller-Duncan procedure, assuming a K ratio of 100.

well as other phenolic acids, is produced from intermediates of respiratory metabolism via the shikimic acid pathway. Ferulic acid has been found in a variety of crop residues by a number of researchers (Guenzi and McCalla, 1966a; Lodhi, 1979; Wang et al., 1967). The reported inhibitory effects of ferulic acid on germination and seedling growth have varied widely. Borner (1960) found that a ferulic acid concentration as low as 10 ppm inhibited the growth of wheat and rye roots. Guenzi and McCalla (1966a), however, reported that a 2500 ppm solution of ferulic acid had no effect on the germination of wheat and reduced the growth of wheat roots by only about 50%. In work with soybean seedlings, Patterson (1981) showed that soybean total dry weight, leaf area, plant height, and number of leaves were all significantly reduced when soybean plants were grown in solution culture containing 194 ppm of ferulic acid. Ferulic acid had no effect on soybean growth when the ferulic acid concentration was reduced to 19.4 ppm. Lodhi (1979) reported that a ferulic acid concentration of 194 ppm was very inhibitory to the seed germination and radicle growth of radish (*Raphanus sativus* L.).

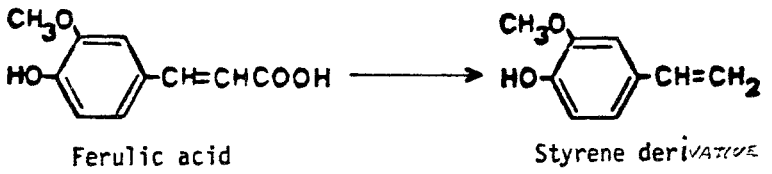
When we compared the inhibitory effects of ferulic acid obtained by TLC from field-grown wheat, and commercially obtained ferulic acid, there appeared to be a discrepancy in the observed phytotoxicity. Using spectrophotometric methods and an absorbance maximum for ferulic acid at 320 nm, the amount of ferulic acid in the wheat straw was determined to be 1243 $\pm$ 21 ppm. Based on a ferulic acid concentration of 1243 ppm in the wheat straw, obtained by alkaline hydrolysis, we calculated the concentration of ferulic acid in the test solution used to germinate morning glory (Table 3) at 435 ppm.

This is approximately half of the highest concentration (5×10^{-3}M or 970 ppm) of ferulic acid used to germinate morning glory in Table 4. Despite the lower concentration of ferulic acid obtained by TLC from the wheat straw, substantially more inhibition of morning glory was observed in this test solution than was observed for a higher concentration of ferulic acid obtained commercially.

It is difficult to explain the reason for such differences. It is possible that impurities present in the sample (early eluting peaks in Figure 1) could have contributed to the greater inhibition observed, even though present at low levels. Also, test solutions of compounds isolated by TLC were not buffered as were authentic ferulic acid solutions. From personal experience we have observed greater phytotoxicity in nonbuffered versus buffered solutions of ferulic acid, probably due to a pH effect. This increase, however, is small and probably could not account for the large difference in phytotoxicity between the two bioassays. In addition, there may be slight differences in properties of ferulic acid produced naturally and that obtained commercially. For example, ferulic acid exists as two isomers, *cis* and *trans*. Commercial ferulic acid is predominantly *trans*. Ferulic acid in plants is believed to be primarily *trans* but can exist as a mixture of both isomers (Engelsma, 1974; Kahnt, 1967). It is not known if one or the other is more phytotoxic.

With the extraction procedure we employed (Guenzi and McCalla, 1966a), ferulic acid was isolated as the most inhibitory component in wheat straw. There could also be other unknown compounds in the straw which would not be evident with this procedure. In addition, we ignored the possible influence of toxin-producing microorganisms. Microorganisms may have influenced the phytotoxicity exhibited by the aqueous wheat extract in Table 2. Although the present study was not concerned with the phytotoxic effects of microbially decomposed wheat straw, an influence of microbial activity on ferulic acid phytotoxicity was observed. From the results of Table 4, it appears that the presence of the prickly sida seed carpel enhanced the inhibitory effects of ferulic acid. At the completion of the ferulic acid bioassay with prickly sida, samples were taken from the ferulic acid test solutions in which prickly sida with and without carpels were germinated.

The samples were chromatographed by TLC as described earlier. When the developed TLC plates were exposed to iodine, the chromatogram spotted with the ferulic acid solution containing prickly sida seed with carpels produced two spots: one was ferulic acid and the other unknown. Only ferulic acid was detected on the chromatogram spotted with the ferulic acid solution containing prickly sida seed without carpels. The additional compound present in the ferulic acid test solution used to germinate prickly sida seed with carpels was determined to be 2-methoxy-4-ethenylphenol from a mass spectrum obtained of the compound. 2-Methoxy-4-ethenylphenol is formed



SCHEME 1.

by the decarboxylation of ferulic acid (Scheme 1). This styrene compound was produced by a bacterium present on the carpel of prickly sida seed. The decarboxylation of ferulic acid was detected in aqueous solutions of ferulic acid inoculated with the bacterium isolated from the carpels of prickly sida seed. No conversion occurred when the bacterium was not present.

It seems most likely that the presence of the styrene compound was at least partially responsible for the inhibition of prickly sida germination and root length, since ferulic acid alone (prickly sida seed without carpels plus ferulic acid) had no effect on prickly sida germination or root length (Table 4). The same mechanism may also explain the differences observed when prickly sida seed with and without carpels were bioassayed with caffeic acid. The decarboxylation of phenolic acids to corresponding styrenes is known from studies on fungi and bacteria (Finkle et al., 1962; Indahl and Scheline, 1968). However, in a number of studies directly concerned with the microbial decomposition of ferulic acid, as well as other phenolic acids, no mention is made of any styrene compounds produced as a result of phenolic acid decarboxylation (DiMenna, 1959; Henderson, 1963; Henderson and Farmer, 1955; Turner and Rice, 1975).

Although ferulic acid was shown to be inhibitory to morning glory in a soil system, levels required to produce phytotoxic effects appear to be relatively high compared with concentrations of ferulic acid and other phenolic acids found in the soil. Workers have postulated, however, that phytotoxic substances are not evenly distributed in the soil, and high concentrations of these substances that might reach toxic levels are probably in localized pockets of decomposing residue fragments (Guenzi and McCalla, 1966b; Patrick et al., 1963). It is likely that relatively high concentrations of phytotoxic substances may accumulate at or near the soil surface in no-till situations as rain would continually leach such chemicals from the mulch into the soil.

It is unlikely that any one particular compound could be responsible for reduced weed growth in no-till. Higher plants and microorganisms produce a myriad of phytotoxic substances. If these substances are present in the right combination and concentration, phytotoxic effects may be observed. With the proper choice and management of various cover crops and plant residues, it

may be possible to supplement if not reduce the number and amount of herbicides used in no-till cropping systems by utilizing natural phytotoxic substances.

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RYE RESIDUES CONTRIBUTE WEED SUPPRESSION IN NO-TILLAGE CROPPING SYSTEMS[1]

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Abstract—The use of allelopathic cover crops in reduced tillage cropping systems may provide an ecologically sound and environmentally safe management strategy for weed control. Growers often plant winter rye (*Secale cereale* L.) for increased soil organic matter and soil protection. Spring-planted living rye reduced weed biomass by 93% over plots without rye. Residues of fall-planted/spring-killed rye reduced total weed biomass over bare-ground controls. Rye residues also reduced total weed biomass by 63% when poplar excelsior was used as a control for the mulch effect, suggesting that allelopathy, in addition to the physical effects of the mulch, did contribute to weed control in these systems. In greenhouse studies, rye root leachates reduced tomato dry weight by 25–30%, which is additional evidence that rye is allelopathic to other plant species.

Key Words—Rye (*Secale cereale*), conservation tillage, allelopathy, mulch.

INTRODUCTION

Concern over destructive effects of current cultural practices and high energy inputs into agroecosystems mandates the need for improved cultural systems for crop production. Zero-tillage, sod planting, mulch planting, plot planting, no-till, reduced tillage, minimum tillage, or conservation tillage are various names given to reduced tillage production systems. In contrast to clean cultivated fields, these sites are generally covered with some plant residue which provides reduced soil erosion and improved water retention. Crop seeds, or transplants, are placed into the soil through the sod or previous crop

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residues by creating a slot wide and deep enough to receive and cover the seed or transplant (Young, 1973). Early attempts with the system were often failures because undecomposed residues in close association with crop seeds appeared to retard germination (Rutherford, 1976).

Basic to the no-tillage (NT) system is the use of a cover crop. Frequently called "smother crops," they have often been planted to help suppress weed growth (Overland, 1966). Potential smother crops include barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.) sorghum (*Sorghum bicolor* L.), buckwheat (*Fagopyrus esculentum*), sudangrass (*Sorghum vulgare* L.) sweet clover (*Melilotus alba* Desr.), and sunflower (*Helianthus annuus* L.). Overland (1966) suggested that allelopathy contributed to barley interference. Almost all the cover crop species listed above have been reported to be allelopathic to certain test species (Rice, 1979).

Allelopathic effects appear to be especially significant in natural communities with a strong dominance of a single species (Whittaker, 1975). Since most agroecosystems consist of vast monocultures of crops, allelopathy may also play a role in these manipulated ecosystems. Many of the early investigations into allelopathy were a result of crop phytotoxicity problems observed in agriculture. McCalla and Duley (1948, 1949) published two papers on the effects of decaying wheat (*Triticum vulgare* var. Mida) residues on corn (*Zea mays* L.) growth. In many instances yields were reduced in stubble mulch farming, suggesting that the detrimental effect of crop residues might be due to a combination of toxins released from residues and from microorganisms that were caused to grow more profusely by substances in the residues. Later, Norstadt and McCalla (1963) isolated fungi from stubble mulched field plots which produced patulin, which is toxic to corn plants. The organism was later identifed as *Penicillium urticae* Bain., and was found to comprise 90% of the total soil fungal population (Ellis and McCalla, 1973).

Hill (1926) noted that the addition of green rye to heavy soils depressed corn growth, while growth was increased in light soils. Roots were more toxic than tops. Patrick and Koch (1958) found that decomposing residues of rye (*Secale cereale* L.) were very inhibitory to respiration of tobacco seedlings. Patrick (1971) identified several compounds toxic to lettuce and tobacco in decomposing residues of rye. DeFrank (1979) noted toxicity from several cover crop residues, including winter rye.

Kimber (1973) found cold extracts of several grasses, including slightly green rye straw that had rotted for periods up to 21 days, to inhibit growth of wheat grown under aseptic conditions. Sterile conditions were used to eliminate the possible interactions of pathogens and microbial products. He found slightly green straw to be more toxic than fully matured residue. The most toxic materials found were from extracts of rye straw which had decayed for four days.

Our field evaluations were aimed at determining the response of weeds and vegetable crops to rye residues in no-tillage situations. Greenhouse studies were primarily concerned with separating the various components of plant interference to determine if allelopathy by rye is responsible for the noted weed reductions. In addition, several studies evaluated two cultivars of rye to determine if allelopathic activity was under genetic control.

METHODS AND MATERIALS

Field Evaluations of Rye Toxicity. In one test, winter rye (MSU-13) was drilled in a Spinks loamy sand on May 21, 1980, at a rate of 140 kg/hectare. To evaluate the effect of a living rye cover on weed density, five areas were sampled 34 days after planting. Forty-one days after planting rye, weeds were harvested from 1.0-m^2 areas, and dried at 50–60° C for biomass determinations.

In another experiment, two selections of winter rye, Wheeler and MSU-13, were drilled in a Marlette fine sandy loam (2–6% slope) on October 10, 1980, at a rate of 168 kg/hectare. Granular ammonium nitrate (168 kg/hectare) and weed seeds (*Setaria lutescens*, 650/m^2; *Chenopodium album*, 1200/m^2) were overseeded across fall-sown cover crop areas with a cyclone spreader before covers were killed in spring. Rye was killed with 1.12 kg/hectare glyphosate (*N*-phosphonomethyl glycine) on May 5, 1981, at the preboot stage. Residue biomass was 4.9 metric tons/hectare. Peas (*Pisum sativum* L. cv. Sparkle) inoculated with *Rhizobium* spp. (38 seeds/m) were planted NT through the early killed rye residues on May 26, 1981, or 21 days after kill. Poplar excelsior (PE) was then laid in control plots to simulate physical impacts of the mulch. It was applied to plots on an equal weight basis. Previous greenhouse experiments had indicated that PE was a suitable control for the mulch effect. Plastic netting (2.5 × 5.5 cm) was used to secure PE to the ground. All plots were 3.1 × 3.1 m with six rows of crop spaced 34 cm apart. Forty-six days after kill (25 days after planting peas), weeds from four sample areas were counted and harvested. Forty-eight days after planting, stand, fresh plant, pod, and pea weights were recorded.

All plots were planted with the Moore-Uni-Drill, a specialized no-tillage seeder. The drill was also pulled through unplanted control plots to provide similar physical influences caused by planting. The experimental design for both tests was a randomized complete block with four replications.

Evaluation of Poplar Excelsior (PE) as a Nontoxic Control Mulch. In a greenhouse study of surface mulches, 30 seeds of both Petoearly tomato (*Lycopersicum esculentum* Mill. cv. Petoearly) and barnyardgrass (*Echinochloa crusgalli* L. Beauv.) were planted in Spinks loamy sand [≃1.0% om (organic matter)] in 10 × 15 cm styrofoam pots. PE, vermiculite, and peat were applied over planted seeds at rates which produced light reductions

equivalent to 4.4 g of rye residue or 450 μE/cm^2/sec. The sensor or a LI-COR quantum/radiometer/photometer were placed in a pot with a glass plate over the top to determine light reduction measurements. Weights of mulches which provided specific light reductions were determined and utilized as a basis for further applications. Plants were grown in the greenhouse under 16 hr of metal halide light. The experimental design was a randomized complete block with four replications. To determine if poplar excelsior was an adequate control for the mulch effect, and whether it adversely affected plant growth, germination counts of tomato and barnyardgrass were taken 7 days after planting (DAP). Rows were also thinned to 10 plants per row at this time. In addition, barnyardgrass and tomato were harvested 14 and 19 DAP, respectively, dried at 50–60°C, and weighed to evaluate the effect of mulches on dry weights of plants.

Evaluation of Residue Toxicity in Rye Killed Back at Different Ages. Two cultivars of rye MSU-13, screened from a portion of the world's collection, and Wheeler rye, a standard cultivar in Michigan, were planted into plastic flats (25 × 25 × 7.5 cm) containing a Spinks loamy sand and grown under metal halide lighting (500 μE/cm^2/sec). Rye was planted at 10-day intervals over a 30-day period. While growing, rye was watered with soluble fertilizer (1.0 g/liter of Peters soluble fertilizer 20% N, 20% P, 20% K) every other day and was weeded prior to herbicide treatment. Unplanted controls of PE were watered, weeded, and fertilized as rye. The youngest rye treatment was 20 days old at the time when 1.12 kg/hectare of glyphosate was applied. Sprayed and unsprayed PE were utilized as controls for the chemical and mulch effects. The rate of PE was determined from residue production in the 50-day-old rye treatment since the mulch effect would be greatest in this treatment.

To evaluate residue toxicity, indicator species of weeds and crops were planted into the residue 10 days after herbicides were applied to rye. It was necessary to develop a system to sow the indicator seed through the residue in a manner similar to a NT situation in the field. To facilitate accurate seed placement and to ensure good seed–soil contact necessary for germination, a planting board was designed to evaluate the response of four test species at one time. Four rows, with ten holes each, were drilled into the board. Plastic syringes (5 cc) from which the tip was cut off, were inserted through the drilled holes. The plungers were used to push seeds down through the residue to a uniform depth in the soil. Control flats were also planted with the board, although the physical characteristics of the PE necessitated removing it during planting. In all cases, the experimental design was a randomized complete block with four replications. To assess germination, the number of plants which emerged out of 30 seeds planted were recorded. Plants were then thinned to 10 plants per row. Later, shoots were harvested, dried at 50–60° C,

and weighed. Rye residue (biomass) was also determined to see if germination or plant growth correlated with the quality of residues present.

Evaluation of Rye Root Leachates on Plant Growth. Generally, rye and indicator species were grown in quartz sand in the greenhouse under metal halide lamps (16-hr days). To ensure no nutrient or water deficiencies, all plants received half-strength Hoagland's solution (Hoagland and Arnon, 1939), adjusted to pH 6.5. All controls were monocultures of indicator species which also received Hoagland's solution.

To evaluate rye root leachate toxicity, rye and indicator species were grown in separate pots (10 cm diameter) and half-strength Hoagland's solution was manually transferred through a four-pot system. The associations consisted of either a monoculture of four pots of the indicator species or a biculture of two pots of rye and two pots of the indicator species. It was necessary to cover the pots with plastic film until emergence because of the low moisture holding capacity of the quartz sand. When growth of algae became a problem, a chelated copper algacide, Cutrene (0.05% v/v), was applied with the half-strength Hoagland's solution. The quantity and quality of light was similar for all pots in the series. Indicator species tested were lettuce (*Lactuca sativa* L. cv. Ithaca) and Petoearly tomato, and they were harvested 30 DAP for biomass evaluation. In all cases, the experimental design was a randomized complete block with four replications. Roots were separated from quartz sand and dried at 50–60° C for biomass determinations. In addition, shoots were harvested and dried for individual biomass determination.

In a second study, the four-pot series of transfers was used to evaluate the effect of age of rye on root leachate toxicity. This involved testing 30-, 20-, 10-, and 0-day-old rye at time of planting indicators. Two selections of rye (Wheeler and MSU-13 selected at Cornell University) were evaluated for their effect on biomass of Petoearly tomato. Pots of rye which had been growing for 20 and 30 days developed algae growth on the outside; therefore all pots were scrubbed with a solution of Cu chelate (0.05% v/v) and rinsed with water prior to planting tomatoes. Tomatoes were harvested 21 DAP.

RESULTS AND DISCUSSION

Field Evaluation of Rye Toxicity. A living cover of spring-planted winter rye reduced early season biomass of common lambsquarter (*Chenopodium album* L.) by 98%, large crabgrass (*Digitaria sanguinalis* (L.) Scop.) by 42%, and common ragweed (*Ambrosia artemisifolia* L.) by 90% over unplanted controls (Table 1). There was no significant difference in the individual and total densities of these weeds, which may have been due to a large variablility in the natural weed population. In contrast, the total weed biomass per square

TABLE 1. EFFECT OF SPRING-PLANTED LIVING RYE COVER CROP ON EARLY SEASON BIOMASS/ M^2 OF LARGE CRABGRASS, COMMON RAGWEED AND COMMON LAMBSQUARTERS IN SPINKS LOAMY SAND

Cover crop	Large crabgrass (g/m^2)	Common ragweed (g/m^2)	Common lambsquarters (g/m^2)	Total[a] (g/m^2)
No rye	12	21	165	265
MSU-13 rye	7	2	4	16

[a] Means are significantly different at the 5% level.

meter was reduced 94% over unplanted plots. Direct rye–weed competition for water, light, and nutrients, in addition to allelopathic chemicals released from rye roots and shoots, may contribute to the marked interference.

Weed control and pea yields were not different under residues of fall planted Wheeler rye, a standard cultivar planted in Michigan, and MSU-13 rye (Table 3). Although densities of barnyardgrass and redroot pigweed did not differ under rye residues when compared to PE mulch, barnyardgrass biomass was reduced up to 74% and redroot pigweed biomass was reduced up to 55% under rye residues (Table 2). Total weed biomass in NT peas was reduced up to 73% under rye residues when compared to the mulch control, while total weed density remained the same. Yields of peas were not affected by rye residues (Table 3). Thus, rye residues appear to suppress total weed growth, but not weed germination or the germination, growth, or yield of peas.

In general, weeds and crops responded similarily to residues of the two rye selections. Fall-sown rye cover-crop residues suppressed weed growth which suggests that allelopathy, in addition to the physical presence of the mulch, is responsible for the weed biomass reductions. Pea yields were not reduced under rye residues, suggesting that they may be successfully managed

TABLE 2. EFFECT OF EARLY-KILLED RYE RESIDUES ON BARNYARDGRASS, REDROOT PIGWEED, AND TOTAL BIOMASS PER 1.0 M^2 IN NO-TILL PEAS ON MARLETTE FINE SANDY LOAM[a]

Cover	Barnyardgrass (g)	Redroot pigweed (g)	Total (g)
Poplar excelsior	50.2 b	1.4 b	51.9 b
MSU-13 rye	22.0 a	0.9 a	23.9 a
Wheeler rye	12.7 a	0.5 a	13.9 a

[a] Means within a column followed by the same letter are not significantly different at the 5% level by Duncan's multiple-range test.

TABLE 3. YIELD RESPONSE OF NO-TILL PLANTED PEAS TO RYE RESIDUES IN MARLETTE FINE SANDY LOAM[a]

Cover	Stand (no.)	Fresh wt (kg)	Pod wt (kg)	Pea wt (g)
Poplar excelsior	141	1.0	0.60	276
MSU-13 rye	132	0.9	0.54	256
Wheeler rye	134	1.1	0.60	283

[a] Four meters of crop row were harvested. Means are not significantly different at the 5% level.

in a NT rye cover-crop system. A fall-sown rye provides soil protection during winter and allows for timely management of crop production in spring. Although spring-sown rye also reduced weed biomass, production of early season crops, such as peas, is not always feasible since rye will not have attained sufficient growth.

Evaluation of Poplar Excelsior (PE) as a Nontoxic Control. The physical presence of the mulch cover (which changes environmental conditions at the seed–soil interface) may influence the growth of other plant species (Phillips, 1973). To separate the allelopathic effects from the physical effects, a control for the mulch effect is desirable. Poplar excelsior (PE) has been used to stabilize new plantings along roadsides while the grass seed becomes established. Its appearance is more similar to rye residues than either vermiculite or peat, two common horticultural mulches, and it can be more easily handled for field application. Therefore, it was necessary to determine if poplar excelsior had any adverse effects on plant growth.

In this study PE did not differentially affect percent emergence of both barnyardgrass and tomato when compared to other mulch treatments or a no-mulch control (Table 4). Similarly, the dry weight per plant under PE was not statistically different from the other treatments. Thus, PE did not appear to adversely affect plant emergence or growth when compared to other commonly used horticultural mulches and was therefore selected as our control for the physical presence of the mulch on the soil surface.

Evaluation of Residue Toxicity in Rye Killed Back at Different Ages. Greenhouse experiments were initiated to define more clearly the nature of interactions of rye residues with weeds and vegetable crops observed in the field. For greenhouse studies to be meaningful, it is important to simulate field conditions as closely as possible. Detection of allelopathic chemicals is difficult due to the ephemeral nature of the products (Patrick, 1971). Since growth regulators are present in plants in extremely small quantities, bioassays are frequently the only methods of analysis sensitive enough for detection of leached compounds (Tukey, 1969). Phytotoxic materials liberated

TABLE 4. PERCENT EMERGENCE AND DRY WEIGHT PER PLANT (mg) OF BARNYARDGRASS AND TOMATO IN SEVERAL SURFACE MULCHES[a]

Mulch	Emergence		Dry wt/plant	
	Barnyardgrass (%)	Tomato (%)	Barnyardgrass (mg)	Tomato (mg)
None	80	68	24	60
Vermiculite	83	63	24	63
Peat	76	83	25	61
Poplar excelsior	88	72	21	63

[a] Means within a column are not significantly different at the 5% level by Duncan's multiple-range test.

by plants or plant residues may gradually accumulate and inhibit further growth of plants (McCalla and Haskins, 1964).

Residues of Wheeler rye reduced emergence of both lettuce and yellow foxtail in a linear fashion as the age of the rye at time of kill increased from 20 to 50 days (Figure 1). There was also a positive correlation between residue biomass and age of rye at time of kill. Percent emergence of lettuce was correlated with rye residue biomass, suggesting that the amount of residue may influence emergence.

There was no significant difference in plant dry weight in controls of glyphosate-sprayed or -unsprayed poplar excelsior. Generally, as the age of rye at time of kill increased from 20 to 50 days, all plant growth was reduced (Figure 2). While the residue biomass increased from 14 to 29 g/flat, there was no significant linear correlation between the quantity of rye residue and plant dry weight. Growth was suppressed the most in 50-day-old rye residues. The presence of rye residues accounted for most of the variability in germination and growth indicators, suggesting that allelopathy may be a component of the total interference noted in the field. Since there were controls for both the chemical and mulch effects, residue toxicity could be the result of either natural toxins leached from rye or from microbial intermediates produced during the decomposition process.

In this study, the NT bioassay for greenhouse evaluation of rye residues appeared to be sensitive enough to detect differences in germination and plant growth of the species investigated. Rye residues reduced germination and growth of several plant species.

All treatments were fertilized on a regular schedule, and thus it was assumed that the nutritional conditions of the soil were not significantly different under rye residues when compared to PE. It is important to remember that conditions associated with greenhouse culture of plants are not identical to field conditions. Direct observations of root and shoot morphology

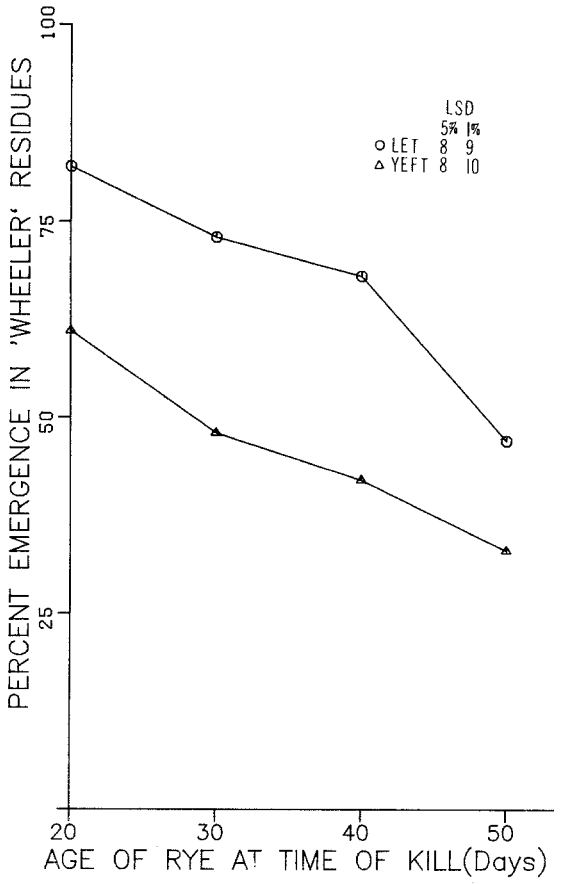

FIG. 1. Percent emergence of yellow foxtail (YEFT) and lettuce (LET) in residues of Wheeler rye killed back at different ages. Yellow foxtail was counted 13 days after planting (DAP) and lettuce 11 DAP.

revealed fall-planted field-grown rye to differ considerably from greenhouse-grown winter rye. Root growth is restricted and soil moisture conditions in pot culture are also radically different from field conditions (Spomer, 1975, 1976). In addition, quantity and quality of light in the greenhouse, diurnal temperature fluctuations, and microbial activity, which may influence allelopathic activity of rye residues, also all vary from field situations (Rice, 1979).

While the greenhouse environment may not be identical to the field, these results suggest that the rye may influence the growth of other plant species through leaching of chemicals from the plant residues or indirectly by microbial products formed upon decay.

Evaluation of Rye Root Leachate Toxicity. An old Swedish agricultural

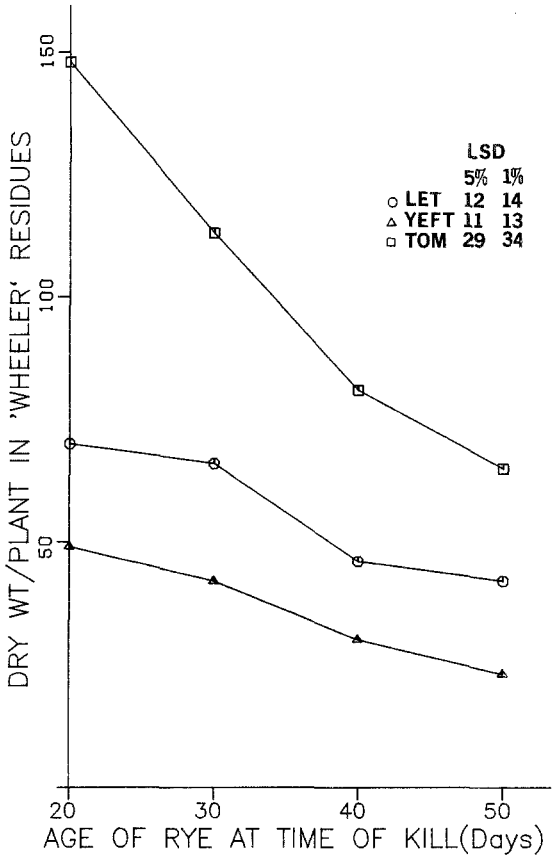

FIG. 2. Biomass of lettuce (LET), tomato (TOM), and yellow toxfail (YEFT) in residues of Wheeler rye killed back at different ages. Lettuce was harvested 19 days after planting (DAP); tomato and yellow foxtail were harvested 22 DAP.

practice to rid fields of infestations of wild oats (*Avena fatua*) is to plant rye. Osvald (1953) found rye root exudates reduced the germination of wild oats, while exudates from barley and wheat had no effect. Root exudate is often used in a broad sense, but generally refers to inhibitors resulting from the presence of living roots where no leachates, volatiles, or residues from the tops of plants are present (Rice, 1979). The potential for root exudation by rye exists and may play a role in its inhibitory activity.

In the initial transfer study, MSU-13 rye and indicator species were grown in separate pots and Hoagland's solution was manually transferred through the four-pot series. In the biculture treatment of rye and test species, indicator pot 1 received leachates from one pot of rye only. In contrast,

TABLE 5. EFFECT OF RYE ROOT LEACHATES ON BIOMASS AS PERCENT OF CONTROL

	Lettuce		Tomato	
Pot	Shoot	Total	Shoot	Total
1	94	100	97	105
2	73[a]	75[a]	86	82[a]

[a] Percent biomass is significantly different from control at the 5% level.

indicator pot 2 received leachates from two pots of rye. Each pot of rye contained four plants with an average dry weight of 142 mg. Root, shoot, and total biomass of lettuce and tomato were unaffected by leachates from one pot of rye (Table 5). In contrast, shoot and total biomass of lettuce were reduced 27% and 25%, respectively, when it had received leachates from two pots of rye. Total biomass of tomato was reduced by 18% where solutions had passed through two pots of rye. This indicates that root leachates of rye are more inhibitory to tomato and lettuce growth than leachates from other tomato and lettuce plants. Since biomass was reduced where solutions had passed through two pots of rye, it may also suggest a concentration effect of rye root toxins.

In a second experiment, where rye root leachates of different aged ryes were manually transferred, root, shoot, and total biomass of tomato were again reduced in pot 2 (Table 6). Both rye cultivars at all ages similarly reduced root and total biomass of tomato in pot 2.

This study suggests that inhibitory compounds from rye roots may be released and taken up by other plants. However, without radioactive tracer studies, it is very difficult to determine the exact origin of the compounds (Rice, 1974). Toxicity of leachates may result from compounds sloughed off from outer cells or produced by microbial acticity, in addition to those actually exuded by the roots. It appears that living rye interferes more with plant growth, rather than with the processes associated with germination.

TABLE 6. EFFECT OF RYE ROOT LEACHATES ON BIOMASS OF TOMATOES AS PERCENT OF CONTROL[a]

Pot	Root	Total
1	68**	70**
2	71**	75*

[a] Means were averaged across cultivars and age of rye. Single asterisk indicates percent biomass is significantly reduced from control at the 5% level. Double asterisk indicates percent biomass is significantly reduced from control at the 1% level.

Since effects of direct plant–plant competition were minimized, adequate nutrients and water were always available, and quality and quantity of light received were equal, the reductions in biomass of tomato and lettuce are evidence that rye root leachates are inhibitory to growth of tomato and lettuce. Thus, in addition to toxicity from residues, allelopathy from living rye root leachates may be a component of the total interference noted between weeds and rye in the field.

Phytotoxicity problems occurring where winter rye was present could be due to the accumulation of growth inhibitors in the soil. According to Chapman (1966), root excretions, leaf washings, plant residues, and microbial activity on plant residues, all contribute to the presence of growth inhibitors in soils. Chou and Patrick (1976) identified nine compounds in decaying rye residues including vanillic, ferulic, phenylacetic, 4-phenylbutyric, *p*-coumaric, *p*-hydroxybenzoic, salicylic, and *o*-coumaric acids, and salicylaldehyde. Most of these were found inhibitory to germination in the lettuce seed bioassay. In addition, it has been clearly shown that numerous kinds of organic compounds can exude from roots of donor plants and can be taken up by adjacent plants (Rovira, 1969; Foy et al., 1971).

Scientists need to integrate methods to provide an ecologically sound and technically feasible weed management approach for vegetable crops. The use of allelopathic cover crops—specifically rye (*Secale cereale* L.)—for weed control in no-tillage systems may provide an environmentally safe and ecologically sound management strategy. The objective is to capitalize on the chemical and physical attributes of the cover crops for weed control, as well as to exploit other water and soil conservation advantages.

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ALLELOPATHIC EFFECTS OF ALFALFA

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Abstract—Several experiments have been conducted at the University of Illinois, Urbana, Illinois, to determine the role of allelopathic (autotoxicity) effects of alfalfa (*Medicago sativa* L.) on the establishment of alfalfa. By means of correcting any soil fertility differences and protecting the alfalfa seedlings from fungal attack, results suggest that there is a release of phytotoxic factors from the previous crop on reestablishing alfalfa. Field results indicate that the best preceding crop for alfalfa establishment is corn (*Zea mays* L.), followed by various small grains, soybeans (*Glycine max* L.), and the worst preceding crop is alfalfa. Results indicate there were no major genetic differences among cultivars for resistance to autotoxicity. There was no evidence supporting saponins as being phytotoxic to alfalfa. Exudates of different saponin level cultivars indicated that saponin content is not the suspected phytotoxic factor.

Key Words—Allelopathy, autotoxicity, establishment, *Medicago sativa* L., *Zea mays* L., *Glycine max* L., small grains, saponins, alfalfa yields.

INTRODUCTION

In the past, plant interactions have been explained in physical terms by assuming that competition for light, space, water, and nutrients were the major factors. More careful measurements tend to suggest that many plants are given selective advantage by means of allelopathic activity. Allelopathy is any direct or indirect harmful effect by one plant on another through the production of chemical compounds that escape into the environment (Rice, 1974).

One of the most studied areas of allelopathy is its role in old field succession and other natural ecosystems (Rice, 1974). In recent years, allelopathy has been implicated in agricultural situations as well. Due to the increased interest in various agricultural systems where plant interactions are

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critical, knowledge of allelopathy has become a necessity. These systems include interplanting, double cropping, conservation tillage, and limited or nonrotational cropping.

Alfalfa (*Medicago sativa* L.), a long-lived perennial, is one of the most important legumes used for livestock feed and soil improvement. Under limited rotation of cropping in central Alberta, Canada, alfalfa was difficult to reestablish (Webster et al., 1967). Typical plant symptoms in the field were: dwarfed, spindly, yellowish green plants with irregular brown-reddish to dark-brown lesions on the tap and lateral roots and a few ineffective nodules (Webster et al., 1967).

Results of field and greenhouse studies (Webster et al., 1967; Webster and DeKock, 1969) indicate that neither macro- nor micronutrient deficiencies were responsible for the stunted growth of alfalfa. Steam sterilization or sterilization with vapam and gamma radiation increased alfalfa yields, suggesting that the inhibitory factor was biotic. Aqueous extracts from affected soils added to alfalfa growing in sand culture significantly reduced the yield (Webster et al., 1967).

Nielsen et al. (1960) investigated the effects of aqueous extracts of alfalfa hay (50% bloom), timothy hay (50% bloom), and mature corn stover, oat straw, and potato vines on seed germination and seedling growth of corn (*Zea mays* L.), soybeans (*Glycine max* L.), peas (*Pisum sativa* L.), oats (*Avena sativa* L.), alfalfa (*Medicago sativa* L.), and timothy (*Phleum pratense* L.). Alfalfa caused the greatest delay in germination of all test species except one; the germination of timothy was delayed the longest with timothy extract. Thus, Nielsen definitely demonstrated that certain crop plants (especially alfalfa) contain water-soluble materials that inhibit seed germination and seedling growth of several crop plants.

In Nebraska (Guenzi et al., 1964), it was found that alfalfa contains water-soluble substances toxic to itself and to other plants, and water extracts of immature alfalfa forage had the highest phytotoxic effects on corn seedlings in a laboratory study. Soil incorporation of young alfalfa forage from old plants while interseeding alfalfa to thicken old stands may introduce water-soluble substances toxic to new alfalfa seedlings.

Guenzi et al. (1964) had implicated saponins as being a possible water-soluble phytotoxic substance released by alfalfa forage. Aqueous solutions of alfalfa root saponin have been shown to inhibit the germination of cotton seeds (Marchaim et al., 1975). However, aqueous extracts of alfalfa root saponin did not inhibit the germination of alfalfa.

Work at Illinois (Klein and Miller, 1980) indicated that alfalfa was difficult to reestablish without rotating to another crop first. When alfalfa was plowed in the fall and reseeded with alfalfa the following spring, yield and stand count decreased each year. The yield and stand counts of alfalfa in the single cropping situation were markedly lower than when rotated with either

corn or corn and soybeans. Pathologists concluded that pathogen infestation could not account for these results.

The purpose of the present investigations was to gain information on the factors responsible for the difficulties in reestablishing a weakened stand of alfalfa and to determine whether such problems are related to improper management, plant diseases, soil fertility, or to the release of phytotoxic substances by alfalfa. If the reestablishment problem could be attributed to autotoxic substances, we hoped to determine whether genetic tolerance for the toxic factor existed, and whether saponins were the toxic factor. A further purpose was to determine whether phytotoxic substances, exuded from fall dormant cultivars, along with the proper environmental stimuli (cold nights and short days) would result in fall dormancy.

METHODS AND MATERIALS

Experiment One. Two sites at the University of Illinois, Urbana-Champaign, Agronomy South Farm were selected for conducting experiment 1. The two sites were separated by a distance of approximately 300 m. At one site an alfalfa variety trial had been conducted the previous five years, while the second site had been planted in soybeans in 1975 and corn in 1976. Soil type at the former alfalfa site was an Aquic Argiudolls, fine, montmorillonite, mesic, while the soil at the corn–soybean site was an Aquic Arguidolls, fine, silty, mixed mesic. The seedbed of each site was prepared with a corrugated field roller producing firm seedbeds. Seeds of the cultivars listed in Table 1 were planted May 12, 1977. The cultivars were mechanically broadcast on plots 152 × 366 cm at the rate of 20 kg/hectare.

Plant populations (plants/meter^2) were determined June 14, 1977; September 8, 1977; and subsequent to the first harvest in 1978. Populations were based on the number of plants in a 1-m^2 area. Three population samples were taken per plot, with one sample taken at each end and one toward the middle of the plot. Forage harvests were scheduled when the alfalfa was $\frac{1}{10}$ to $\frac{1}{2}$ bloom. The center 92 cm of each plot was harvested and dry matter yields determined. Harvest dates were September 19, 1977; June 6, 1978; and August of 1978. Additional harvests were scheduled in 1978, but yield data were not recorded due to the weakened condition of the stands at both sites.

Soil pH, P, and K levels, carbon–nitrogen ratios, soil moisture, and the incidence of the diseases, *Phytophthora megasperma*, *Pythium* sp., and *Fusarium oxysporum*, were determined from soil samples from each site (Table 2). Pathogen incidence was determined by procedures employed by the University of Illinois Plant Clinic. Diseased seedlings were planted on both potato–dextrose agar and Schmidhenner's–water agar and were subsequently inspected for signs of the three mentioned diseases (Graham et al., 1979). The

TABLE 1. CHARACTERISTICS OF VARIOUS ALFALFA STRAINS, EXPERIMENT ONE

Strains[a]	Saponin content	Fall growth[b]
Ladak	Low	Pronounced fall dormancy
Ladak	High	Pronounced fall dormancy
Uinta	Low	Slight fall dormancy
Uinta	High	Slight fall dormancy
DuPuits	Low	Slight fall dormancy
DuPuits	High	Slight fall dormancy
Ranger	Low	Moderate to pronounced fall dormancy
Ranger	High	Moderate to pronounced fall dormancy
BWR synthetic	Low	[c,d]
BWR synthetic	High	[c,d]
Saranac AR	[c,d]	Moderate to pronounced fall dormancy
Vernal	[c,d]	Pronounced fall dormancy

[a] Seed source: M.W. Pedersen, Logan, Utah.
[b] Fall growth characteristics according to Smith (1975).
[c] Not included in comparative trials.
[d] Information not available.

percentage of seedlings exhibiting signs of any of the three diseases was recorded. Soil moisture was determined by weighing the soil samples, placing them in an oven at 100° C for 65 hr, and reweighing.

In the spring of 1978, 272 kg/ hectare of P_2O_5 and 710 kg/ hectare of K_2O were applied to the alfalfa variety trial site and 141 kg/ hectare of P_2O_5 and 471 kg/ hectare of K_2O were applied as a top dressing to the soybean–corn site.

The insecticide Sevin was applied at a rate of 454 kg/ hectare active ingredient one week after each harvest.

Prior to the first harvest in 1977, substantial grass and broadleaf weed infestation occurred at both sites. Hand cultivation and the grass herbicide Dowpon M were employed to control weed infestation. Dowpon M was applied June 30, 1977, as a postemergence treatment at a rate of 2270 kg/ hectare at 84% active ingredient. Both sites were irrigated July 17, 1977, with approximately 3.8 cm of water.

Experiment Two. A site was selected at the University of Illinois Agronomy South Farm on which forage management practices had been investigated the previous four years. Soil type at this site was Aquic Argiudolls, fine, silty, mixed mesic. In 1974, eight tiers were planted—three were solid seeded to Vernal alfalfa, one to a smooth brome–Vernal alfalfa mixture, and four were planted in forage grasses, resulting in a stand density of 43%, 32%, and 21% alfalfa. Various cutting schedules were conducted on the eight tiers resulting in forage stands of varying densities and levels of weed infestation.

TABLE 2. SOIL TEST RESULTS FOR SOIL SAMPLED FROM ALFALFA VARIETY TRIAL AREA AND SOYBEAN–CORN ROTATION AREA, EXPERIMENT ONE[a]

Area	pH	P_1 test (kg/hectare)	K test (kg/hectare)	C–N ratio	Soil moisture (%)	Pathogen incidence (%)
Alfalfa variety trial	6.40 ± 0.15	58.6 ± 15.4	247.7 ± 36.3	12.4 ± 0.5	17.1 ± 2.2	5.5 ± 0.04
Soybean–corn rotation	6.65 ± 0.15	54.7 ± 15.4	261.4 ± 36.3	12.3 ± 0.5	21.2 ± 2.2	3.8 ± 0.04

[a] Mean values and standard errors presented, $N = 12$.

Soil samples were collected from the site and tested as described in experiment 1. Prior to seeding, maintenance and build-up of P and K fertilizers were applied according to accepted guidelines. The herbicide Tolban was applied as a preplant treatment at a rate of 2.3 liters/hectare to control grassy weeds. The seedbed was prepared in the same manner as in Experiment 1. Cultivars Vernal, WL318, and WL306 were treated with captan and subsequently planted June 15, 1978, in 152 × 457-cm plots. The plots were positioned within the boundaries of the previous year's cutting treatments (tiers). The broadleaf herbicide, 2,4D-B, was applied as a postemergence treatment at an accepted rate prior to the first harvest in 1978. Plots were irrigated on June 15 and 16 with approximately 2.5 cm of water each day.

Seedling stand densities per square meter were recorded June 27, 1978. Forage harvest dates were September 28, 1978, and May 31 and August 15, 1979. One week after each harvest, the insecticide Sevin was applied.

Prior to the first harvest in 1978, heavy grass infestation occurred. Hand cultivation was employed to control the grass.

Statistical Analyses. The experimental designs for the two experiments were: (1) experiment 1 was a randomized complete block design for both the former alfalfa area and the soybean–corn rotation area with five replicates in 1977 and four remaining in 1978; and (2) experiment 2 was conducted as a randomized complete block design for each of the eight tiers with four replicates per tier. A combined analysis over areas (or tiers) was conducted for each experiment, to determine the significance of rotational differences and the significance of the interaction of the cultivars with rotations. For experiment 2, the following rotation (tier) comparisons were made: (1) the linear and quadratic effects of stand density of the previous alfalfa crop; and (2) the comparison of those tiers formerly established in alfalfa versus those tiers formerly established in forage grasses.

A stepwise regression procedure was used for experiment 1 to explain the variation in alfalfa performance among those plots from which soil was sampled June 15, 1977. Independent variables used in the equations were: soil pH, P and K levels, pathogen incidence, C–N ratios, and the location itself. Cultivar effects were not included in the regression procedure since plots of all the cultivars were not sampled. However, an equal number of plots of the selected cultivars were sampled from each field to eliminate any cultivar-location confounding. Variation associated with the location may include rotation differences or other confounding factors. A model was selected to best explain the variation in plant population and dry matter yields among the sampled plots.

Experiment Three. Soil samples were collected from 13 sites within each of the two field locations of field experiment 1. Soil sampling sites within each

location were: (1) the root zones of the 12 alfalfa strains planted in 1977 at each location (Table 1), and (2) the fallow area for each location.

Eight to 10 plants of each strain were excavated and the soil from the root zone of each plant recovered and placed into separate, labeled containers. Fallow soil was collected in similar manner from both locations. Soil was allowed to air dry in a greenhouse for four months. Soil pH, phosphorus, and potassium were determined for the soil samples. On January 26, 1978, 15 rhizobial-inoculated seeds of Vernal alfalfa were sowed in each pot at a depth of 2 mm. A 1-cm layer of quartz sand mulch covered the sand. A 300-ml solution of the fungicide Shield was applied immediately after planting. Subsequently, Shield was applied at three-day intervals until 50% of the seedlings were at the first trifoliate leaf stage.

Percent emergence was recorded 4 and 16 days after planting. Seedling height measurements were obtained 20 days after planting. Heights of individual seedlings were measured from the cotyledonary node to the first trifoliate leaf node. Densities were then reduced to four plants per pot. At 60-day intervals, leaves and shoots were harvested three times and oven-dried at 60° C for at least 48 hr prior to weighing.

Following each harvest, a solution of KH_2PO_4 was added to the soil in each pot to build up K and P to levels required for alfalfa growth. The amount of maintenance KH_2PO_4 to be applied was based on the quantity of K removed per pot rather than P, since greater quantities of K are removed per unit dry matter of alfalfa (Smith, 1975).

Diurnal temperature limits of 15/32° C and 16 hr of light were provided throughout the length of the experiment. Supplemental radiation was supplied by fluorescent tubes and incandescent bulbs.

The experiment was designed as a 2×13 factorial in a randomized complete block design with three replicates. The factors were: (1) the two locations from which soil was collected—locations cropped in alfalfa (1972–1976) or cropped in soybeans (1975) and corn (1976); and (2) the 13 excavation sites within each location—sites including the fallow area and the root zones of the 12 strains planted in 1977 at each location. The phytotoxic properties of the following soils were compared: (1) root zone soil of high saponin versus low saponin alfalfa strains; (2) root zone soil of fall dormant versus slightly fall dormant alfalfa strains; (3) root zone versus the fallow soils; and (4) soil cropped in alfalfa during the period from 1972 to 1976 versus soil cropped in soybeans in 1975 and corn in 1976.

RESULTS

Experiment One. Plant populations and dry matter yields were consistently greater where alfalfa was grown in rotation with corn and soybeans

TABLE 3. DRY MATTER YIELDS AND PLANT POPULATIONS OF ALFALFA IN TWO ROTATION SEQUENCES

Rotation sequence	Plant population (plants/m^2)			Yield (metric tons/ha)		
	6/14/77	9/8/77	6/6/78	9/19/77	6/6/78	9/15/78
Alfalfa–alfalfa	61.5	23.8	16.7	1.52	3.57	2.47
Soybean–corn–alfalfa	90.4	29.8	22.3.	2.24	4.29	3.04
Difference	−28.9**[a]	− 6.0**	− 5.6**	−0.74**	−0.72**	−0.57**

[a]**Significant, based on one-tailed T test, at the 0.05 level.

(Table 3). Populations and dry matter yields differed among the cultivars. These differences were similar for both rotation sequences except for the August harvest date. August yields of most cultivars were slightly greater for the soybean–corn–alfalfa rotation (Table 4). The exceptions were Vernal alfalfa which yielded essentially the same for both rotations, and DuPuits (low saponin) and BWR syn (low saponin) alfalfa which yielded substantially less in the continuous cropping situation.

The regression models selected by the stepwise regression procedure are presented in Table 5. Seventy-five percent of the variation in seedling populations was attributable to four independent variables: soil pH, soil P,

TABLE 4. DRY MATTER YIELDS (METRIC TONS/HECTARE) OF 12 ALFALFA CULTIVARS FOR TWO ROTATION SEQUENCES.

	Rotation sequence	
	Alfalfa–alfalfa[a]	Soybean–corn–alfalfa[a]
Ladak (low saponin)	1.8	2.5
Ladak (high saponin)	2.6	2.9
Uinta (low saponin)	2.6	3.2
Uinta (high saponin)	2.4	3.1
DuPuits (low saponin)	1.6	2.6
DuPuits (high saponin)	2.1	2.7
Ranger (low saponin)	2.5	3.1
Ranger (high saponin)	2.9	3.1
BWR syn. (low saponin)	2.3	3.6
BWR syn. (high saponin)	2.8	3.3
Saranac AR	3.1	3.6
Vernal	3.0	3.0

[a] Metric tons per hectare.

TABLE 5. INDEPENDENT VARIABLES SELECTED BY STEPWISE REGRESSION PROCEDURE AND CORRESPONDING PROPORTION OF VARIATION ACCOUNTED FOR BY VARIABLES, EXPERIMENT ONE[a]

Dependent variable	Soil factors							Location (rotation)	
	pH	P	K	C–N ratio	Moist.	Path.	R^b(%)		R^b(%)[c]
Plant pop. (6/77)	x	x				x	46.5	x	26.3
Plant pop. (9/77)						x	16.5		
Plant pop. (6/78)						x	29.5	x	31.0
Yield (9/77)								x	62.2
Yield (6/78)	x						34.2		
Yield (9/78)								x	32.1

[a] Marks under a given factor indicate selection by the stepwise regression procedure.
[b] The proportion of the variation attributable to the soil factors selected by the stepwise regression procedure.
[c] The proportion of the variation attributable to location (rotation) after adjusting for the selected soil factors.

pathogen incidence, and location. The location effect, which may be the result of a difference in rotation sequence, accounted for 26% of the variation in seedling population.

A mere 16% of the variation in the fall of 1977 alfalfa populations was accounted for by soil pathogen incidence (Table 5). No other independent variable accounted for a significant percentage of the variation. In contrast, soil pathogen levels and location together accounted for nearly 61% of the variation in 1978 plant populations with location accounting for 31% of that. Sixty-two percent of the variation in 1977 yields and 32% of the variation of the September 1978 yields can be assigned to locations with none of the other independent variables accounting for an appreciable amount of the variation. In contrast, the location did not account for a significant percentage of the variation of the June 1978 yields, while soil pH was able to account for 34%.

Experiment Two. Seedling populations and 1979 yields increased as stand densities of the previous alfalfa crop increased from 21 to 32% and subsequently decreased as previous stand densities increased from 32 to 43%. In contrast, there was a linear increase in 1978 yield as stand densities of the previous alfalfa crop increased.

The three cultivars, WL306, WL318, and Vernal, responded similarly to the change in stand density of the previous alfalfa crop.

Seedling populations and 1979 yields were greater where the previous crop was a forage grass rather than alfalfa (Table 6). However, dry matter yields in 1978 were similar for those tiers previously established in forage grasses and alfalfa. The response to the type of forage species previously established was similar for the three cultivars tested.

Experiment Three. Early growth of Vernal alfalfa was generally more

TABLE 6. ALFALFA DRY MATTER YIELDS AND SEEDLING POPULATIONS UNDER TWO ROTATION SEQUENCES, EXPERIMENT THREE

Rotation sequence	Seedling populations (plants/m^2)	Dry matter yields (metric tons/hectare) 9/78	5/79	8/79
Alfalfa-alfalfa	171.9	2.0	5.0	4.6
Forage grasses-alfalfa	197.4	2.2	5.5	5.3
Difference	− 25.5*[a]	−0.2	−0.5*	−0.7*
Average	184.6	2.1	5.2	5.0

[a] *Significant, based on one-tailed T test, at the 0.05 level.

TABLE 7. PERCENTAGE OF EMERGENCE OF VERNAL ALFALFA, FOUR TO SIXTEEN DAYS AFTER PLANTING

Previous crop	Percentage of emergence (days after planting)	
	4	16
Soybean (1975)–corn (1976)		
Root zone average	76.6	80.1
Fallow soil	95.6	93.3
Difference	−19.0**[a]	−13.2*
Overall average	77.8	79.8

[a] *,**Significant, based on one-tailed T test, at the 0.1 and 0.05 levels, respectively.

vigorous in the fallow soil as compared to the root zone soil previously cropped in soybeans (1975) and corn (1976). Percentage of emergence (Table 7), seedling height (Table 8), and first harvest yields (Table 9) were greater in the fallow soil. Soil potassium and phosphorus levels were also substantially higher in the fallow soil previously cropped in soybeans (1975) and corn (1976).

TABLE 8. SEEDLING HEIGHTS OF VERNAL ALFALFA 20 DAYS AFTER PLANTING

Previous crop	Seedling height (mm)
Soybean (1975)–corn (1976)	
Root zone—fall dormant	18.8
Root zone—slight fall dormant	21.9
Difference	− 3.1*[a]
Root zone average	21.2
Fallow soil	32.6
Difference	−11.4*
Alfalfa (1972–1976)	
Root zone average	15.5
Fallow soil	19.6
Difference	− 4.1*
Soybean (1975)–corn (1976) average	22.1
Alfalfa (1972–1976) average	15.8
Difference	6.3*
Overall average	18.9

[a] *Significant, based on one-tailed T test, at the 0.05 level.

TABLE 9. DRY MATTER YIELD OF VERNAL ALFALFA HARVEST 60, 90, AND 120 DAYS AFTER PLANTING

Previous crop	Dry matter yield (g) (days after planting)		
	60	90	120
Soybean (1975)–corn (1976)			
Root zone average	0.22	2.01	15.64
Fallow soil	0.46	2.54	16.03
Difference	−0.24*	−0.53*	− 0.39
Alfalfa (1972–1976)			
Root zone—low saponin	0.17	1.58	13.37
Root zone—high saponin	0.19	1.99	15.24
Difference	−0.02	−0.41*	− 1.87
Soybean (1975)–corn (1976) average	0.19	1.81	14.45
Alfalfa (1972–1976) average	0.24	2.05	15.67
Difference	−0.05*	−0.24*	− 1.22
Overall average	0.21	1.92	14.96

[a]*Significant, based on one-tailed T test, at the 0.05 level.

Except for seedling height differences, the growth of Vernal alfalfa was similar in the root zone and fallow soils previously cropped in alfalfa. Soil potassium and phosphorus levels were higher in the fallow soil but the magnitude of the difference was not as great as was observed for the soils previously cropped in soybeans and corn. Later stages of alfalfa growth were similar in the root zone and fallow soils.

The growth of Vernal alfalfa in the root zone soils of high and low saponin strains was similar except for second harvest yields. Second harvest yields were greater for root zone soils of high saponin strains previously cropped in alfalfa (1972–1976) (Table 9). Soil phosphorus levels were slightly higher for the root zone soil of high saponin strains while potassium levels were essentially the same.

Except for seedling height differences, the growth of Vernal alfalfa was similar for the root zone of fall dormant and slightly fall dormant strains. Seedlings were approximately 3 mm taller in root zone soils of slightly fall dormant strains. Height differences were observed only for soil previously cropped in alfalfa. Soil K levels were substantially higher for the root zone soils of slightly fall dormant strains.

Seedling height alone with first and second harvest yields differed for soil previously cropped in alfalfa (1972–1976) and soil previously cropped in soybeans (1975) and corn (1976). Height and yield were greater for the soil

previously cropped in soybeans and corn (Tables 8 and 9). Soil P and K levels were substantially greater for those soils previously cropped in soybeans and corn.

DISCUSSION

Alfalfa yields and stand densities were greater where alfalfa was rotated with soybeans and corn, compared with continuous cropping of alfalfa. A considerable portion of the variation in alfalfa performance was attributable to soil fertility, pH, and soil pathogen incidence. Results tend to suggest that some of the variation in alfalfa performance was attributable to the release of phytotoxic factors from the previous alfalfa crop.

When P and K deficiencies were corrected and alfalfa seedlings were protected against fungal attack, seedling emergence was still poorer with the continuous cropping of alfalfa. Having eliminated disease and P and K deficiencies as probable causes, phytotoxic factors are strongly implicated as the cause of the reestablishment difficulties. Second year dry matter yields for experiment 2 were substantially lower when alfalfa was grown following alfalfa. Differences in second-year yields may be due to the release of phytotoxic factors by the previous alfalfa crop.

In general there were no detectable cultivar–rotation interactions. It would appear that genetic differences among cultivars in response to this trait do not exist. These results are in agreement with those of Goplen and Webster (1969) which indicate that genetic selection against the suspected toxic soil factor was ineffective. A selection program may be unsuccessful unless more definitive information on the cause of this problem can be discovered.

Even though the results are not conclusive, alfalfa seedling establishment was more successful where alfalfa had not been grown for at least two years. The difficulty in reestablishing alfalfa without rotating may be ascribed to the accumulation of phytotoxic substances. Another possible explanation is soil fertility deficiencies due to the heavy feeding of P and K by the previous alfalfa crop. The differences in seedling growth between the root zone soils of fall dormant and slightly fall dormant alfalfa would tend to suggest an involvement of phytotoxic factors associated with fall dormancy.

There is little evidence to support saponins as being phytotoxic to alfalfa. While various studies indicate that they are toxic to cotton, they apparently are not toxic to alfalfa (Marchaim et al., 1975). This interpretation is based on the assumption that saponins are released from alfalfa tissue in proportion to their tissue content. It is known that saponins are released by alfalfa roots (Leshem and Levin, 1978), but whether they are released in proportion to their tissue content is unknown.

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ALLELOPATHIC EFFECTS OF WESTERN RAGWEED ON SEED GERMINATION AND SEEDLING GROWTH OF SELECTED PLANTS

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Abstract—Western ragweed (*Ambrosia psilostachya* DC.) top growth and rhizome extracts were inhibitory to germination and growth of seedling shoots and roots of test plants in almost all cases. Germination of all plants tested was reduced an average of 19.5% by the ragweed extracts. Shoot and root growth of plants tested was reduced an average of 56.8% by the ragweed extracts. The combined effects of germination plus growth reductions resulted in the tested plants producing only 34.8% in the extracts compared to production in distilled water.

Key Words—Allelopathy, inhibitors, western ragweed, *Ambrosia psilostachya* DC., germination, shoot growth reduction, root growth reduction.

INTRODUCTION

Double cropping, i.e., producing one crop before or after another during the same year, is a relatively common practice. In southern Oklahoma winter small grain crops are often produced in bermudagrass (*Cynodon dactylon* [L.] Pers.) sods following bermudagrass summer forage production. During 1965–1968 in some double cropping research studies, it was noted that rye (*Secale cereale* L.) established and grew poorly in areas where good stands of western ragweed (*Ambrosia psilostachya* DC.) occurred in the bermudagrass.

Soil moisture and plant nutrition was adequate, so it was hypothesized that western ragweed may have allelopathic characteristics causing the negative response of rye, and possibly negative responses in other species. The negative response of rye and the possible allelopathic effects of western ragweed led to a germinator study of the effect of western ragweed extracts on selected plant species. The objective was to determine the degree of influence, if any, of the extracts on germination and early seedling growth of the selected plants.

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METHODS AND MATERIALS

Western ragweed was collected from the field study area during late summer when the ragweed was nearly full grown and in the inflorescence blooming stage. Top growth was separated from root growth at the point where the plants emerged from the soil surface. Rhizomes included the rhizome and the crown up to the point where the crown emerged from the soil surface. All fibrous roots were removed from rhizomes. These two plant components, top growth and rhizomes, were oven dried at 43° C for 12 hr.

Extracts of western ragweed top growth and rhizomes were prepared based upon the procedures of Guenzi et al. (1967). Ten grams of each western ragweed component were extracted with 150 ml of distilled water agitated by magnetic stirring for 2 hr with water temperature at 25–30° C. The extract was then filtered through No. 2 filter paper in a ceramic perforated funnel with vacuum, and it was not tested for possible differences in osmotic pressure.

The extracts were used to test germination and seedling growth on 16 different selected species (Table 1). Ten healthy-appearing seeds of each species per each of three replications were placed on extract-saturated blue blotters in a germinator operated at 25°C. Blotters were maintained at saturation throughout the study. Percent germination was determined at 7 days. Shoot and root growth of the seedlings were measured to the nearest millimeter at 14 days after placing the seeds in the germinator. It appeared that

TABLE 1. CROP AND WEED SPECIES USED TO DETERMINE ALLELOPATHIC EFFECTS OF WESTERN RAGWEED EXTRACTS

Species	Variety and kind	Classification
Avena sativa L.	Ora oat	Crop
Eragrostis curvula (Schrad.) Nees	Ermelo weeping lovegrass	Crop
Lespedeza stipulacea Maxim.	Korean lespedeza	Crop
Lycopersicum esculentum Mill.	Sioux tomato	Crop
Medicago sativa L.	Okla. origin alfalfa	Crop
Melilotus officinalis (L.) Lam	Yellow sweet clover	Crop
Panicum virgatum L.	Caddo switchgrass	Crop
Secale cereale L.	Elbon rye	Crop
Triticum aestivum L.	Kaw 61 wheat	Crop
Vicia villosa Roth	Hairy vetch	Crop
Amaranthus spinosus L.	Rough pigweed	Weedy forb
Ambrosia psilostachya DC	Western ragweed	Weedy forb
Aristida oligantha Michx.	Annual threeawn	Weedy grass
Bromus secalinus L.	Cheat	Weedy grass
Digitaria sanguinalis (L.) Scop.	Large crabgrass	Crop/Weed
Helianthis annuus L.	Annual sunflower	Weedy forb

most of the seedling growth from the seed food storage was accomplished by that time.

RESULTS AND DISCUSSION

Germination. Germination of Korean lespedeza, hairy vetch, western ragweed, annual threeawn, crabgrass, and sunflower was too low in all treatments to consider for evaluation. This low germination was apparently due to the seed being fresh and still dormant during the test period.

There was a definite trend for seeds of most other species to germinate less in western ragweed extracts compared to germination in distilled water (Table 2). This general result is also cited by Rice (1974) in information published since this study was done. Germination in the extracts was less than in distilled water in 80% of the cases.

Weeping lovegrass was the only species showing a statistically significant difference in percent germination in the extracts compared with distilled water. The lack of statistically significant differences in germination of the other species in the extracts compared with distilled water appeared to be due to variation in data between replications likely caused by limited number of seeds per replication.

Rye seed germination averaged 25.0% less in extracts than in distilled water, thus supporting the field observation of a negative influence of western ragweed on rye. Rye residue has also been shown to be autoallelopathic

TABLE 2. PERCENT GERMINATION OF CERTAIN SEEDS IN DISTILLED WATER AND WESTERN RAGWEED EXTRACTS[a]

Seed	Distilled water	Top growth extract	Rhizome extract
Alfalfa	93.3a	70.0a	70.0a
Cheat	73.3a	76.7a	66.7a
Oat	96.7a	96.7a	100.0a
Rough pigweed	20.0a	0.0a	6.7a
Rye	86.7a	66.7a	63.3a
Switchgrass	60.0a	53.3a	70.0a
Tomato	100.0a	56.7a	76.7a
Weeping lovegrass	80.0a	53.3b	53.3b
Wheat	100.0a	93.3a	93.3a
Yellow sweet clover	40.0a	30.0a	10.0a
Overall averages	75.0	59.7	61.0

[a] Means on the same line followed by a different letter are statistically different at the 0.05 level of probability.

(Scholte and Kupers, 1978). There was no consistent difference in germination of the species tested in top growth or rhizome western ragweed extracts.

Percent germination in western ragweed extracts averaged 80.0% and 81.0%, respectively, in top growth and rhizome extracts in relation to percent germination in distilled water. There was an overall average 19.5% reduction in germination in the western ragweed extracts. The most pronounced degree of germination reduction due to the extracts was with rough pigweed and yellow sweet clover. Cheat, oat, and switchgrass germinated slightly better in the extracts than distilled water in some trials but this cannot be credited to the extracts due to the small differences.

Shoot and Root Growth. Sweet clover was the only species exhibiting equal or greater growth in extracts of western ragweed top growth compared to growth in distilled water (Table 3).

Seedling shoot and root growth of all other species was consistently less in the western ragweed extracts compared to growth in distilled water and these differences were statistically different for almost half the species. Shoot and root growth reduction was more pronounced than the reduction in germination.

Percent shoot and root growth of all species tested averaged 45.0% and 41.0%, respectively, in top growth and rhizome extracts in relation to growth in distilled water. These reductions in shoot and root growth were most severe for rough pigweed and weeping lovegrass, although all species showed great suppression in the extracts. Rough pigweed produced only 20.0% of shoot and root growth in western ragweed top growth and rhizome extracts compared to distilled water. Weeping lovegrass shoot and root growth was more suppressed in western ragweed top growth extract than rhizome extract.

Considering all species, shoot and root growth was more suppressed in western ragweed rhizome extract than it was in top growth extract. There did not seem to be consistent major differences in suppression of shoot over root growth in the two extracts.

Alfalfa and tomato also had short, stubby deformed root development in western ragweed rhizome extract. There was no attempt to qualitatively or quantitatively determine the allelopathic compounds.

Rye shoot and root growth averaged 52.0% in the western ragweed extracts compared to distilled water, again supporting the field observation of negative influence of western ragweed on rye. The reduction of rye germination plus the reduction in growth amounted to a total productive rye reduction of 61.0% in the extracts compared to production in distilled water.

All species and all extract treatments produced a total suppressed production equal to only 34.8% of the production of all species in distilled water. This stresses the total effect of inhibitory action of western ragweed extracts and the importance of controlling western ragweed in forage and crop

TABLE 3. SHOOT AND ROOT GROWTH OF CERTAIN SEEDLINGS IN DISTILLED WATER AND WESTERN RAGWEED EXTRACTS[a]

	Growth per germinated seed (mm)								
	Distilled water			Top growth extract			Rhizome extract		
Seedling	Shoot	Root	Total	Shoot	Root	Total	Shoot	Root	Total
Alfalfa	27a	36A	63a	16b	12B	28b	10b	11B	21b
Cheat	30a	47A	77a	9a	15A	24a	11a	14B	25a
Oat	51a	51A	102a	15b	24A	39b	16b	20A	36b
Rough pigweed	20a	10A	30a	1a	1A	2a	2a	1A	3a
Rye	67a	79A	146a	32b	47B	79b	31b	42B	73b
Switchgrass	14a	7A	21a	12a	5A	17a	9a	3A	12a
Tomato	32a	54a	86a	6a	22b	28b	10b	17b	27b
Weeping lovegrass	24a	5A	29a	1a	1A	2a	14a	2A	16a
Wheat	56a	105A	161a	32a	58B	90b	25a	59C	84b
Yellow sweet clover	14a	5A	19a	14a	8A	22a	1a	1A	2a
Overall averages	34	40	73	14	19	33	13	17	30

[a]For statistical information, compare only figures on a given line followed by the same type of lettering (a, A, a), i.e., shoot growth against shoot growth, etc. Therein means on the same line followed by a different letter are statistically different at the 0.05 level of probability.

production circumstances. The data also indicate that the negative effects of the weed are more complex than simple competition for light, nutrition, and moisture.

SUMMARY

Extracts of western ragweed top growth and rhizomes were tested on germination and seedling top growth and root growth of selected plant species. The extracts had a negative effect on germination, shoot, and root growth of almost all species. Alfalfa and tomato exhibited malformed root development due to western ragweed rhizome extract. The total cumulative negative effect of the extracts on germination and seedling growth was that all species in the extracts averaged only 34.8% of their production in distilled water.

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EVALUATION OF ALLELOPATHIC POTENTIAL OF DOMINANT HERBACEOUS SPECIES IN A COFFEE PLANTATION

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Abstract—Several shaded coffee plantations in Coatepec, Veracruz (Mexico) are characterized by a dense cover of herbaceous vegetation mainly dominated by species from the Commelinaceae which protect the soil from erosion and presumably contribute to regulating the abundance of other weeds. To detect their alleopathic potential, leachates from fresh, air-dried, or oven-dried plants and litter collected during different months of the year were tested upon *Brassica campestris*, *Bidens pilosa*, and *Rumex* sp. seeds. Significant radicle growth inhibitions were obtained mainly from dried plants and litter collected during the rainy season (August). Drainage water collected from pots with fresh, chopped plants and litter produced no inhibitions until the third week of recycling the water. Concentrated soil extracts from chopped plants and litter collected after seven weeks of decomposition produced significant inhibitions on radicle growth of *Rumex* sp. Dry weight of *Bidens pilosa* was significantly reduced when grown in soils treated with fresh and chopped plants and litter exposed to natural field conditions for five weeks.

Key Words—Coffee plantation, weeds, dominance, Commelinaceae, allelopathy, leachates, soil extracts, litter, inhibition.

INTRODUCTION

Coffee plantations are agrosystems which are kept in many different ways, from intensively managed cultivation to natural forest-like ecosystems. These latter are characterized by an arboreal stratum of shade trees and a specific

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herbaceous layer whose composition depends mainly on the amount of light that reaches the soil (Jiménez, 1981). In Coatepec, Veracruz (Mexico) several coffee plantations are characterized by a dense cover of herbaceous vegetation dominated by Gramineae and Compositae in sunny plantings and by species from the Commelinaceae in shaded plantations. Although in some orchards several grubbing practices are carried out, in many of them, mainly in the shaded coffee communities, weeds cover the soil during most of the year.

Wellman (1961) states that in some cases Commelinas are encouraged to grow in lightly shaded coffee, presumably to protect the soil and keep a natural layer of mulch in perpetual production. These shallow-rooted perennial weeds keep up a pronounced dominance which seems to contribute toward regulating the establishment of other weeds. Grime (1982) analyzed the nature of dominance and stated that the deletereous effects that big plants can exert upon their smaller neighbors mainly derive from light reduction, mineral and water depletion in the soil, litter deposits, and the release of phytotoxic compounds. In shaded coffee plantations many of these restrictions may be suspected from the arboreal stratum up to the herbaceous layer. However, coffee growers still benefit from the advantages of a forest-like planting. Sometimes bananas and citrics are planted as shade trees, and Commelinas are used to feed pigs (Jiménez, 1981) or to protect the soil from erosion. In this way they achieve multiple use and keep a certain equilibrium within their plantations. Shade trees favor the selection of certain weeds which, similarly, create a complex mosaic of microhabitats. Besides, both strata are a constant supply of organic matter to the soil. To the edaphic variations, selective predation, local perturbation of the soil, and the redistribution of minerals by the animals, Grime (1982) adds the spatial differences which come from plant activities, such as the variation in nutrient availability (Snaydon, 1962), water supply, amount of shade, litter accumulation, organic toxins (Abdul-Wahab and Rice, 1967), and modification of natural field microflora. These circumstances, together with temporal variations, determine in each case the coexistence of herbaceous vegetation (Grime, 1982).

The dominant weeds in the shaded or semishaded coffee plantations have an effective dominance mechanism which is expressed as a high regenerative capacity and a great ability to expand over great areas. Anaya et al. (1982) studied the allelopathic potential of species from the different strata in a coffee plantation. It was found that some of the species, including the coffee trees, possess a phytotoxic potential when tested against some weed seeds in laboratory conditions. One of the species which proved to have the highest phytotoxicity was a member of the Commelinaceae. This suggested that the release of toxic substances (allelopathy) by these species could be another factor which contributes to the maintenance of its dominance. Putnam and

Duke (1978) assume that allelopathy may be implicated in shifts in species distribution and increases of dominance by certain annual and perennial weeds in a variety of agroecosystems. Parker and Muller (1979) demonstrated that *Pholistoma auritum* has a dominance mechanism which can severely limit the presence and growth of other understory species. Hence, the object of this study is to evaluate the allelopathic potential of the Commelinas by: (1) detecting the potential from different "origins," that is, from fresh and dried plants and litter; (2) comparing the potential in different months of the year (rainy season or dry season); (3) testing soil extracts (natural and concentrated) where the origins are incorporated; and (4) testing phytotoxicity in the soil under greenhouse and natural field conditions.

METHODS AND MATERIALS

In order to quantify the relative importance of the herbaceous species, a systematic point-intercept (Levy and Madden, 1933) field sampling was designed. Frequency, cover, and production were calculated for each species, following Riepma's (1963) equations, in October and in April.

To detect the allelopathic potential of the dominant weeds, field collections of fresh live plant material were made in a coffee plantation with *Inga leptoloba* as a shade tree in December 1980 (after the rainy season), in April 1981 (during the dry season), and in August 1981 (during the rainy season). Plant material was separated into different "origins": fresh, air-dried, oven-dried at 30°C, oven-dried at 40°C, and litter. This latter was collected from pots with live plants, as it was difficult to collect and separate it from other material in the field. Leachates from each origin were made in each season, by soaking 10 g of fresh entire plants (as they are shallow-rooted species there was no need to separate into plant parts) (1 g of dried plants and litter) in 100 ml of distilled water for 3 hr. After filtering the leachate, 10 ml of each (distilled water for the control) were melted with 10 ml of agar (2%) in each of three Petri dishes where 20 test seeds were set. Test seeds used were *Brassica campestris*, *Bidens pilosa*, and *Rumex* sp; the last two are usually found in the coffee plantation although in small clusters or outside the coffee plots. All Petri dishes were maintained at 27°C with a 12-hr photoperiod. After 3–5 days the percentage of germination and radicle growth were calculated and statistically analyzed under a split-plot design. The osmotic pressure of each leachate was previously determined in a freezing-point osmometer.

In order to have a quantitative value of the allelopathic potential in each season, a relative percentage of phytotoxicity was calculated as follows:

$$\text{Allelopathic potential} = \frac{\text{TNI}}{\text{TNT}} \times 100 \frac{\text{Total number of inhibitions}}{\text{Total number of treatments}}$$

where: TNI is the number of cases in which the leachates from the different origins produced an inhibitory effect on radicle growth, and TNT is the total number of leachates tested in each season, that is, leachates from three different species in five different origins, upon three test seeds (total = 45). The percentages are shown graphically in Figure 4.

To test whether the rainwater collected after a rainfall in August produced any inhibitory effect, glass bottles with funnels covered with a gauze were put inside stands under Commelinaceae without the influence of coffee or *Inga* cover, and in an open stand for the control. Bioassays in Petri dishes as described above were carried out with *Rumex* sp. as test seeds.

As some of the litter and air-dried leachates proved to have an inhibitory effect upon the seeds, an experiment was designed in order to detect if the leachates were still effective once they had reached and passed through the soil. Rectangular blocks (10 cm depth) of soil (2000 g) from the coffee plantation were collected in 32 × 22-cm plastic pots to which 500 g of fresh live plants, 200 g of litter, and 500 g of chopped plants (the three species together) were added in the greenhouse. Chopped plants refers to plants which are cut in the field with a cane knife, as a grubbing practice, and left covering the soil until they are incorporated as organic matter. The amount of plant material added to the soil was the mean weight biomass from 20 field plots of the same size as the plastic pots. As leachates from litter and 40°C oven-dried plants did not differ significantly, it was decided to oven-dry fresh plants at 40°C so as to accelerate the natural decomposition of the plants. Once dried, the material was incorporated into the soil pots as litter. Each treatment consisted of five replicates, with a control of soil with no plants. The pots were irrigated three times a week with a fine mist of tap water up to their field capacity (500 ml), and the drainage water was collected in plastic trays located at the bottom of each pot. Every third day the pots were irrigated with the drainage water collected in the trays in order to concentrate it. In this way, the drainage water was recycled during three weeks. The recycled drainage water obtained at the end of the first, second, and third week was first filtered, then measured to assess its osmotic pressure, and finally used for bioassays in Petri dishes with agar. Percentage of germination and radicle length were measured and statistically analyzed as a 3 × 4 factorial experiment.

To test whether any toxins were left in the soil with the treatments, four little pots from each of three replicates per treatment were sowed with five test seeds (*B. pilosa* and *Rumex* sp). After three weeks of irrigating the pots daily with tap water, three seedlings chosen at random from each pot were dried and weighed. Dry weight data were analyzed under a randomized complete block design.

As soil produced no inhibitory effects, we decided to test natural and artificial concentrated soil extracts. The holes at the bottom of each of three pots were sealed, and the soils with litter and chopped plants as well as the control were irrigated up to their field capacity. Treatment with fresh plants was now eliminated because they had become infested with aphids.

Another treatment of soil which had been stored without being irrigated was incorporated into the experiment as another control to test it against the leached original control. Soils were soaked for 24 hr at room temperature (22°C), and then the extracts were filtered and half of each was concentrated in a Buchler flash evaporator at 22°C to two times the original concentration. Both extracts were used for Petri dish bioassays with *B. campestris* and *Rumex* sp. and to measure the osmotic pressure.

Two months after the beginning of the greenhouse experiment, litter as well as chopped plants were almost all incorporated into the soil. For this stage of decomposition, a final soil extract was obtained as described above, but this time it was concentrated in the Buchler flash evaporator to three times the original concentration. Radicle growth and germination of *B. campestris* and *Rumex* sp. were calculated after bioassays and analyzed as a 2×3 factorial experiment.

Finally, to test if the effect was observed from treatments occurring naturally in the field, an experiment was designed in which homogeneized soil from the coffee plantation was put in rectangular plastic pots with the following treatments were given: (1) fresh plants transplanted directly from the area (the species together as found naturally) (400 g); (2) chopped (or cane knife grubbed) plants (400 g); (3) litter (200 g of crumbled dry plants oven-dried at 40°C for 24 hr); and (4) dry peat moss (200 g) as control.

A previous bioassay with leachates of peat moss was carried out in order to detect its phytotoxic potential. Test seeds used were *B. campestris* and *Rumex* sp. Neither radicle growth nor germination were significantly different from the control (distilled water), as proved by an F test at the 1% level.

The pots were placed in an open area inside the coffee plantation in a randomized complete block design with four replicates per treatment. They were covered with a thin gauze to prevent other undesirable material from being incorporated into the treatments. After 40 days under field conditions, the pots were taken to the greenhouse where, after removing all plant material from each treatment, 60 seeds of *B. pilosa* were sowed in one half of the pots and 80 seeds of *Rumex* sp. in the other half. After 25 days, the seedlings were counted, harvested, dried, and weighed. As *B. pilosa*'s leaves had been predated, the few seedlings left with leaves were defoliated so that only the stem and roots were considered for the measurement of dry weight. Mean dry weight data per repetition and percentage of germination were statistically analyzed under a randomized complete block design.

TABLE 1. POPULATION PARAMETERS AND IMPORTANCE VALUE FROM DOMINANT SPECIES IN A SEMISHADED COFFEE PLANTATION (COATEPEC, VERACRUZ, MEXICO)

	Frequency		Cover		Production		Importance value	
Species	October	April	October	April	October	April	October	April
C. difussa	0.83	0.60	0.50	0.23	0.89	0.35	2.22	1.18
Zebrina sp.	1.0	1.0	0.78	0.68	1.92	1.40	3.70	3.08
T. serrulata	0.96	0.90	0.45	0.58	0.72	0.95	2.13	2.43

RESULTS AND DISCUSSION

The species with the higher importance value were members of the Commelinaceae in both samplings (October and April). *Zebrina* sp. was the species with the highest values of frequency, cover, and production during the rainy and the dry season, followed by *Commelina diffusa* in October and *Tripogandra serrulata*, which is the most succulent, in April (Table 1). *Zebrina* sp. was the species which produced the highest number of inhibitions. This could reflect a more effective mechanism of dominance as expressed by its importance value.

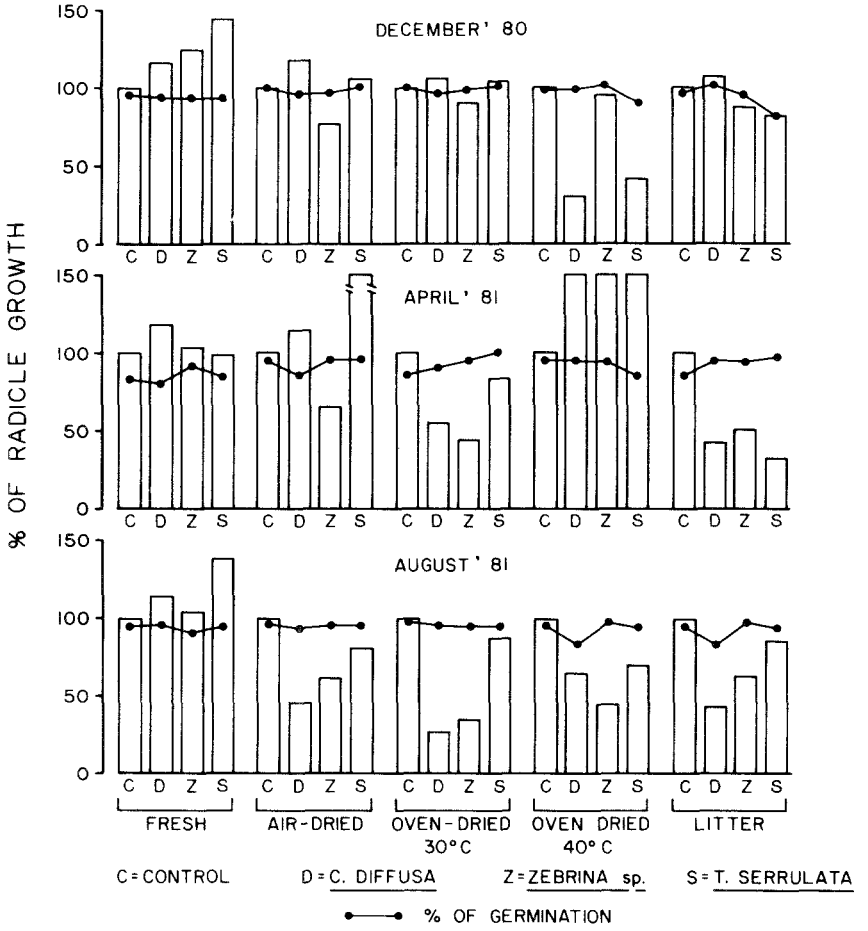

FIG. 1. Effect of the leachates on the germination and radicle growth of *Brassica compestris*.

The osmotic pressure of the leachates ranged from 0 to 30 mosm/liter, values which according to Anaya and Rovalo (1976) are not likely to account for an inhibition of germination or radicle growth.

Figure 1 shows the effect of the leachates upon *Brassica campestris*, which is more inhibited by litter and oven-dried plant leachates, particularly from plants collected during the rainy season (August). *Bidens pilosa* (Figure 2) was mainly affected by leachates from dry material and litter from plants collected after the rainy season (December). The greatest effects upon germination were given by oven-dried (30°C) plant leachates, litter leachates, and fresh plant leachates from *Zebrina* sp. from August. The effect upon *Rumex* sp. (Figure 3) seems to be more uniform, although leachates from

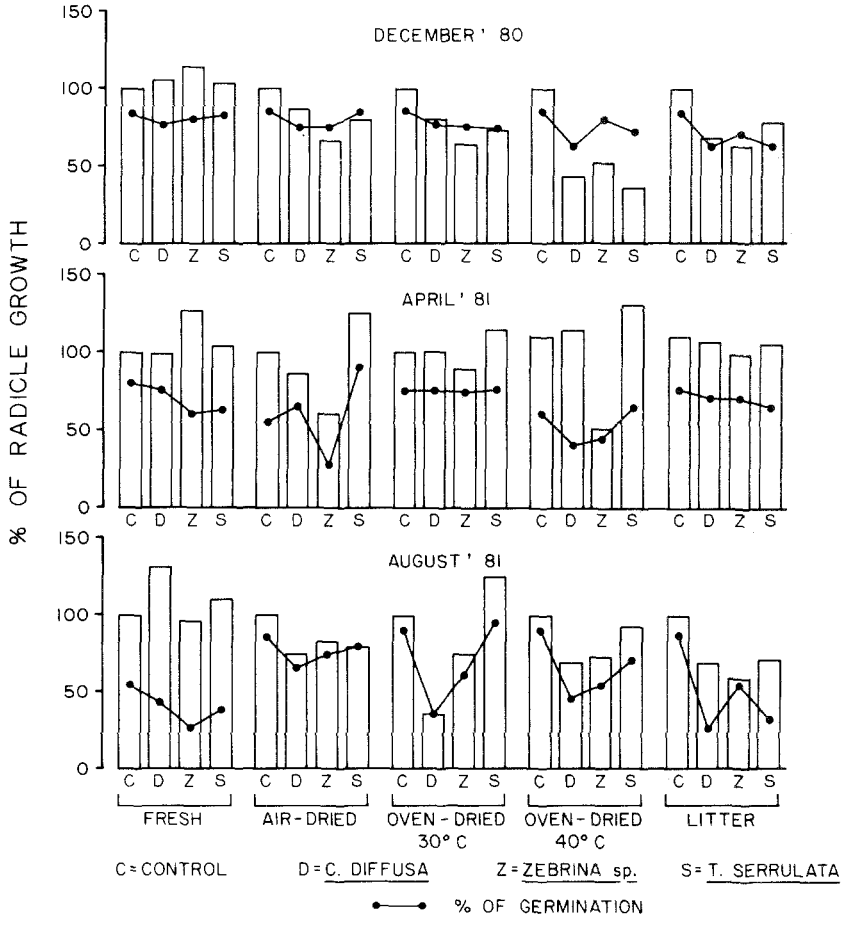

FIG. 2. Effect of the leachates on the germination and radicle growth of *Bidens pilosa*.

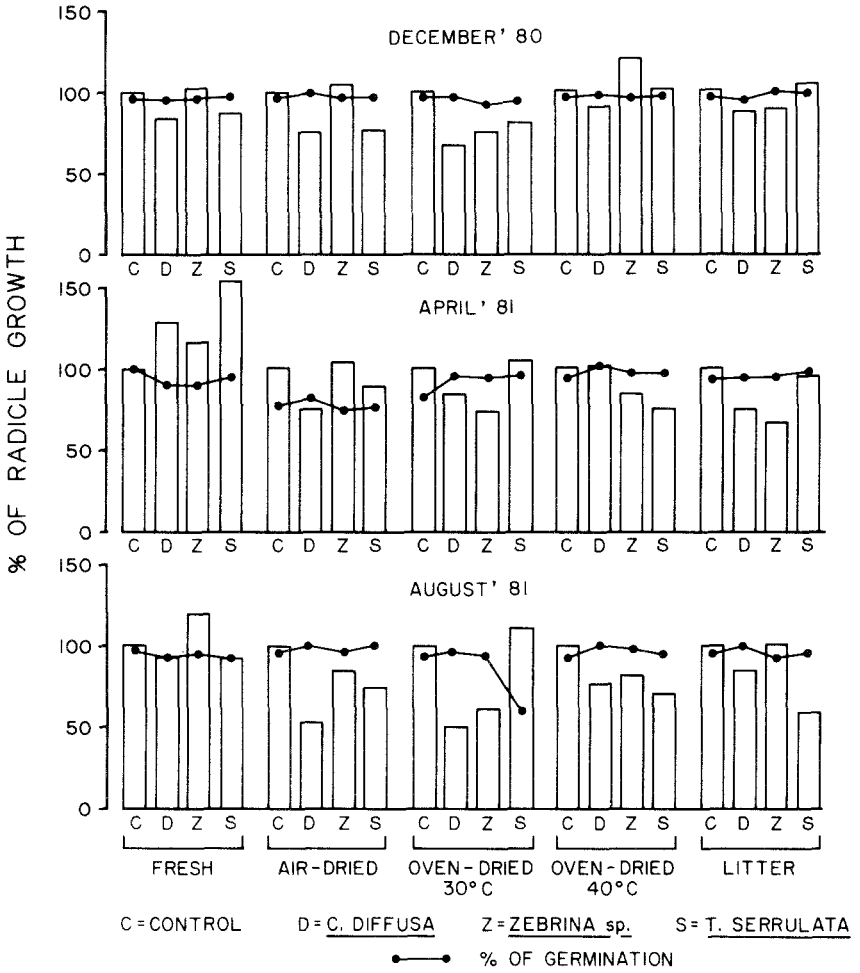

FIG. 3. Effect of the leachates on the germination and radicle growth of *Rumex* sp.

August produced the greatest radicle growth inhibition. Germination was not significantly affected.

Fresh leachates produced mainly stimulation of radicle growth, while dried material and litter leachates inhibited it. Comparison of the split-plot analysis showed that leachates from air-dried plants are vey different from 30°C oven-dried plant leachates as are these from 40°C oven-dried plant leachates (Table 2). It is important to consider these differences when determining an allelopathic potential. Drying the plants naturally or artificially can lead to different conclusions. Of course, it is desirable to use the

TABLE 2. PERCENTAGE OF INHIBITION OF RADICLE GROWTH OF *Brassica compestris, Bidens pilosa,* AND *Rumex* SP. WITH LEACHATES OF COMMELINAS COLLECTED IN DECEMBER, APRIL, AND AUGUST

	Fresh			Air-dried			Oven-dried (30°C)			Oven-dried (40°C)			Litter		
Test seed	Dec.	Apr.	Aug.	Dec.	Apr.	Aug.	Dec.	Apr.	Aug.	Dec.	Apr.	Aug.	Dec.	Apr.	Aug.
B. campestris															
C. diffusa	+[a]	+	+	+	+	52.4[b]	+	43.7[b]	73.4[b]	70.0[b]	+	35.7[b]	+	57.3[b]	56.5[b]
Zebrina sp	+	+	+	21.5[b]	35.0[b]	38.3[b]	9.8[b]	53.2[b]	65.3[b]	4.4	+	53.2[b]	11.3[b]	49.0[b]	36.4[b]
T. serrulata	+	0.5	+	+	+	18.2[b]	+	15.5[b]	11.1[b]	58.7[b]	+	29.5[b]	18.2[b]	67.4[b]	12.4[b]
B. pilosa															
C. diffusa	+	5.3	+	11.2[b]	15.0[1]	24.2[b]	19.2[b]	+	63.4[b]	55.6[b]	+	30.7[b]	30.9[b]	1.6	30.4[b]
Zebrina sp.	+	+	4.6	32.8[b]	40.0[b]	17.9[b]	36.0[b]	10.7[b]	24.1[b]	46.3[b]	48.2[b]	26.8[b]	37.7[b]	10.2[b]	40.2[b]
T. serrulata	+	+	+	18.4[b]	+	21.0[b]	25.6[b]	+	+	61.8[b]	+	6.6	20.4[b]	5.9	28.2[b]
Rumex sp.															
C. diffusa	19.5[b]	+	6.1	23.2[b]	24.4[b]	45.7[b]	31.9[b]	15.2[b]	47.3[b]	11.8[b]	+	21.3[b]	11.4[b]	24.3[b]	12.2[b]
Zebrina sp.	+	+	+	+	+	14.5[b]	23.2[b]	26.3[b]	37.7[b]	+	4.4	17.2[b]	10.5[b]	32.4[b]	+
T. serrulata	11.8[b]	+	7.0	21.1[b]	10.5[b]	23.2[b]	18.2[b]	+	+	+	21.8[b]	29.5[b]	5.1	5.10	40.5[b]

[a] Plus indicates stimulation of radicle growth; control corresponds to 0.
[b] *F* test significant at the 1% level.

material fresh, dry, or decomposed, as we find it naturally in the field, if we are looking for ecological significance.

Drying plants at 40°C caused changes in their chemical composition so that their leachates inhibited radicle growth almost in the same proportion as litter leachates. Perhaps under natural conditions in the field the decomposition process reaches temperatures as high as 40°C under the mat of litter, which is very thick and exposed to a great amount of precipitation throughout the year (and a mean temperature of 19.5°C).

It was found, also, that there is a great interaction between the "origin" of the leachate, the season when the plants were collected, and the test seed used (Table 2). Furthermore, the greater number of inhibitions were from August plant leachates, followed by the ones from April. Gliessman (1978) found that leaves from *Quercus eugeniaefolia* collected in the wet season were not inhibitory to the root growth of *Bromus rigidus*, while those collected in the dry season were significantly toxic. On the contrary, in Commelinas, it seems that the allelopathic compounds are mainly produced during the rainy season and that in April, having no means to be released into the environment, they may be produced in lesser proportions. Figure 4 shows a hypothetical relation between the relative percentage of phytotoxicity of the Commelinas and the month of the year when plants were collected and leached.

Rain drops collected under stands of live intact plants in the field proved to be nontoxic to *Rumex* sp. (F test at the 5% level), so it was realized that if

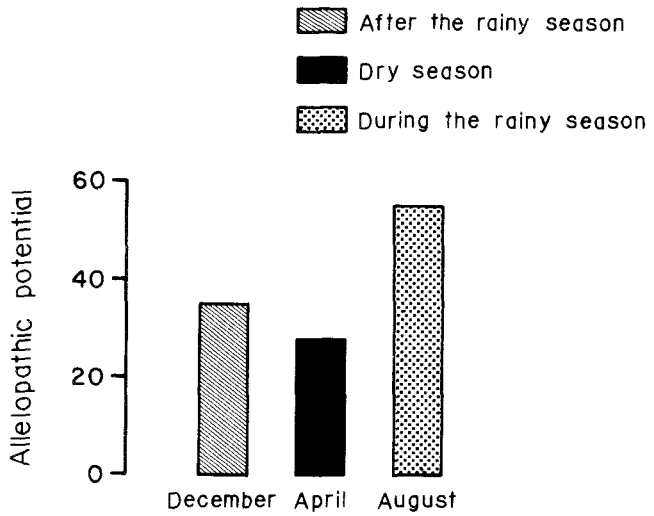

FIG. 4. Hypothetical relation between the allelopathic potential (relative phytotoxicity) of the Commelinas and the month of the year when plants were collected and leached.

there were any phytotoxicity, it came mainly from chopped plants or litter and should be found in the soil.

Drainage water collected from soils treated with the different "origins" produced no significant inhibitory effect on *B. pilosa* after the first and second week of recycling the water (Figure 5). It was not until the third week that the inhibitory effect was observed (F test at the 1% level). These results show that

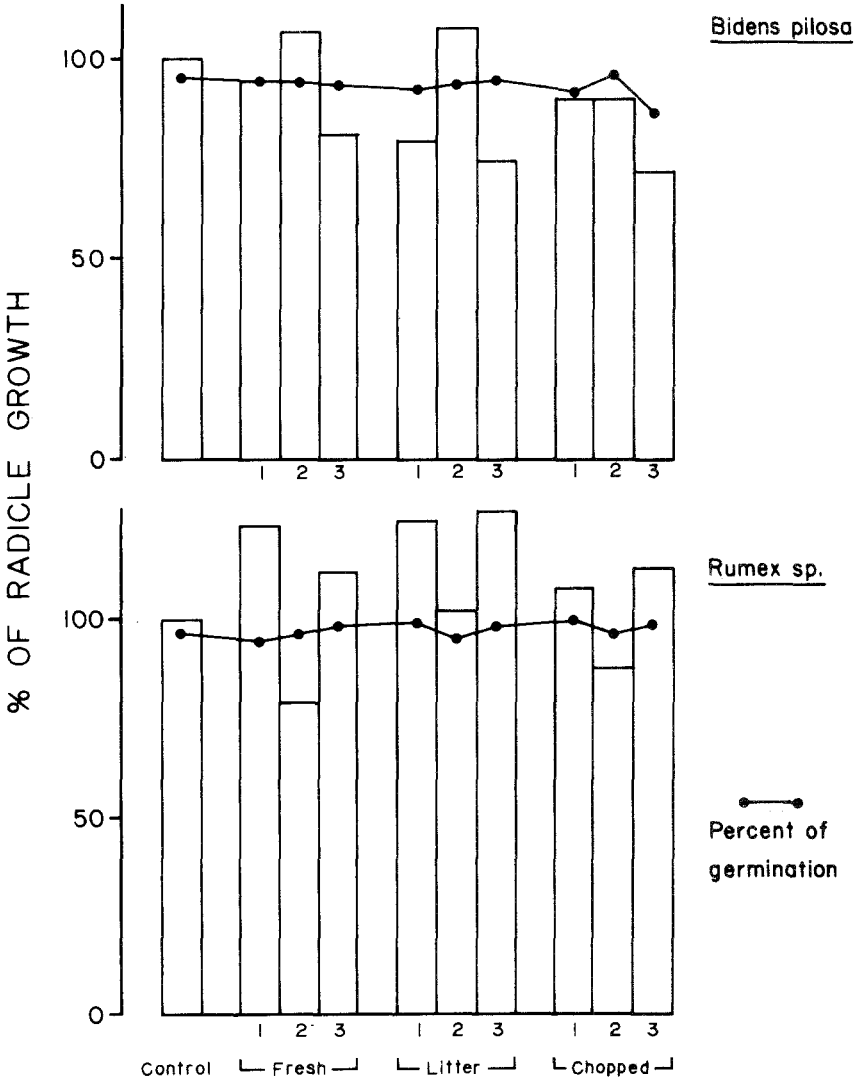

FIG. 5. Effect of recycled drainage water collected after 1, 2, and 3 weeks of leaching.

leachates have to be concentrated for three weeks to produce their toxic effect. In no instance was the percentage of germination significantly reduced, as shown by an F test.

In *Rumex* sp. the only significant difference found (F test at the 1% level) was the one from litter; however, it was not an inhibitory effect, as shown in Figure 5.

Soils treated with fresh, litter, and chopped plants in the greenhouse produced no inhibitory effect on the radicle growth of test seeds. Although there were significant differences (F test at 1% level) between treatments and control, these were due either to stimulation (Figure 6) or to the possibly lower level of nutrients in the soil of the control as compared with that of the treatments, which still had the organic matter contribution.

Although *Brassica campestris* had always been the seed most susceptible to the leachates, concentrated soil extracts collected seven weeks after the beginning of the experiment produced no inhibitory effects on its radicle

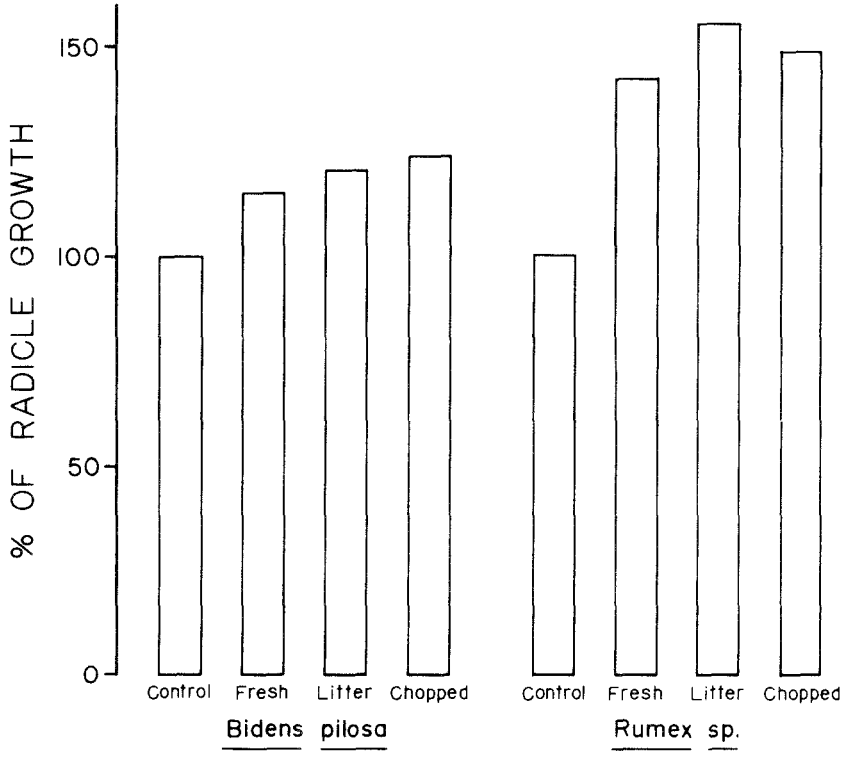

FIG. 6. Effect of soils treated with different "origins" on the radicle growth of *Bidens pilosa* and *Rumex* sp.

growth or in germination. The F test showed no significant differences (5% level) among treatments or between the original and the 2× concentrated extract (Figure 7).

However, as shown in Figure 8, original and 2× concentrated soil extracts did produce significant inhibitions on radicle growth of *Rumex* sp. Litter and chopped plant treatments produced the highest inhibitions (Table 3).

Concentrated litter soil extracts (3×) greatly inhibited the radicle growth of *Brassica campestris* as well as *Rumex* sp. (Tables 4 and 5), although in general they were less toxic than the first ones (Figure 9). This may be due to the constant washing of plant material by that time (eight weeks since the first leaching), or because the most toxic activity is found during a previous stage in the decomposition process. Guenzi et al. (1967) found that after eight weeks of decomposition, the toxicity of water extracts from wheat straw and oat straw residues had disappeared, while that of corn remained at a relatively high level for 22 weeks. The results obtained from our study agree with those of Chou

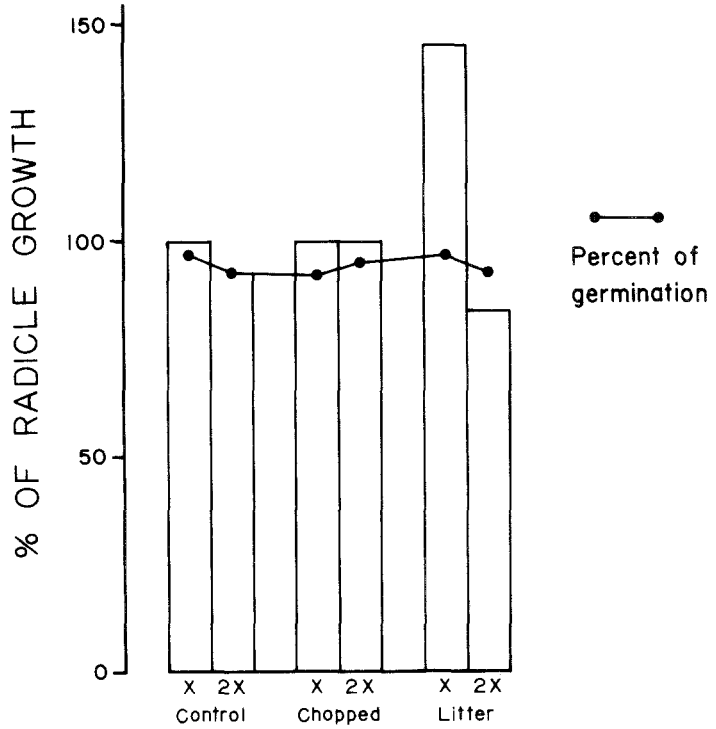

FIG. 7. Effect of the concentrated soil extracts (×, 2×) on the germination and radicle growth of *Brassica campestris*.

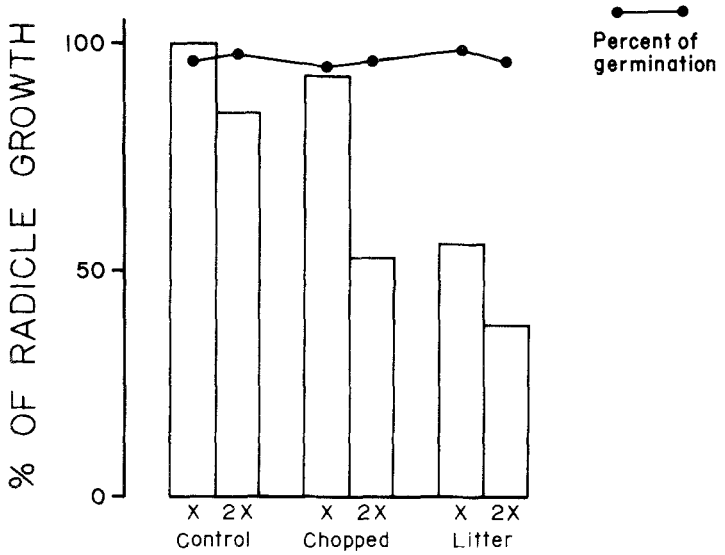

FIG. 8. Effect of the concentrated soil extracts (×, 2×) on the germination and radicle growth of *Rumex* sp.

and Patrick (1976) in that phytotoxicity depends on the amount of organic substrate decomposing in the soil and that toxicity decreases as the period of decomposition increases. Horsley (1976) states that a number of authors (McCalla et al., 1963; Mojé, 1966; Norstadt and McCalla, 1968) have reported the development of toxic conditions when soil is saturated with moisture, because anaerobic conditions permit only limited decomposition of organic material by soil microorganisms (Patrick and Koch, 1958; Wang et al., 1967). However, in our study soils were saturated only for 24 hr before the extract

TABLE 3. EFFECT OF CONCENTRATED SOIL EXTRACTS ON RADICLE GROWTH OF *Rumex* SP.[a]

Origin	Radicle growth (cm)	
	Original extract	2× extract
Control (soil without plants)	1.60[b]	1.33[b]
Soil with chopped plants	1.51[b]	0.90[b]
Soil with litter	1.08[b]	0.81[b]

[a] Numbers are the mean from three replicates in cm.
[b] *F* test significant at the 1% level.

TABLE 4. EFFECT OF SOIL EXTRACTS ON RADICLE GROWTH OF *Brassica campestris*[a]

Origin	Radicle growth (cm)	
	Original extract	3× extract
Control (soil without plants)	3.71[b]	2.98[b]
Control 2 (stored soil)	3.15[b]	2.75[b]
Soil with chopped plants	3.45[b]	3.24[b]
Soil with litter	2.55[b]	2.05[b]

[a] All numbers are the mean from three replicates in cm.
[b] *F* test significant at the 5% level.

was assayed. These conditions are very similar to those occurring in the field when, after several days of continuous rainfall, soil is practically flooded.

From the last experiment, in which seeds were sowed in soils with the treatments exposed to natural field conditions, the statistical analysis showed that there were no differences between the control and the treatments' percentage of germination from either of the two test seeds (*F* test at 1% level). However, the allelopathic potential from fresh plants, chopped plants, and particulary litter, was demonstrated to be effective in the soil, as *B. pilosa*'s dry weight was significantly reduced (Table 6). Unfortunately, we do not know the behavior of this seed in the two previous experiments because the seed's viability was too low to be used for the assays; however, it had already been observed with the first bioassays that it was more susceptible to the leachates than *Rumex* sp.

In *Rumex* sp., however, there were no differences between treatments and control (*F* test at 5% level), but it is believed that, as shown by the results from the concentrated soil extracts, the soil needs a longer supply of leachates. Maybe the treatments had to be kept under environmental conditions for a

TABLE 5. EFFECT OF SOIL EXTRACTS ON RADICLE GROWTH OF *Rumex* SP.[a]

Origin	Radicle growth (cm)	
	Original extract	3× extract
Control (soil without plants)	1.64[b]	1.61[b]
Control 2 (stored soil)	1.88[b]	1.73[b]
Soil with chopped plants	1.99[b]	1.47[b]
Soil with litter	1.68	1.27[b]

[a] All numbers are the mean from three replicates in cm.
[b] *F* test significant at the 5% level.

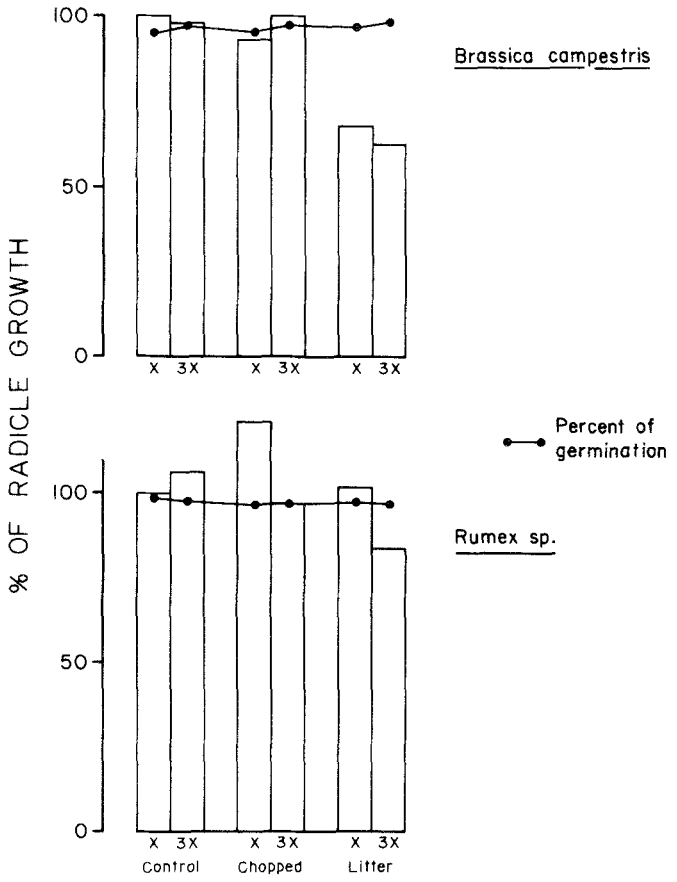

FIG. 9. Effect of the concentrated soil extracts (×, 3×) on the germination and radicle growth of *Brassica campestris* and *Rumex* sp.

longer period of time, or maybe the pots, once sowed, should have been irrigated with leachates from each origin, an event more likely to occur under natural field conditions.

It is obvious that the Commelinas, as dominant competitors, create certain microclimatic conditions that favor a selective germination of other weeds. In the coffee plantation, Commelinas cover the soil throughout the year, either as a stand of living plants or as a big mat of chopped plants. In both ways, they provide the soil with a continuous supply of litter which seems to be rapidly decomposed. In this study the phytotoxic potential of litter was manifested in the laboratory and in the soil. There is a stage in decomposition (one month under field conditions) in which litter can reduce the growth of *Bidens pilosa.* The phytotoxic compounds need to be concentrated in the soil

TABLE 6. EFFECT OF COMMILINACEAE ON DRY WEIGHT OF *Bidens pilosa*

	Individual dry weight (g)[a]
Control	0.0031[b]
Fresh plants	0.0019[b]
Chopped plants	0.0019[b]
Litter	0.0017[b]

[a]Numbers are the mean from four replicates in g.
[b]*F* test significant at the 1% level.

to produce an inhibitory effect on *Rumex* sp. Although the immediate physical or chemical effect of litter may be transitory or associated with a high rate of decomposition (Grime, 1981), a continuous supply of Commelinas organic matter might be enough for the toxins to accumulate.

Moreover, the soils might contain other toxins in addition to those of Commelinas, for Anaya et al. (1982) have found that some shade trees (*Inga*) as well as coffee have a phytotoxic potential upon the same seeds used in this study. Research on the combined effect of coffee, shade trees, and Commelinas upon other weeds and upon coffee itself, and on its behavior in the field or in other cultivations, must continue as Commelinas might be an alternative in biological weed control.

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CAFFEINE HAZARDS AND THEIR PREVENTION IN GERMINATING SEEDS OF COFFEE (*Coffea arabica* L.)[1]

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Abstract—The inhibition of growth of seedlings of coffee (*Coffea arabica* L.) exposed to 10 mM caffeine was found to occur in the rootlet: mitosis and cell plate formation were also inhibited. Since concentrations of endogenous caffeine in the imbibed seed are 40–60 mM, 4–6 times as high as in the seedlings, we conclude that coffee embryos have specific means of avoiding caffeine autotoxicity. Observations indicate that cell divisions in root tips start only after the latter are pushed away from the caffeine-rich endosperm by elongation of the hypocotyl and maintained through cell elongation. Caffeine is introduced into the embryonic cotyledons mostly after cell division is completed there. Thus, coffee seedlings may avoid autotoxic effects of endogenous caffeine by separation between sites where mitosis is occurring and those where caffeine is stored. This is achieved in root tips by separation is space but in the cotyledons by separation in time. Caffeine is liberated from the tree litter in coffee plantations and eventually will produce autotoxic effects, resulting in some degeneration.

Key Words—*Coffea arabica*, coffee, caffeine, theophylline, germination inhibitors, avoidance of autotoxicity.

INTRODUCTION

Some secondary metabolites stored in seeds exhibit toxicity against various seed predators and thus function as natural protectants of the quiescent embryo (Bell, 1978). A number of these compounds may also have phytotoxic activity (Rice, 1974), i.e., inhibit growth or germination of various species, but

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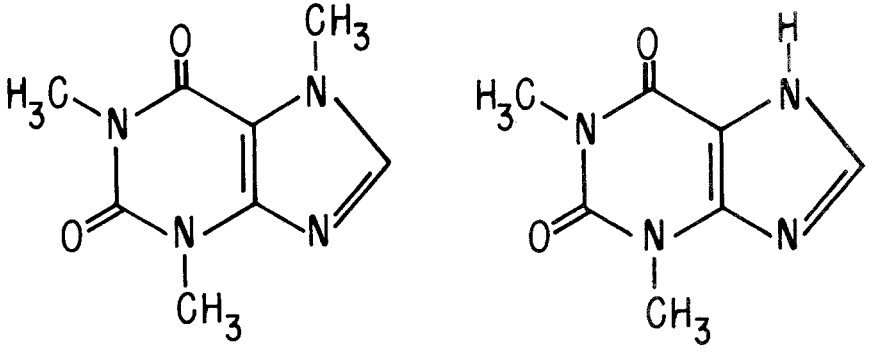
may also adversely affect their own species. Phytotoxins with autotoxic activity are often present in the outer parts of seeds or diaspores. If these toxins are not sufficiently leached out by rainfall or metabolized by soil microflora, they hold germination in check and ensure that this will occur only after the amounts of rainfall are sufficient for the establishment of seedlings (Evenari, 1949). Other toxins, however, are stored within the seeds and are hardly leachable; these are natural protectants, e.g., caffeine in *Coffea arabica* and strychnine in *Strychnos nux vomica* (Evenari, 1949). When either of these compounds is applied exogenously to the seed that stores it, germination is inhibited, even when the concentration of the exogenous inhibitor is much lower than that found endogenously in the imbibed seed (Evenari, 1949). This suggests that such seeds have the means to avoid the effects of their own phytotoxins. Fowden and Lea (1979) describe mechanisms by which plants avoid autotoxicity by their phytotoxic secondary metabolites, especially by nonprotein amino acids. This work was undertaken to quantify the effect of exogenous caffeine and one of its degradation products, theophylline, on germination and growth of seedlings of coffee, to identify in the coffee embryo sites either susceptible or resistant to caffeine, and to speculate on the strategies by which the coffee embryo avoids autotoxic hazard during germination (Scheme 1).

METHODS AND MATERIALS

Seeds of *Coffea arabica* cv. Bourbon, 3–6 months after collecting, containing $32 \pm 3\%$ water (on dry weight basis), were obtained from Conafruit, Jalapa, Mexico. As described by Valio (1976), endocarp-free seeds



CAFFEINE THEOPHYLLINE

SCHEME 1.

were allowed to germinate at 27 ± 0.1°C in darkness. Seeds were initially soaked and shaken in 0–20 mM aqueous solutions of caffeine, pH 5.2–6.0, for 48 hr at 27°C, and later washed and set in the middle of four layers of strips (10 × 50 cm) of Whatman chromatographic paper, 0.3 mm thick. These were rolled and placed in glass tanks (30 × 30 × 10 cm) each with a different concentration of caffeine. In each tank, 150 seeds, divided into three groups (replicas) of 50 each, were set. To avoid gross changes in caffeine concentration by either uptake or evaporation of water, 300 ml of solution per tank were used and tanks were sealed. The effect of exogenous theophylline was also similarly tested. Lengths of rootlets and hypocotyls were periodically measured. Extraction and quantitative determination of caffeine were conducted by a method of Chou and Waller (1980b), slightly modified. Caffeine content was determined for each treatment in each of the five seedlings weekly and was measured separately for the rootlet, hypocotyl, cotyledons, endosperm, and the germination medium.

The effect of caffeine on cell division was studied in cells of root tips of coffee seedlings. The root tips were allowed to develop on filter paper in distilled water for 4 weeks, at 27°C in the dark, and then placed on filter paper immersed in 10 mM caffeine in a tightly covered glass tank (30 × 30 × 9 cm). Root tips were removed after 24 hr, fixed, and prepared for microscopic examination, using a modification of Warmake's method (Warmake, 1935).

RESULTS

When seeds of coffee were allowed to germinate in aqueous solutions of caffeine of various concentrations, elongation of hypocotyls was reduced in all cases and growth of rootlets was almost completely inhibited by 10 mM caffeine (Figure 1, Table 1). Root tips darkened and 4–5 days later deteriorated. A similar although milder inhibition was observed in response to theophylline. Suppression of growth occurred, although concentration of the endogenous caffeine in the imbibed coffee seeds was 40–60 mM. This suggested that embryos of germinating coffee seeds must be able to avoid autotoxic hazards from their endogenous caffeine. Following the level of caffeine in germinating coffee seeds showed that in the quiescent seeds, the embryos were nearly caffeine-free, containing only about 0.6% (on dry weight basis), whereas the adjacent endosperm contained 1.4% caffeine. However, during germination (ca. 4 weeks after seed wetting), substantial amounts of caffeine (ca. 75% of the total amount) were found in the developing embryo (Figure 2). Other portions of the caffeine were distributed as follows; 5.6 ± 3.2% of the total amount was found in the leachate, and 6.4 ± 2.2% was left in the residual, unutilized endosperm. The rest, 13.0 ± 4.1%, could not be located, but the appearance of some metabolic products, including theo-

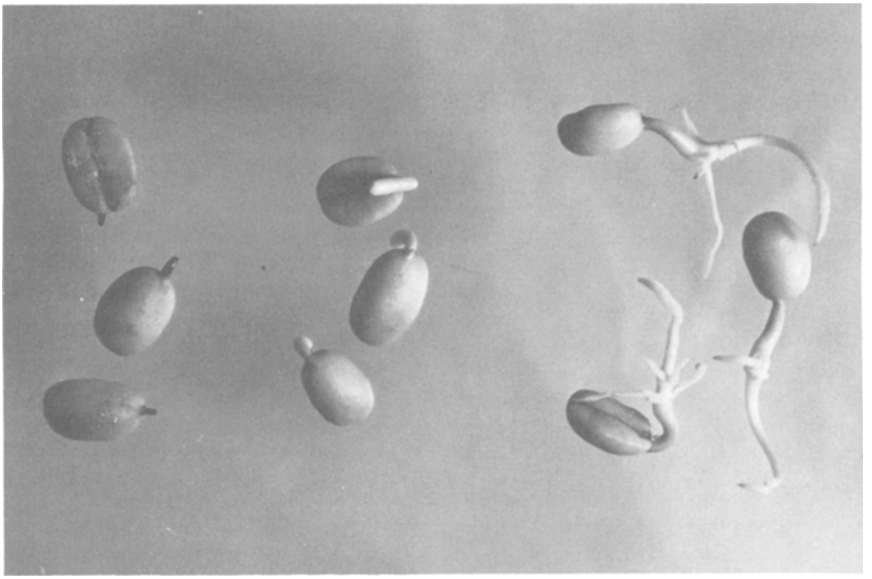


FIG. 1. Seedlings of coffee germinated in distilled water (left), in 2.5 (center) and 25 mM (right) caffeine, three weeks after wetting, in dark. In all caffeine-treated seeds, emergence of the hypocotyl did occur but rootlet growth was arrested (×2.5 natural size).

TABLE 1. GROWTH OF ROOTLET AND HYPOCOTYL OF 4-WEEK-OLD COFFEE SEEDLINGS GERMINATED IN VARIOUS CONCENTRATIONS OF CAFFEINE OR THEOPHYLLINE[a]

	Concentration (mM)		
	5	10	20
Caffeine			
Rootlet	72 ± 8	3 ± 2	3 ± 3
Hypocotyl	52 ± 7	61 ± 19	39 ± 12
Theophylline			
Rootlet	59 ± 7	27 ± 4	14 ± 7
Hypocotyl	58 ± 4	48 ± 11	54 ± 6

[a]Data presented as percentage of growth (length) of the control (distilled water), average of 150 seedlings (± SE)

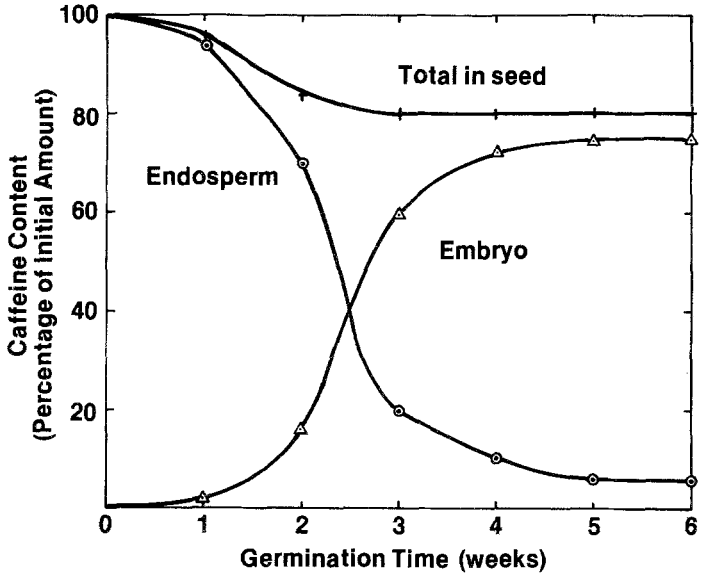


FIG. 2. Changes in localization of endogenous caffeine during germination of coffee. Calculated as percentage of the initial amount (100%) found in the quiescent seed.

phylline and theobromine, in the hypocotyl and in the cotyledons, during the third week of germination (Figure 2), suggested that this portion was catabolized by the embryo. Of caffeine within the embryo, most (83.8 ± 8.7%) was found in the cotyledons, lesser amounts (14.2 ± 4.3%) in the hypocotyl, and the remaining 2.0 ± 1.1% in the rootlet. This pattern of distribution of caffeine was nearly constant during seed germination, starting at the third week after seed wetting. From either of the seedling organs caffeine could be easily extracted by water and was therefore believed to be stored in a soluble form. The rootlet of a coffee embryo may be more susceptible to caffeine damage than the cotyledons, as caffeine is known to interfere with mitosis in root tips of several higher plants (Kihlman, 1977). We therefore tested effects of exogenous caffeine on mitosis in root tips of seedlings of coffee and followed the relation between accumulation of endogenous caffeine and mitosis in the cotyledons. Cell division was inhibited in root tips that were exposed to 10 mM caffeine for 24 hr. Also cell plate formation was blocked, yielding some binucleate cells (Figure 3). Similar results have been obtained on onion root tips (Kihlman, 1949). Tetranucleate cells could not be detected in our study, even after exposure for 48 or 72 hr or to higher caffeine concentration.

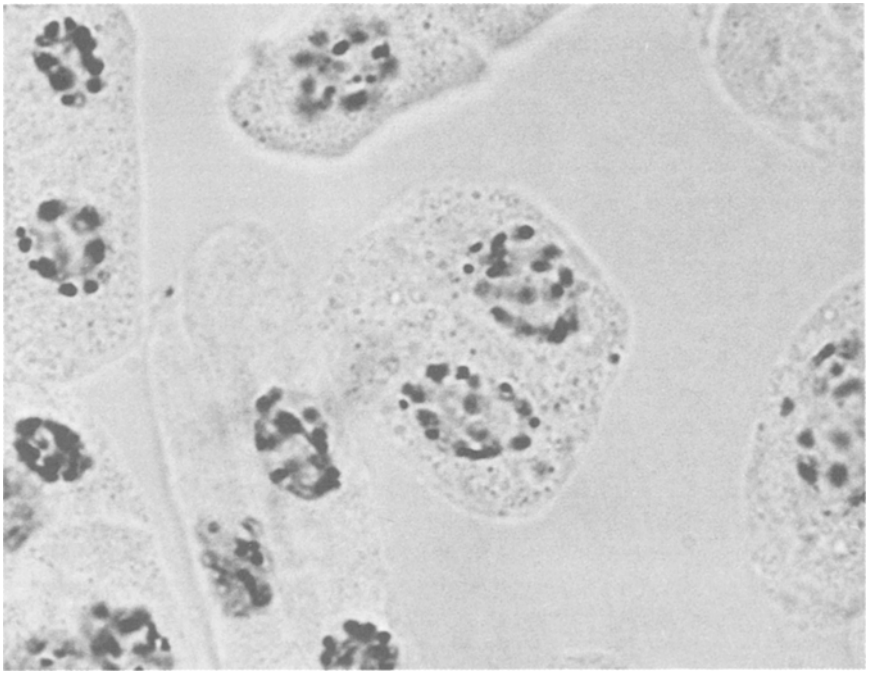


FIG. 3. Binucleate cells in root tips of coffee seedlings after exposure to 10 mM caffeine for 24 hr (×1200).

DISCUSSION

The embryo in the quiescent coffee seed is about 3 mm long, including two cotyledons and a long hypocotyl terminating with a minute radicle (ca. 0.2–0.5 mm long). Initial stages of germination (1–4 days after seed wetting) were characterized by elongation of the hypocotyl and were observed under all treatments, but at this stage no cell division could be detected in either the hypocotyl or the radicle. In the root tips grown in distilled water, cell division started only after the rootlet was pushed by elongation of the hypocotyl 1–3 mm out of the endosperm, which had occurred 5 or 6 days after seed wetting. This growth of the hypocotyl is achieved by cell elongation only after the tip of the rootlet has become separated from the caffeine-rich endosperm before cell division starts in this part. The zone where mitosis occurs is thus separated in space from that where caffeine is localized. The means by which caffeine is sequestered in the endosperm apart from the embryo are not clear.

In the cotyledons of the germinating embryo, such a separation cannot occur. In the quiescent seed, these small organs (1.1–1.5 mm in diameter) are

embedded in the caffeine-rich endosperm and serve as the embryo's haustoria, expanding remarkably (16–20 mm) until germination is completed (5–6 weeks after seed wetting). We therefore supposed that mitosis in the cotyledons was completed at stages of embryonic differentiation while the seed was still on the mother plant. However, observations during germination disproved this view. Cell division in the embryonic cotyledons started 9–11 days after seed wetting; as translocation of endogenous caffeine progressed (Figure 2), mitosis was much reduced, and almost stopped completely 3 weeks after seed wetting. Therefore in the very young cotyledons, the process of mitosis and accumulation of caffeine appear to be separated in time, and thus caffeine autotoxicity is avoided. Whether caffeine is the signal to stop mitosis, or whether each process is separately regulated, is unknown. Separations between caffeine and mitosis either by space, as in the root tips, or by time, as in the cotyledons, are the plant's means of avoiding autotoxicity.

Catabolism of caffeine in germinating coffee embryos was indirectly measured, both by disappearance of about 13% of the amount of caffeine initially present within the quiescent seed, as well as by the presence of some products of biodegraded caffeine (theobromine and theophylline) (Suzuki and Waller, 1981) in the cotyledons and hypocotyl, as already noted. The small amount of catabolism suggests that it plays a minor role in the prevention of autotoxic effects. Conversely, about 75% of the caffeine stored in the endosperm is later found in the embryo, most of it in the cotyledons. This amount did not decrease even in seedlings kept in continuous dark and starved for 84 days at 27°C. The fact that caffeine is preserved despite starvation suggests that the alkaloid is not easily utilized either for energy or as a nitrogen source. Nevertheless, uptake of the alkaloid into the cotyledons, despite its potential hazard, suggests that caffeine is an important agent for the plant. Its wide spectrum of biological activity, e.g., inhibition of fungi (Rizvi et al. 1980a) and bacteria (Kihlman, 1977), and its chemosterilant effect on the seed beetle (*Callosobruchus chinensis*) (Rizvi et al., 1981), as well as its possible allelopathic effects on weeds (Anaya-Lang et al., 1978; Chou and Waller, 1980a; Rizvi et al., 1980b), all support the view that it is a natural plant protectant.

Although the toxic effects induced by caffeine on coffee seedlings in the laboratory were not extremely dramatic, autotoxicity may occur in nature. Around old coffee trees, considerable amounts of caffeine may be released from the tree's own litter and accumulate in the vicinity of roots over the years. The annual amount of litter leaves produced, plus about 10% of lost fruits, in old coffee trees is 150–200 g dry matter/m^2/year (Epifanio, 1981). We reason that this litter may release 1–2 g caffeine/m^2/year, with some additional amounts of caffeine derivatives. The antimicrobial activity of caffeine may reduce catabolism of the alkaloid in the soil and thus prolong its retention and increase caffeine accumulation. Since most roots of coffee develop in the

upper soil layer immediately under the tree's own litter, autotoxicity can be manifested. Thus it is possible that the worldwide phenomenon of early degeneration of coffee plantations at 10–25 years of age (Wellman, 1961) is due in part to autotoxicity, and caffeine, the factor which allows for survival of coffee plants in a hostile environment, is also responsible for shortening their lives.

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SEEDS AS ALLELOPATHIC AGENTS

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Abstract—Inhibitors of germination or of growth, highly diversified chemicals are commonly found in higher plants. They occur in vegetative organs as well as in seeds or other dispersal units. Nonprotein amino acids, when present, are mainly found in seeds where they can occur in extremely high concentrations. Density of seeds, rate of emanation of inhibitors, their amount and effectiveness, all determine allelopathic potential of seeds. To induce allelopathy, rate of emanation of inhibitors must be fast and of sufficient duration. Our observations in coffee seeds *Coffea arabica* L. indicate that rate of emanation of the inhibitor caffeine is highly enhanced during senescence of seeds, suggesting that when allelopathic potential of seeds is evaluated the presence of both young and old seeds should be considered. In many plants seeds are liberated close to the parent plant, the zone where seed-induced allelopathy may occur. Large numbers of seeds are usually produced in order to ensure establishment; greater number and mass of seeds may also increase allelopathic inhibition of competing vegetation.

Key Words—Seed allelopathy, germination inhibitors, emanation of inhibitors from seeds, caffeine, *Coffea arabica* L.

INTRODUCTION

Many seeds, fruits, and other dispersal units contain inhibitors of germination or growth, which belong to widely diversified chemical groups (Evenari, 1949; Billings, 1957; Ketring, 1973). Such inhibitors control seed germination in a variety of ways in concert with external conditions. For instance, they may prevent premature germination when the seed is on the parent plant, extend germination over a longer period of time, or allow germination to occur only after sufficient amounts of rainfall, thereby improving the chances of seedling

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establishment. Considerable efforts have been made during the last few decades to detect germination inhibitors in seeds, but these efforts were largely devoted to the study of the physiological rather than the ecological features of such inhibitors. Both the survivability of some seeds in soil and the occurrence of germination inhibitors led Went (1950) to suggest that seeds may possibly influence the chemical equilibrium in soils. However, analysis of the effects of allelopathy is extremely complicated by interactions between various chemicals in soils and the difficulties of their extraction, isolation, and purification. Seed allelopathy has been left largely unexplored (Rice, 1974).

The purpose of this paper is to critically review the limited literature in this area, present some new information on the effect of senescence on emanation of endogenous inhibitors from seeds, and to point out necessary considerations for determining the allelopathic potential of seeds through research.

OBSERVATIONS AND EXPERIMENTAL DATA

Chemical Nature of Seed Inhibitors, Rate of Activity, and Localization. Heterogenous chemical groups of inhibitors are found in seeds (Table 1). Most groups of inhibitors from vegetative plant parts (Putnam and Duke, 1978) are also encountered in seeds. However, in some seeds amino acids normally incorporated in proteins are found in a free state as are some uncommon, nonprotein amino acids (Table 1). Both types of amino acids may induce inhibition of seed germination or of seedling growth. Over 200 nonprotein amino acids have been detected in lower and higher plants, either in a free state or as condensation products such as γ-glutamyl, acetyl, and oxalyl derivatives (Fowden, 1974; Bell, 1972, 1976). In seeds, especially in the Leguminosae, they may accumulate in amazingly high quantities. Thus, 14% of the seed weight in the West African legume *Griffonia simplicifolia* is comprised of 5-hydroxy-L-tryptophan (Bell et al., 1976). As much as 10% of the seed weight in *Dioclea megacarpa* consists of canavanine, and 8% of the seed weight of *Mocuna mutisiana* is L-3,4-dihydroxyphenylalanine (Bell and Janzen, 1971; Bell, 1972, 1976; Bell et al., 1976). Nonprotein amino acids have also been reported to occur in seeds in plants other than the Leguminosae, as, for example, L-α-amino-β-methylaminopropionic acid in *Cycas circinalis* (Nunn et al., 1967), or selenostathione in seeds of *Lecythis ollaria* (Aronow and Kerdel-Vegas, 1965).

The extent of inhibition that a certain chemical exerts on a given seed can be expressed by the time needed to obtain 50% germination in a given concentration of the inhibitor as compared to the germination in distilled water (Gressel and Holm, 1964) or by the percentage of germination per day compared with the controls, as measured from the onset of germination until

TABLE 1. SOME EXAMPLES OF INHIBITORS OF GERMINATION OR GROWTH IN SEEDS, FRUITS, OR OTHER DISPERSAL UNITS BY CHEMICAL GROUPS

Chemical group	Inhibitor	Species producing	References
Phenolic acids	Ferulic acid	*Solanum lycopersicum*	Akkerman and Veldstra (1947)
	Abscisic acid (ABA)	*Corylus avellana*	Williams et al. (1973)
		Fraxinus americana	Sondheimer et al. (1968)
Courmarins and furocoumarins	Coumarin	*Trigonella arabica*	Lerner et al. (1959)
	8-Metoxypsoralen	*Ammi majus*	Friedman et al. (1982)
	Heraclenol	*Petroselinium crispum*	Kato et al. (1978)
	Momilactone	*Oryza sativa*	Kato et al. (1977)
Terpenes and terpenoids	Volatile terpens (unidentified)	*Foeniculum vulgare*	Evenari (1949)
	Nonvolatile terpens (unidentified)	*Pinus pinea*	Corrillon and Martinez-Honduwilld (1980)
Flavonoids	Myrcetin	*Trifolium repens*	Fottrell et al. (1964)
	Glucoflavonoids	*Beta vulgaris*	Kudryavtsev (1979)
Alkaloids	Caffeine	*Coffea arabica*	Evenari (1949), Friedman and Waller (1983)
Cyanogenic glucosides	Amygdalin	*Prunus amygdalus* (bitter almond)	Laibach and Keil (1937)
Mustard oils	Allyl-isothiocyanate	*Brassica nigra*	Evenari (1949)
Protein amino acids	Arginine	*Abutilon theophrastii*	Gressel and Holm (1969), Elmore (1980)
Nonprotein amino acids	*N,N*-Dimethyl-L-tryptophan	*Abrus precatorious* (jequirity bean)	Mandava et al. (1974)
Proteins	Ribonucleo protein and a lipoprotein	*Echinochloa crus-galli*	Rusev and Atanasova (1981)
Di- and tricarboxylic acids and miscellaneous aromatic compounds	(4*R*, 3*R*)-*threo*-1,3,4-pentane tricarboxylic acid and dimethyloxybutyl benzene	*Avena sativa*	Lohaus et al. (1982)

its termination (Friedman et al., 1977). Different species may differ in their susceptibility in response to a given inhibitor. For instance, psoralen, a linear furanocoumarin, arrests germination of radish seeds at a concentration of 2×10^{-5} M (Fukushi, 1960) and of lettuce achenes at 2×10^{-6} M (Shina-Roy and Chakraborty, 1976).

Collection of data on the allelochemical inhibition by seed on plant growth poses no great problem, but differences in the methods employed by various laboratories often lead to different conclusions. Inhibition of one plant species by seed allelochemicals would be irrelevant where the response of other plant species is concerned. In evaluation of an inhibitor in a particular habitat, the susceptibility of each of the indigenous species has to be determined. It is true that many inhibitors that regulate germination of seeds of one species may also be effective against those of other species, thereby acting both as germination regulators and allelopathic agents. However, the seeds of some species contain highly potent germination inhibitors that arrest only the germination of adjacent seeds of other species. Such substances do not induce autoinhibition, but function merely as allelopathic agents. Examples include 8-methoxypsoralen in fruits of *Ammi majus* (Friedman et al., 1982), amino acids in *Abutilon theophrastii* (Gressel and Holm, 1964), and nonprotein amino acids in various Leguminosae (Wilson and Bell, 1978a). Additionally, some of these inhibitors may also exhibit toxic effects other than inhibition of germination, and hence function as natural seed protectants (Bell, 1978).

Inhibition of germination is sometimes reported to be associated with the arrest of mitosis in the root tips. Thus volatile terpenes, when applied to various seedlings (Muller, 1965, 1966), or caffeine when applied to young coffee seedlings (Friedman and Waller, 1983), block mitosis. Some inhibitors are considered to be antigibberellins (Wurzburger and Leshem, 1969). However, the precise mechanisms of inhibition of germination are, for the most part, unknown.

Localization of the inhibitors may affect the rate and duration of their efflux and subsequently their allelopathic potential. Germination inhibitors can be localized in the various envelopes that encase the embryo, as well as in the embryo itself, e.g., lignans (Cooper et al., 1979); in glumes and hulls of various species of *Aegilops* (see Waisel and Adler, 1959); coumarins in pods of *Trigonella arabica* (Lerner et al., 1959); psoralen in the seed coat of *Psoralea subacaulis* (Baskin et al., 1967); caffeine in the endosperm of coffee seeds (Friedman and Waller, 1983); or the nonprotein amino acid *N,N*-dimethyl-L-tryptophan in the embryo of the jequirity bean (*Abrus precatorius*) (Anderson et al., 1972; Mandava et al., 1974). Reports of localization in the cellular and subcellular levels are rare.

Emanation of Inhibitors. Seeds or other dispersal units containing inhibitors of germination or growth will induce allelopathy only when

discharged into the environment. The common method for isolating inhibitors relies on their extraction with solvents at elevated temperatures—conditions which are significantly different from those in the natural habitat. Consequently, seeds containing large amounts of highly reactive inhibitors are not necessarily allelopathic. The rate and duration of efflux of inhibitors has to be sufficient to induce allelopathic inhibition. Furthermore, the efflux has to be demonstrated experimentally under conditions similar to those in the natural habitat. Some work in this direction has already begun.

Gressel and Holm (1964) have shown that the leachate of seeds of various weeds (*Abutilon theophrastii*, *Ambrosia artemisiifolia*, *Barbarea vulgaris*, *Datura stramonium*, and *Echinochloa crusgalli*), obtained after setting in Petri dishes to germinate for 24 hr, inhibited the germination of some crop seeds, especially those of alfalfa. Inhibitory effects similar to those obtained in Petri dishes occurred also in soils. Inhibitors in the leachate of *Abutilon theophrastii* seeds were preliminarily characterized by the authors as amino acids. Subsequently, studying the same extract, Elmore (1980) identified 21 common amino acids, the most prevalent of which were glutamine and glutamic acid. Germination and growth of lettuce seedlings were found to be inhibited by common amino acids, but the suppressive activity of uncommon, nonprotein amino acids was, in most tests, much higher (Wilson and Bell, 1978a).

In seeds of *Glycine wightii* both common and uncommon amino acids were emanated during the course of germination (Wilson and Bell, 1978b). During the first 30 hr, such release was high; the materials included glutamic acid, tricarboxytyrosine, other ninhydrin-reactive compounds, and small amounts of canavanine; subsequently, the emanation almost ceased for another 30 hr, to recommence at about the 60th hour. The relative amount of canavanine, in this case, increased whereas glutamic acid and other ninhydrin-reactive compounds decreased, suggesting that the emanation in these seeds was an active process, controlled by the germinating seed.

An allelopathic effect can be expected when inhibitors are discharged from seeds at a rate which will yield, on the one hand, a concentration sufficient for inhibition of adjacent seeds/seedlings and, on the other hand, will be slow enough to ensure emanation for a sufficiently long period. These conditions were found to prevail, at least in part, in fruits of *Ammi majus* (Bishop's weed, Umbelliferae) (Friedman et al., 1982). Here the efflux of one of the inhibitors (8-methoxypsoralen) into the germination medium during 24 hr amounted to 2% of its total content in the fruit, and the inhibitory effect of such fruits was not weakened even after they were submerged in a large volume of water for a 10-day period. The findings suggested that a mechanical barrier in the outer part of the dry fruit ensures a low rate of efflux. In the long run, either the content of inhibitors within a seed will decrease, or its germination will occur and emanation will cease. Interestingly, the rate of

germination of fruits of *Ammi majus* planted in the soil is 3–10% in the first year, which suggests that fruits of this species may function as allelopathic agents for more than a year (Friedman et al., 1982).

Since the soil contains a bank of seeds of various ages, we deemed it of interest to study the effect of seed senescence on the rate of emanation of germination inhibitors. Available literature data had already shown that leakage of ionic solutes from germinating seeds increases with seed age, thereby increasing the electrical conductivity of the medium (Harman and Granett, 1972; Parish and Leopold, 1978). Young, viable seeds often reabsorb sugars, amino acids, and other metabolites by active uptake (Osborne, 1981), but as senescence progresses, the initial loss of cytoplasmic solutes is correspondingly greater and the extent of subsequent uptake is lower (Roberts, 1972). Based on these findings, as well as on the limited data dealing with leakage of secondary metabolites from seeds, we tested the hypothesis that leakage of a secondary metabolite which also inhibits germination will increase with seed age.

For this purpose coffee seeds were selected, because they are known to lose viability some months after collection (Valio, 1976; Bewely and Black, 1982) and contain caffeine, an active germination and growth inhibitor of various plant species (Evenari, 1949; Rizvi et al., 1980), as well as autotoxic to coffee itself (Friedman et al., 1981, Friedman, 1982; Waller et al., 1982; Friedman and Waller, 1983). The rate of emanation of caffeine was determined both in new, viable seeds of coffee (*Coffea arabica* cv. Bourbon) 3 months after their collection (in Jalapa, Mexico), having 38 ± 4% water (on dry weight basis) and showing 87 ± 6% germination during 10 days, as well as in one-year-old dead seeds of the same variety and locality, with 18 ± 8% water on dry weight basis. Seeds of both types contained 0.8% caffeine. Twenty-five seeds of each type were immersed in 50 ml of glass-distilled water, then shaken for 24 hr at 27 ± 0.5° C. The eluate was replaced by distilled water every 24 hr for a period of 12 days, and the caffeine content determined according to a slightly modified method of Chou and Waller (1980). Experiments with both seed types were replicated five times. The results are shown in Figure 1. The averge leakage of caffeine is expressed as a percentage of the amount initially present in the seed. During a period of 24 hr, the viable seeds showed an average rate of caffeine efflux comprising 1.8% of the total caffeine; the average rate of leakage from the old, nonviable seeds was more than five times higher than in the young seeds.

Our incidental observations in coffee plantations in Jalapa, Mexico, have established that only a small number of lost, uncollected coffee seeds germinate. This leads us to conclude that in nature most of the caffeine incorporated in seeds of coffee will be discharged into the soil around the seed layer within 1–2 years after seed dispersal. Rate of caffeine efflux will be expected to increase as the last seed crop ages. Hence, if the allelopathic

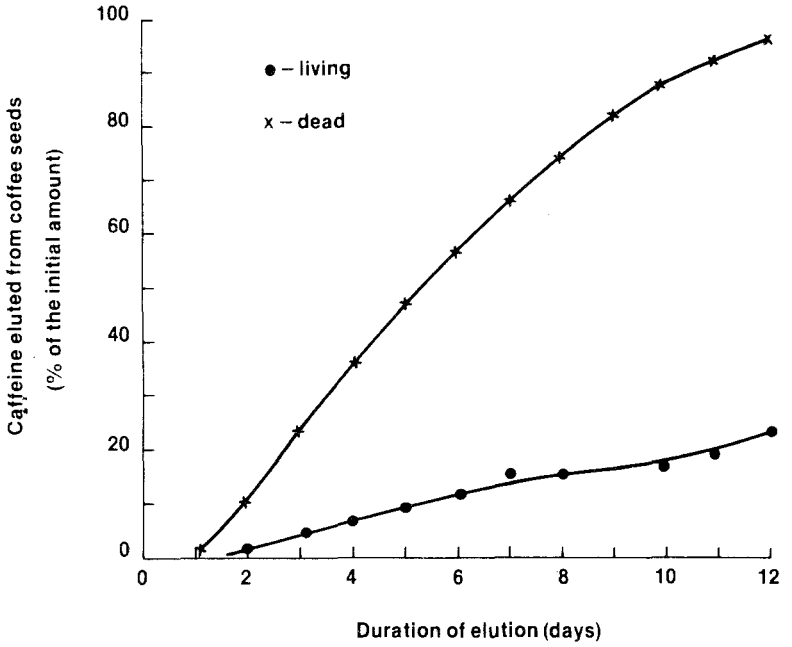

FIG. 1. Average rate of caffeine emanated from young viable seeds (●) and from old dead seeds (x) of coffee (*Coffee arabica* cv. Bourbon) shaken in distilled water.

potential of seeds is to be properly evaluated, both the old nonviable and the young seeds in the soil should be taken into consideration.

Seed Yield and Patterns of Seed Dispersal. The amount of inhibitors released into the soil is dependent also on seed yield and dispersal. Despite the variety of devices in plants to ensure dispersal of the seeds (Pijl, 1974), in many species the dispersal of the majority of seeds takes place in the immediate vicinity of the parent plant, so that the seed density diminishes steeply with distance (Harper, 1977). Thus, in many species whose plants do not continuously populate an area, seed dispersal will not counteract the possible seed-induced allelopathy. An example of such a situation can be derived from a study of *Ammi majus* (Friedman and Rushkin, 1980a, 1980b; Friedman et al., 1982). The numerous minute fruits (1.5–2.0 mm) of this annual are dispersed under the plant's canopy in a density of 5–15 fruits/cm^2/year, that is, 2.5–7.5 mg/cm^2/year. The fruits contain an average of 0.8% (dry weight basis) of 8-methoxypsoralen (8-MOP), a germination inhibitor. Two percent of the total content of this subsurface emanates during the first 24-hr, as already noted, which amounts to a minimum of 20 μg/cm^2/day. Collected in 1 ml of water, this will produce a solution whose concentration is 1×10^{-4} M—in excess of the concentration (0.75×10^{-4} M) which, under experimental

conditions, reduced germination of a test plant by 50%. The same fruits emanated other, as yet unidentified, inhibitors which were six times as reactive as 8-MOP. The slow efflux of 8-MOP and the consequent inhibitory effect may last for weeks or even months. It seems that the more arid the environment the higher the retention of the inhibitors and the longer-lasting their effect.

Of the total amount of dry matter produced by a single plant, only a limited portion is incorporated in seeds and their various envelopes, e.g., in *Senecio vulgaris* about 10% (Harper and Ogden, 1970) or in *Medicago laciniata* var. *laciniata* about 20% (Friedman and Elberse, 1976). This is probably why the role of seeds as allelopathic agents has not yet been sufficiently investigated. This review and the original work on the effect of senescence of seeds on emanation of inhibitors suggest that the allelopathic potential of seeds may be greater than previously believed. Thus the large number of seeds produced by some plants may ensure the establishment of seedlings not only by increasing the chance of seed germination on sites fitted for establishment, but also by reducing the germination and growth of competing vegetation.

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ALLELOPATHIC EFFECTS OF BLACK WALNUT ON EUROPEAN BLACK ALDER COPLANTED AS A NURSE SPECIES

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Abstract—Black alder trees were interplanted with black walnut on a 2.4 × 3.7-m spacing at four locations in Illinois and Missouri. In every plantation, the alders suddenly declined and died after 8–13 years. Alternative causes for the mortality were investigated—competition from walnut, adverse soil properties, frost, insects, disease, unsuitable seed source, and allelopathy—but only allelopathy could be substantiated. The decline in alder survival and diameter growth was strongly related to walnut above-ground dry biomass and walnut crown competition factor. We propose that the black walnut allelopathy most likely results from a combination of (1) sufficient walnut biomass to contribute substantial amounts of juglone to the environment, and (2) wet soil that greatly restricts aerobic metabolism by soil microorganisms, allowing juglone to build up to toxic levels.

Key Words—*Alnus glutinosa*, juglone, decline, *Juglans nigra*.

INTRODUCTION

European black alder [*Alnus glutinosa* (L.) Gaertn.] is recommended as a nurse species to stimulate growth of other deciduous and coniferous species planted on spoil banks in the eastern United States (Plass, 1977). It grows rapidly on a range of spoil types and contributes to soil enrichment by fixing nitrogen and providing a leaf fall rich in nutrients. Black alder was considered a promising candidate species in a study begun in 1969 to test how interplanted woody nurse species affected the growth of black walnut (*Juglans nigra* L.) trees in four intensively cultured plantations in Illinois and Missouri.

In three of the four plantations after three years, walnut trees interplanted with alder were significantly larger than walnut in pure plots at the same spacing (Schlesinger and Williams, 1984). However, the beneficial relationship of alder and walnut was interrupted at every location after 8–13 years by the sudden decline and death of the alders. Healthy trees began showing twig and branch dieback, mostly at the top of the tree; as dieback intensified, trees became increasingly sparsely foliated with small leaves. Some shedding of green leaves occurred beginning in early summer, and varying amounts of epicormic branching occurred from the bole. An individual vigorous tree typically declined and died in 1–2 growing seasons, and the majority of the alders in the plantation were dead within 2–3 years. There was no basal sprouting of declining trees. The interplanted alders are now nearly gone in two plantations and are rapidly declining and dying in the remaining two plantations.

Because of the absence of other conspicuous causal agents, we began to suspect allelopathy as a possible cause for the death of the alders. The preexisting study provided us with an excellent opportunity to investigate the interactions between walnut and alder. Existing growth data were analyzed with the following objectives: (1) to determine the most probable cause for the alder mortality, and (2) if walnut allelopathy were determined to be the cause, to attempt to relate the alder decline with walnut density.

METHODS AND MATERIALS

Details of the 1969 study and description of plantations are published elsewhere (Funk et al., 1979); therefore, only pertinent information will be summarized here. Four plantations were established on upland and bottomland locations with soil types considered suitable and types considered questionable for growing walnut (Table 1). A randomized complete block design was used: two blocks contained 33 interior measurement trees at study locations AC and HC and three blocks contained 21 interior measurement trees at study locations BW and CC. Walnut and alder were alternated at a 2.4-m spacing within rows and 3.7-m spacing between rows. Total height and stem diameter at breast height (dbh, 1.37 m above ground) of each tree were measured at the end of each growing season.

We investigated the following possible causes for the alder mortality at the four locations: (1) competition from walnut, (2) adverse soil properties, (3) frost, (4) insects, (5) disease, (6) unsuitable seed source, and (7) allelopathy. In applying the scientific method, we attempted to reject the null hypothesis for each cause, i.e., H_0: the candidate cause has no effect on alder survival and growth. For study locations HC and BW, where the alders have been dead for several years, we consulted study and weather records for events that

TABLE 1. DESCRIPTION OF STUDY AREAS

Name	Location		Soil type	Topographic situation	Subsoil permeability[a]	Onset of alder mortality[b]
	County	State				Growing season
Ambeer Creek (AC)	Alexander	IL	Haymond silt loam (S)[c]	Bottomland	Moderate	12
Camp Cadiz (CC)	Hardin	IL	Hosmer silt loam (Q)	Upland	Mod. slow to slow	13
Hogthief Creek (HC)	Hardin	IL	Belknap silt loam (Q)	Bottomland	Mod. slow	8
Busch Wildlife (BW)	St. Charles	MO	Putnam silt loam[d]	Bottomland	Very slow	9

[a] Permeability ratings are from Fehrenbacher et al. (1967) and Scrivner et al. (1966). Moderate permeability is considered the most desirable; soils with slow to very slow permeability have restricted water flow.
[b] First year that survival declined noticeably following establishment.
[c] Considered suitable (S) or questionable (Q) for black walnut (Losche et al., 1980).
[d] Not listed in Losche et al. (1980) but considered suitable based on an assessment of soil properties and topographic location.

coincided with the failure of the alders. At study locations AC and CC, which are presently declining, we inspected and sampled the trees for evidence of probable cause. Because the study did not contain plots of alder without walnut to use for comparisons, we similarly inspected and sampled trees from a vigorous 22-year-old pure alder plantation on alkaline stripmine spoils near Lynnville, Indiana. In particular, we examined trees for unusual morphology, evidence of insect attack, and presence of pathogens. Twig and root samples were taken from apparently healthy trees and from declining trees at study location AC, and from healthy trees at Lynnville, Indiana. In the laboratory, root tissues were observed for discoloration and cultured to determine the presence of pathogens.

The relation between walnut density and alder condition was determined for each area by separate nonlinear regressions of percent survival (Y_1) and mean annual diameter growth (Y_2) of alders on mean crown competition factor (X_1) and mean above-ground dry biomass (X_2) of the walnuts. Crown competition factor (CCF, Krajicek et al., 1961) is a measure of crowding that provides an objective method for assessing plantation crowding; at CCF = 100, the sum of the tree crown areas equals the area of the plantation. Walnut biomass was calculated by the equation:

$$BM = 5.45056 + 0.02307\ d^2h$$

where BM = total above-ground dry biomass in kg, d = tree diameter at breast height in centimeters, and h = tree height in meters.[1] The period tested covered the time when the alders attained a measurable diameter at breast height to the time when the alders died, or to the present.

RESULTS

Walnut Competition. The outward signs of tree competition were the relative position and condition of the tree crowns. Vigorous trees that have outgrown their neighbors occupy superior positions in the crown canopy and normally have the best chance of surviving competition in the future (Smith, 1962, p. 33). Graphs of black alder and black walnut tree height over time (Figure 1) reveal that in all of the plantations the alders were the same height (BW) or much taller (AC, CC, and HC) than the walnut when they began to die. This rules out the possibility that the alders succumbed to shading from the walnuts.

Root competition was more difficult to assess. Both walnut and alder possess a vertical-horizontal root form consisting of extensive surface and

[1]Equation developed by Richard C. Schlesinger, data on file at North Central Forest Experiment Station, Carbondale, Illinois 62901.

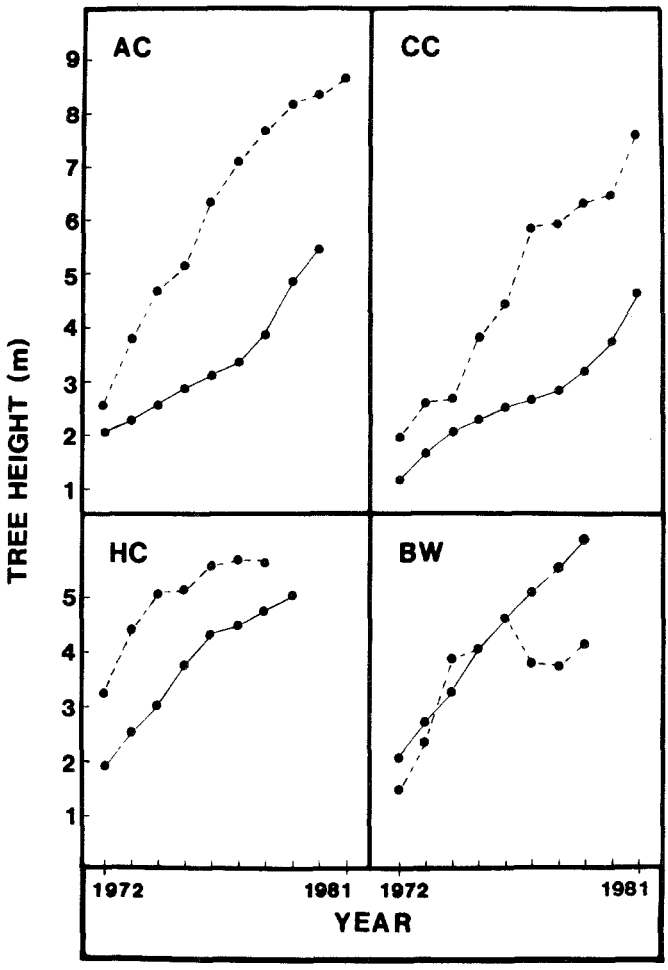

FIG. 1. Mean total height of black alder •---• and black walnut •——• trees alternately planted at a 2.4 × 3.7-m spacing at four locations: AC = Ambeer Creek, CC = Camp Cadiz, HC = Hogthief Creek, and BW = Busch Wildlife.

deep root systems that enable the trees to survive and grow under a variety of conditions (McVean, 1953; Yen et al., 1978). Black walnut is characterized as a taprooted species, yet its well-developed surface root system in the upper 60 cm of soil accounts for more than 80% of its roots (Yen et al., 1978). Thus, we can probably safely assume that strong root competition occurred between alder and walnut and that the effects were similar to crown competition.

Soil Properties. The most important soil properties that affect tree growth are soil fertility, moisture regime, and aeration. Soil fertility is

primarily a factor that limits tree growth, when other factors are favorable, and has little relation to tree survival. The remaining two soil properties are intimately related, and their variations are primarily responsible for the differences between good and questionable soils for growing walnut (Losche et al., 1980) The key soil problems are droughtiness caused by excessive drainage and poor aeration caused by poor internal drainage. Two of the study plantations (CC and HC) were established on soils rated as questionable for walnut because of their fine texture and restricted drainage.

In its natural range, black alder is noted for its capacity to ameliorate the physical and chemical properties of soils and to tolerate soil wetness, although it may gradually decline when planted on excessively wet or dry soils (Goncalves and Kellison, 1980). However, black walnut, a species considered more sensitive to soil drainage than alder, has shown good survival and fair to good growth in all of the plantations. If soil drainage were a causal factor for the alder mortality, then we would expect the walnuts to be even more affected and conspicuous differences to exist among the plantations. Thus, soil properties per se can be discounted as a possible primary cause for the alder mortality.

Frost. Boyette and Brenneman (1978) reported apparent freeze damage to black alder trees in North Carolina plantations during the severe winter of 1976–1977. Most of the trees suffered partial to complete dieback. Regrowth the following summer consisted of dense epicormic branching and basal sprouts, giving the trees a bushy appearance. Although many trees continued to have low vigor, few trees died from the incident. The plantations of our study were exposed to the same severe winter conditions, but only the decline at BW coincided; the other plantations, which were planted with the same nursery stock, were unaffected. Moreover, the symptoms described by Boyette and Brenneman differ markedly from the sudden decline and death of alder in our study. In our experience, there was no basal sprouting from declining trees. Therefore, frost or freezing damage does not account for the sudden and consistent alder mortality in the four plantations.

Insects. No insects thought to be responsible for the sudden alder mortality were isolated from root, bole, or limb tissue. However, all of the study plantations and the pure alder plantation at Lynnville, Illinois, were infested with the wooly alder aphid [*Prociphihis tessellatus* (Fitch)], which may have weakened the trees. Leaf miners and scale insects are also common insect pests on alder (Anonymous, 1981). Although they do not kill the trees, they often slow growth and result in dieback of tops.

Disease. Dozens of diseases have been observed on black alder, but few have been confirmed, or even strongly suspected, of causing serious trouble (Funk, 1983). Several fungus species have been isolated from alder trees weakened by wooly aphid infestations. They include *Botryodiplodia theobromae* (Purnell 1981, cited by Funk, 1983) and *Phomopsis alnea* (Oak

and Dorset, 1983), but neither has been confirmed to be aggressively pathogenic. Oak and Dorset concluded that *P. alnea* is a weak canker pathogen of European black alder. Trees severely infected with *Phomopsis* typically develop vigorous root collar sprouts after top-kill[2]; no such stump sprouting was observed in our studies. Munch (1936) reported that *Valsa oxystoma* was once thought to be responsible for alder death in Europe but, after repeatedly failing to artificially infect trees with the organism, concluded that it is predominantly a secondary saprophyte. Alder trees infected with wooly aphids are sometimes subsequently attacked by sapsuckers (Funk, 1983). We observed some trees with dieback caused by sapsucker girdling, but it was of only incidental importance.

In our examination of declining and recently dead alder trees, we found no bole or branch cankers, sporophores, or other fruiting bodies consistently associated with the decline. Cultured samples of tissues from dying twigs and roots showed no evidence of agressively pathogenic organisms.

In summary, we could find no evidence supporting the supposition that the alder were killed by a disease.

Seed source. Much racial variation for climatic tolerance occurs over the broad natural range of European black alder, but within regions variation is sometimes slight (Funk, 1983). Thus, alder seed must be obtained from a region with climate comparable to that where it will be grown. Several reports in the literature describe the consequences of planting black alder that is not genetically suited to the local climate. Munch (1936) detailed the history of the "alder death" problem in Europe from 1899 to 1936 and reported convincing evidence that the cause was almost total reliance on seed collected from a small area in Belgium. That seed was found to be unsuitable for most other sites. Planted alder trees typically grew well for approximately 12 years, then gradually declined and died over the following 6–8 years. The declining trees were characterized by heavy fruiting, poor form, twig and branch dieback from the top down, and epicormic branching from the bole. In reporting 16-year results of black alder provenance trials in Ohio, Funk (1979) mentioned that one source from Peiting, Germany, demonstrated initial vigorous growth, then gradual decline and failure. Verweij (1977, cited by Funk, 1979) reported similar results from black alder trials in the Netherlands where trees from three German locations grew rapidly for 7 years then fell off sharply in the following 3 years. Goncalves and Kellison (1980) reported that some provenances of black alder being tested for suitability in the Southeast exhibited symptoms similar to those described by Munch (1936). They speculated that the dieback was caused by early- and late-season growth being killed by frosts.

Alder planting stock used for establishing the four plantings and for

[2]Letter from R.B. Hall, Iowa State University, dated August 11, 1982.

replacing dead seedlings was obtained from the Union Forest Nursery, Illinois (lat. N 37° 30′, long. W 89° 20′). The original seed, of unknown origin, was obtained in the early 1960s through a supplier. At that time a small stand of black alder was established at the nursery from which subsequent seed collections were made to supply nursery needs. The planted alder trees at the nursery are now approximately 20 years old, average approximately 20 cm in diameter at breast height and 12-15 m tall, and are apparently healthy. Thus, the seed source of the alder trees used in our study is apparently adapted for growth in Illinois. Moreover, except for the twig dieback, the gradual decline of alder trees from unsuitable seed sources is inconsistent with the rapid decline observed in our study. We conclude that an unsuitable seed source is not a valid explanation for the alder mortality observed.

Allelopathy. Allelopathy from herbaceous species in the affected plantations is unlikely. Larson and Schwarz (1980) found that growth of black alder was unaffected by litter from goldenrod, broomsedge, crownvetch, wild carrot, tall fescue, and timothy that was mixed with the potting medium.

However, increasing walnut density was found to be inversely related to alder survival (Figure 2). Nonlinear regressions of alder survival on walnut

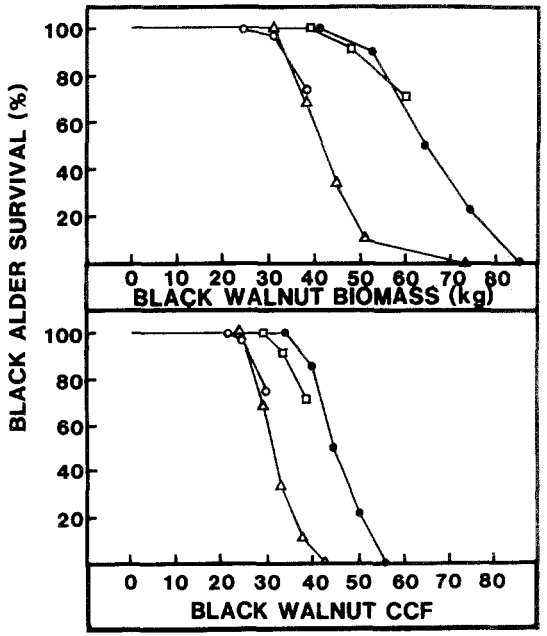

FIG. 2. Black alder survival in relation to black walnut above-ground biomass, and black walnut CCF for two questionable black walnut sites (CC = ○, HC = △) and two acceptable black walnut sites (AC = □, BW = ●).

TABLE 2. NONLINEAR REGRESSIONS OF ALDER SURVIVAL ON BLACK WALNUT ABOVE-GROUND DRY-BIOMASS AND WALNUT CCF FOR FOUR PLANTATIONS[a]

Independent variable	Plantation	Coefficients and standard errors								
		a	(SE_a)	b	(SE_b)	c	(SE_c)	d	(SE_d)	r^{2b}
Walnut biomass	BW	32.71	(42.14)	4.780	(2.597)	−0.1124	(0.0467)	0.00061	(0.00025)	0.83
	CC	203.50	(76.20)	−14.907	(8.630)	0.6447	(0.3173)	−0.00919	(0.00377)	0.87
	HC	335.03	(168.54)	−15.801	(11.916)	0.2757	(0.2605)	−0.00166	(0.00177)	0.70
	AC	26.02	(29.85)	5.723	(2.471)	−0.1512	(0.0635)	0.00109	(0.00051)	0.85
Walnut CCF	BW	14.43	(44.75)	8.506	(4.324)	−0.2626	(0.1250)	0.00195	(0.00110)	0.82
	CC	55.74	(74.47)	3.685	(11.032)	−0.0686	(0.5256)	−0.00151	(0.00805)	0.74
	HC	110.31	(132.97)	0.673	(14.335)	−0.1480	(0.4740)	0.00182	(0.00487)	0.78
	AC	111.61	(24.17)	−3.982	(3.111)	0.2334	(0.1268)	−0.00435	(0.00164)	0.95

[a]Model: $Y = a + bx + cx^2 + dx^3$; where: Y = black alder survival (arcsin transformation of percent); x = black walnut above-ground dry biomass in kg, or black walnut CCF.
[b]Coefficient of determination (r^2) significant at $\alpha = 0.05$.

biomass and walnut CCF showed that alder survival decreased as walnut density increased (Table 2). Both independent variables were significantly related to alder survival at all four plantations ($P \leq 0.05$).

Alder diameter growth was similarly found to be inversely related to walnut density (Figure 3). Nonlinear regressions of alder diameter growth on black walnut above-ground dry biomass and black walnut CCF at two locations (BW and AC) similarly showed that alder diameter growth decreased as walnut density increased (Table 3). The independent variables

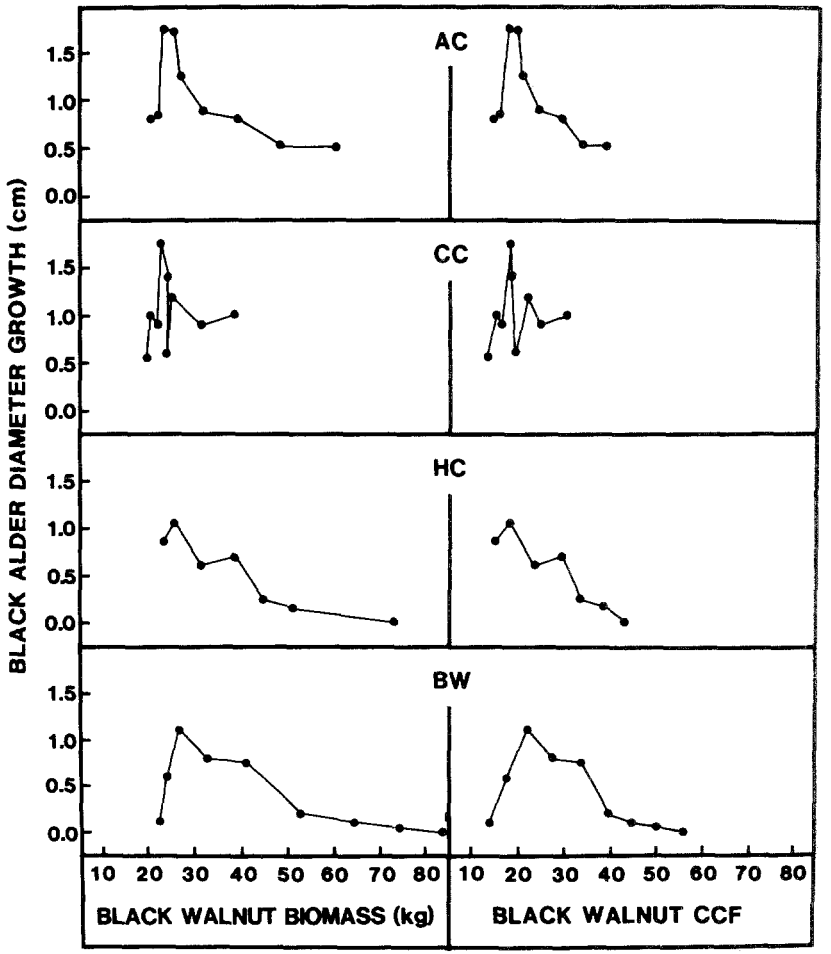

FIG. 3. Black alder diameter growth in relation to black walnut above-ground dry biomass and black walnut CCF for four plantations. Plotted points are means of surviving trees from 1973 to 1981.

TABLE 3. NONLINEAR REGRESSIONS OF ALDER DIAMETER GROWTH ON BLACK WALNUT ABOVE-GROUND DRY BIOMASS AND WALNUT CCF FOR FOUR PLANTATIONS[a]

Independent variable	Plantation	Coefficients and standard errors				
		a	(SE_a)	b	(SE_b)	r^2
Walnut biomass	BW	0.7437	(0.1868)	−0.0274	(0.0041)	0.77[b]
	CC	0.1908	(0.1968)	−0.0062	(0.0069)	0.05
	HC	0.0585	(0.2406)	−0.0095	(0.0063)	0.24
	AC	0.5345	(0.1183)	−0.0155	(0.0031)	0.68[b]
Walnut CCF	BW	0.9145	(0.2840)	−0.0408	(0.0084)	0.65[b]
	CC	0.2698	(0.1986)	−0.0117	(0.0091)	0.09
	HC	0.1177	(0.2508)	−0.0151	(0.0090)	0.29
	AC	0.6604	(0.1120)	−0.0261	(0.0041)	0.77[b]

[a]Model: log $Y = a + bx$; where: Y = black alder diameter growth in centimeters; x = black walnut above-ground dry biomass in kg, or walnut CCF.
[b]Coefficient of determination (r^2) significant at $\alpha = 0.05$.

were significantly related to alder diameter growth ($P \leqslant 0.05$). Mean diameter growth does not always clearly indicate tree vigor because as trees die, the mean is based on fewer, more vigorous survivors. The response is apparently not due to competition because the alders were the same height or taller than the walnuts when the decline began (Figure 1).

The four plantations differed in the timing of alder decline and death, but the general pattern was similar (Figures 2 and 3). Alder diameter growth peaked after 6–8 growing seasons, then declined steadily at all locations (Figure 3). The initial decline occurred when the biomass of the four black walnut trees surrounding each alder was approximately 25 kg and the CCF was approximately 20. The alder decline occurred at slightly lower values at the two locations (CC and HC) with soils rated as questionable for walnut. The rate of diameter growth decline varied among the four plantations, presumably due to site differences.

DISCUSSION

Unlike other reported cases of alder failures, in our study the decline and death of alders was sudden and acute rather than gradual and chronic. The observed pattern is typical of disease, severe insect attack, or a poison. Because we found no evidence to support the possibility of insect or disease causes, and strong evidence supporting the occurrence of allelopathy from surrounding black walnut trees, we deduce that the alders were posioned. The

principal chemical responsible for walnut allelopathy is juglone (5-hydroxy-1,4-naphthoquinone) (Davis, 1928), which is found in the leaves, fruit hulls, inner bark, and roots of walnut (Lee and Campbell, 1969). Rain washes it from living leaves and carries it to the soil; it is released also, along with tannins, from dead leaves and fruits to the soil. There is also significant evidence that juglone is transferred to other species through root contacts (MacDaniels and Pinnow, 1976). When other species are coplanted with walnut, there is apparently a buildup period—the time required for walnut trees to grow to sufficient density to have a significant chemical effect on the environment (Rietveld, 1982).

Alder trees were typically observed to be healthy one year, then decline and die the following year. The first symptoms usually appeared in the spring. We could expect the decline and dying process to be more gradual if we assumed that as walnut biomass increased, the alder trees would be exposed to steadily increasing amounts of juglone. Then why did the alders decline and die so suddenly? We propose the following explanation. The key factor appears to be soil drainage, specifically soil aeration. Fisher (1978) found a strong relationship between soil moisture and the persistence of juglone in soil. As soil moisture increased, the amount of extractable juglone and allelopathic activity increased. The microorganisms responsible for decomposition of aromatic compounds in the soil are aerobic heterotrophs (Alexander, 1977, pp. 214–221). In well-drained, well-aerated soil, these organisms, predominantly bacteria, rapidly break down organic compounds added to the soil. However, under certain conditions where aerobic metabolism is hindered, such as wet soil conditions in the spring, such compounds may accumulate in soil to levels that injure higher plants. Thus, we propose that black walnut allelopathy results from the interactions of two factors: (1) an accumulation of sufficient walnut biomass to contribute substantial amounts of juglone to the environment, and (2) variations in soil aeration that control the capacity of aerobic microorganisms to decompose juglone. Differences in sensitivity observed in the field may also be due to rooting characteristics, presence of detoxifying mechanisms, or differences in sensitivity with age (Fisher, 1979).

Available literature on the chemical nature of juglone indicates that it is a potent inhibitor. Jensen and Welbourne (1962) found that juglone inhibited mitosis in test plants. Koeppe (1972) reported that juglone inhibits oxidative phosphorylation even when it is applied in low concentration. Shoot elongation and dry weight accumulation of alder seedlings were significantly inhibited by juglone concentrations as low as 10^{-5} M, without the appearance of toxicity symptoms; seedlings were severely retarded or killed by 10^{-4} M juglone (Rietveld, 1982). Based on this information, we speculate that no allelopathy would occur in mixed plantations on well-drained sites, some growth reduction may occur on moderately well-drained sites, and growth may be severely reduced on imperfectly drained sites. As soil aeration

decreases and walnut biomass increases, we would expect toxic juglone levels to be more common. When soil juglone levels reach the lethal level, the trees are poisoned and irreversible sudden decline and death occurs. Even brief periods of soil anaerobiosis, which may occur during rainy periods, may be sufficient to bring about lethal juglone levels if sufficient walnut biomass is present. Our research did not include measurements of soil juglone concentrations in the walnut plantations under different moisture–aeration conditions and at different times of the year. Until such data are available, our proposed mechanism for the occurrence of walnut allelopathy must be considered speculative, as a working hypothesis for further research.

This proposed mechanism is supported by the timing of the occurrence of allelopathy in relation to soil permeability among the four plantations (Table 1). We attribute the variable response to differences in (1) soil texture and drainage, (2) soil moisture regimes, and (3) walnut growth rates among the plantations. From a survey of 46 mixed, even-aged plantations of various species with black walnut, Rietveld (1982) observed that soil drainage was a consistent factor affecting the occurrence of allelopathy. For example, eastern white pine (*Pinus strobus* L.), a sensitive species, planted with walnut on well-drained, coarse soils in Michigan, was growing well after 44 years, while the same species growing on moderately drained, finer textured soils elsewhere was killed in as little as 13 years. These observations agree with those of Fisher (1977), who found that planted white pine and red pine (*P. resinosa* Ait.) often suppressed walnut on well-drained sites. In laboratory studies he found that juglone and its inhibitory activity rapidly disappeared from soil that underwent a drying cycle from field capacity before being rewet. When soil moisture levels were maintained between field capacity and one-half field capacity, juglone and its inhibitory activity persisted.

In addition to the direct effects of juglone on alder, effects on essential microorganisms associated with alder roots have been documented. Dawson and Seymour (1983) found that 10^{-3} M juglone completely inhibited nitrogen fixation by the symbiotic bacteria *Rhizobium* that resides in alder root nodules, as well as the actinomycete *Frankia* that symbiotically resides in root nodules of autumn olive (*Elaeagnus umbellata* Thunb.) and other species. Growth of the organisms increased exponentially as juglone concentration decreased to 10^{-6} M. Our own observations of alder roots revealed that nodule clusters from declining trees contained more senescent nodules than did nodule clusters from healthy trees. The consequences of these effects on essential microorganisms, in terms of tree survival and growth, are unknown.

Based on the lack of substantiative evidence to reject null hypotheses for alternative causes of the alder mortality, and the strong relationships found between increasing black walnut density and decreasing alder survival and growth, we conclude that black walnut allelopathy is the most probable cause of alder mortality in our study.

Acknowledgments—We wish to express our appreciation to the Shawnee National Forest, the Missouri Department of Conservation, and the Pierson-Hollowell Co., Inc., for providing experimental areas, and the many people who assisted in data collection over the years, especially Ray Vinyard, Mike Cook, and Elbert Rendleman.

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ALLELOPATHIC EFFECTS OF *Pinus densiflora* ON UNDERGROWTH OF RED PINE FOREST

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Abstract—Correlation between the distributional frequency of undergrowth species of red pine forest and their germination and growth effected by pine extracts and leachates was found. It was made clear by germination and growth tests that pine toxic substances inhibit the germination and growth of low frequency species more than high frequency species in a red pine forest and that these substances are contained in descending concentration in fresh and fallen leaves, roots, pine forest soil, and pine rain. The concentration of pine toxic substances in extracts or leachates was affected by extracting or leaching within a given period of time, requiring a few hours for extracts or a few days for leachates. The amount of dry weight inhibition of the undergrowth treated by pine leachates was expressed as a growth inhibition index (GII) for the comparison of tolerance in various species. GII is a relative value (%) of the test groups against the control and it is an exponential function of the amount of pine toxic substances affecting the dry weight of the undergrowth. The substances were analyzed by paper and gas chromatography. Benzoic acid and 11 phenolic acids were identifed by gas chromatography. Benzoic acid was considered to be a key factor of allelopathy in the red pine forest.

Key Words—Allelopathy, floristic composition, pine extracts, germination inhibition index, gas chromatography, phenolic compounds, benzoic acid, relative germination percentage.

INTRODUCTION

The distribution of species in a plant community is dependent upon various factors, including interference between plants. Interference encompasses both allelopathy and competition (Rice, 1979). The role of chemical inhibition, allelopathy, in vegetational composition and allelopathy as a factor in

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ecological processes has been investigated extensively (Muller, 1966). A variety of allelopathic chemical substances from plant sources has been reported (Abdul-Wahab and Rice, 1967; Corcoran et al., 1972; Jackson and Willemsen, 1976; Stowe, 1979; Bell, 1980; Kobayashi et al., 1980; Lynch et al., 1980).

Molisch (1937) designated the term 'allelopathy' as referring to harmful biochemical interactions among all types of plants including microorganisms. Rice (1974) described allelopathy as a direct or indirect harmful effect of one plant on another through the production of chemical compounds that escape into the environment. While others have defined the term in alternative ways (Numata, 1978; Putnam and Duke, 1978), the definition of Rice (1974) provides the most complete expression of an allelopathic phenomenon.

Initial work on the allelopathic effects on the floristic composition of undergrowth in the red pine (*Pinus densiflora*) forest suggested that *p*-coumaric acid may be an important allelopathic chemical from red pine (Lee and Monsi, 1963). However, many questions in this respect remain unanswered.

In this study, a series of field and laboratory experiments was performed in order to clarify the extent of allelopathic effects of red pine substances on the undergrowth. A comprehensive analysis consisted of: (1) a survey of floristic composition in the undergrowth of the red pine forest, (2) germination and seedling growth tests, (3) bioassays, and (4) identification of phytochemicals from red pine.

METHODS AND MATERIALS

Field Survey

Twelve stands of red pine forest in the southwest of the Korean peninsula were selected and 6–10 quadrants of 10 × 10 m in each stand, totaling 107 quadrats, were taken from 1977 through 1981 (Figure 1). The stems of the pine trees were counted and dbh (diameter at breast height) was measured together with an estimation of crown coverage. From these measurements the structure of the pine forest was determined and the distributional frequency of different species in the undergrowth was calculated. They were divided into three groups: the first group represent inside species growing well in the red pine forest (more than 10% in frequency); the second group represent outside species occurring in the field but not observed in the forest; and the third group, intermediate species, showing lower frequency (less than 10%) in the forest than in the field.

Various seeds in the stands were collected and kept at −5°C in a refrigerator for a germination test the next year.

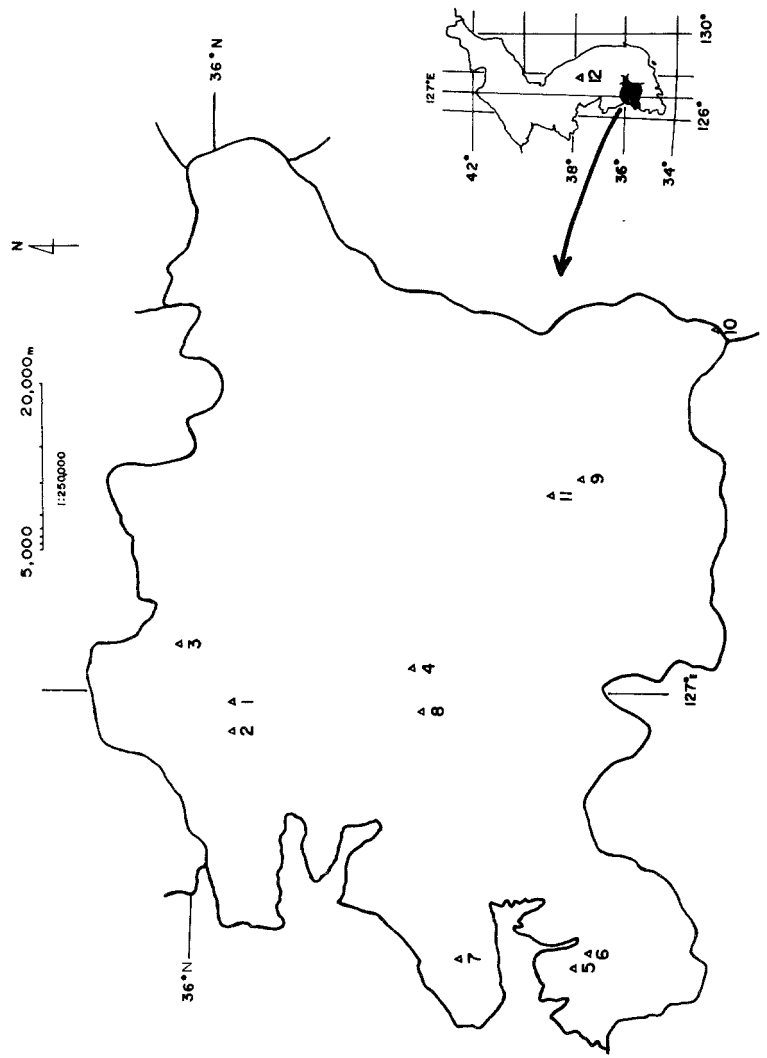

FIG. 1. Investigated localities in Jeonra Bugdo province, South Korea: 1 = Mt. Sora; 2 = Mt. Bae; 3 = Mt. Cheonho; 4 = Mt. Guseong; 5 = Mt. Dosol; 6 = Mt. Seonun; 7 = Mt. Naeso; 8 = Mt. Meonag; 9 = Mt. Bagseog; 10 = Mt. Jiri; 11 = Mt. Osu; 12 = Mt. Obong.

The forest soil of sample sites and soil outside the forest (field soil) were sampled for analysis.

Germination and Growth Tests

Test of Extracts of Pine Materials. Two sample aqueous extracts were made from fresh and fallen leaves and roots, respectively. One liter of water was added to each of 200 g material and half of each extract was kept at 18° C for 24 hr (solution A) and the other half at 80° C for 24 hr (solution B). The extracts were adjusted to pH = 6.0–7.0 and in 50 mosmol less in osmotic pressure (Ungar and Horgan, 1970; Chou and Young, 1974; Anderson et al., 1978). In the preliminary germination test on the seeds of 78 species, 34 species with higher germination percentage were tested with various pine extracts.

Fifty seeds of selected species were planted on filter paper in Petri plates (12 cm diam) with 10 ml of each extract at 20° C; water was used for the control. The extracts or water were applied to each pot every two days and seed germination was recorded every day.

Test of Leachates of Soil and Pine Materials. Fresh and fallen leaves of red pine were put in platic pipe (15 cm diam, 150 cm long), supported by a strong column and each connected to a funnel by a rubber tube with pinch cock. Two hundred grams each of fresh and fallen leaves were leached with 1000 ml water. The leachates of pine forest and field soil were prepared in the same way and kept in a refrigerator for further testing.

Germination boxes (each 75 × 13 × 9 cm in size) covered with vinyl were filled with vermiculite and divided into four sections. The leachates of fresh and fallen leaves, soil, and water were added to each section. Seeds collected in the previous year were planted and 2.5 liters of leachate was poured into each section. Five hundred milliliters of leachate was supplied every two days and each section was fertilized with 2.5 liters of Boysen Jensen's solution for a week.

Since the concentration of leachates was affected by leaching time, pine material leachates of different leaching times were prepared. Four bunches of 200 g fresh leaves were placed in individual 1000-ml water baths in order to make a solution for each leaching time, i.e., 12, 24, 48, and 72 hr. Germination and seedling growth were tested with leachates by applying the same procedure as described above.

The concept of growth inhibition index (GII) is introduced here for quantitative evaluation of the effect of pine chemical substances on the seedling growth of the undergrowth. GII represents the growth inhibition rates of the test groups along the gradient of chemical substances. GII was calculated as follows:

$$\text{GII} = \frac{\text{Dry weight of test group}}{\text{Dry weight of control group}} \times 100$$

Isolation and Identification of Chemical Substances

Isolation by Thin-layer Chromatography and Bioassay. Samples of fresh pine leaves were extracted with ether. Ether extracts were separated into two fractions, sodium bicarbonate and ether, with 2% $NaHCO_3$ solution. Then sodium bicarbonate fraction was adjusted to pH 2.9 with 15% tartaric acid, filtered, and reextracted with ether (30 ml × 3). The ether extracts were concentrated to dryness under vacuum and dissolved in 1 ml abolute methanol (acid fraction). While the ether fraction was evaporated to 10 ml under vacuum, 40 ml distilled water was added and shaken, then filtered and reextracted with ether (30 ml × 3). The ether extracts were also concentrated to dryness under vacuum and dissolved in 1 ml absolute methanol (neutral fraction).

For thin-layer chromatography, plates (20 × 20 cm) precoated with silica gel G (250–300 μm) were used and developed with a solvent system of isopropyl alcohol–25% NH_4OH–distilled water (IAW, 10:1:1, v/v/v). The developed chromatograms were dried and treated with ferric chloride solution, Ehrlich reagent, and UV lamp (2537 Å) in order to detect phenolic compounds (Hong, 1969).

Bioassay was attempted with seed germination tests of *Bidens bipinnata* and *Achyranthes japonica* which occurred in both pine forest stands and fields.

Isolation by Paper Chromatography. Based on the method used by Lodhi and Rice (1971), 25 g fresh and fallen leaves soaked in 500 ml water at 60° C for 2–3 hr were filtered and acidified to pH 2 using 2 N HCl, and extracted twice with half of diethyl ether. Ether and water fractions were evaporated to dryness and were taken up in 3 ml of 85% ethanol and 10 ml of distilled water, respectively.

On the other hand, 10 g each of fresh and fallen leaves were ground and screened by 10-mesh sieve and the hydrolyzed with 150 ml of 2 N NaOH in an autoclave for 45 min. They were filtered by filter paper and acidified to pH 2.0 with 1 N HCl, then reextracted twice with 75 ml of 5% $NaHCO_3$, and the ether portion was discarded. The alkaline portion was acidified again to pH 2.0 and reextracted twice with 75 ml of ether. The ether fraction was evaporated to dryness and the residue put in 3 ml of 95% ethanol.

These fractions were chromatographed in two dimensions on Whatman No. 3 paper with *n*-butanol–acetic acid–water (BAW, 63:10:27, v/v/v), followed by 6% aqueous acetic acid (6% AA). The chromatograms were inspected with short (2537 Å) ultraviolet light and dipped in various reagents.

Identification by Gas Chromatography. The analysis of various extracts was performed on a Varian 2440 gas chromatograph equipped with 6-ft × 1/8-in i.d. (inner diameter) stainless steel column packed with 2.5% SE-30 on ABS, 90–100 mesh. The helium carrier gas flow was 40 ml/min. The flame ionization detection was set at a sensitivity of 2 × 10. Injection port and

detector temperature were held at 230° C and 205° C, respectively. The column temperature was programmed at a rate of 6°/min from 100° C to 250° C. One-microliter sample injections were made with a 5-ml SGE syringe employing the solvent flush technique. Extraction procedures are shown in Figure 2. Details of thin-layer and gas chromatography on pine leaves were described previously (Kil, 1981).

RESULTS

Undergrowth of Red Pine Forest. The red pine forest surveyed was 20 m in average stand height, about 80% in crown coverage, 7–21 stems/100 m^2 in

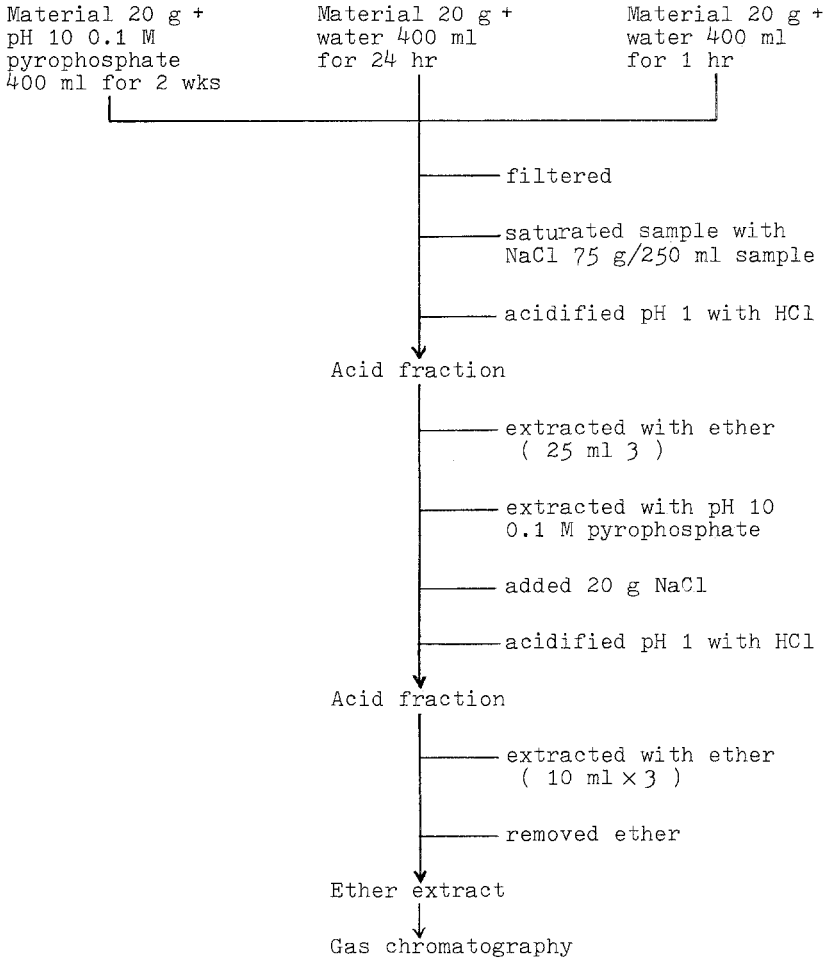

FIG. 2. Flow diagram for the separation of phenolic substances in pine by gas chromatography.

TABLE 1. SOIL ANALYSIS OF SITE 1 (SEE FIGURE 1)

Soil	pH	P_2O_5 (ppm)	SiO_2 (ppm)	OM (%)	Lime req. (kg/10 a)	Ca	Mg	K	H	CEC
Inside pine forest	4.5	25	43	5.4	196	1.1	1.0	.24	1.76	4.10
Outside of pine forest	6.0	21	67	1.4	98	5.4	1.3	.16	+	6.89

TABLE 2. RELATIVE GERMINATION PERCENTAGE (RGP) OF INSIDE SPECIES, INTERMEDIATE SPECIES AND OUTSIDE SPECIES[a]

No.	Species	Frequency	Solution A			Solution B		
			L	Fl	R	L	Fl	R
Inside species								
1	*Pinus densiflora*	44.3	102	99	99	94	89	97
2	*Arundinella hirta*	67.2	102	94	95	86	87	95
3	*Miscanthus sinensis*	68.9	103	57	77	84	64	70
4	*Cymbopogon tortilis* var. *goeringii*	44.3	81	99	88	99	78	66
5	*Themeda triandra* var. *japonica*	67.2	78	80	82	65	58	99
6	*Achyranthes japonica*	11.5	87	60	86	35	33	51
7	*Phytolacca americana*	13.1	55	35	70	48	45	44
8	*Albizzia julibrissin*	19.7	82	91	87	104	98	96
9	*Lespedeza bicolor*	21.3	60	90	90	58	75	97
10	*Peucedanum terebinthaceum*	18.0	80	56	75	19	24	43
11	*Rhododendron schlippenbachii*	16.4	101	87	87	73	90	82
12	*Justicia procumbens*	26.2	104	114	91	44	36	65
13	*Leibnitzia anandria*	18.0	82	54	79	23	56	37
14	*Aster tataricus*	37.3	98	86	82	55	95	75
15	*Atractylodes japonica*	24.6	97	95	95	71	94	104

Intermediate species								
16	*Setaria viridis*	8.2	80	71	88	66	50	63
17	*Melandryum firmum*	1.6	39	25	28	11	7	13
18	*Celastrus orbiculatus*	1.6	109	89	114	78	99	101
19	*Plantago asiatica*	3.3	78	53	89	31	34	48
20	*Erigeron annuus*	9.8	97	98	88	36	43	47
21	*Bidens bipinnata*	4.9	86	81	88	44	42	58
Outside species								
	Digitaria sanguinalis		73	70	83	23	22	71
	Persicaria hydropiper		75	40	10	30	10	15
	Chenopodium album var. *centrorubrum*		67	70	63	37	60	52
	Amaranthus mangostanus		5	14	77	0	16	30
	Celosia argentea		47	69	107	36	42	47
	Sedum erythrostichum		98	93	93	54	88	89
	Cassia tora		35	8	31	70	31	33
	Amorpha fruticosa		97	81	79	57	66	74
	Geranium sibiricum		11	10	89	6	5	7
	Triumfetta japonica		37	51	53	10	31	41
	Oenothera odorata		76	62	65	38	41	54
	Leonurus sibiricus		92	60	68	21	28	47
	Serratula coronata var. *insularis*		60	73	77	16	35	29

[a] L, fresh leaves; Fl, fallen leaves; R, roots, extracted for 24 hr at 18° C (solution A) and at 80° C (solution B).

density, and 35–50 years old in tree age. The disturbance of the forest by human activities was obvious and its soil showed a marked difference compared with field soil in many measurements including nutrient salt (Table 1).

Species with relatively higher frequency (above 41.0%) among 194 species listed in 107 quadrats were *Miscanthus sinensis* (68.9%), *Quercus serrata* (68.9%), *Arundinella hirta* (67.2%), *Themeda triandra* var. *japonica* (62.3%) and seven other species. Comparing the data obtained from the present study with those of Yoshioka (Lee and Monsi, 1963) in the Kanto area of Japan, 23 undergrowth species were common and 11 of them showed a similar frequency between the two regions.

Germination and Seeding Growth in Extract and Leachate of Pine Materials. The percentage of seed treated by extract or leachate against the control was defined as relative germination percentage (RGP). The species high in RGP (more than 90%) recorded were 14 from solution A and 8 from solution B, respectively. The species of lower RGP (less than 50%) recorded were 6 from solution A and 14 from solution B. The inhibitory effect of each different pine extract on the RGP of fresh leaves, fallen leaves, and roots was significant in that order (Table 2, Figure 3).

The mean RGP of species in the germination test with leachate was 78% in fresh leaves, 82% in fallen leaves, and 97% in soil. Some differences appeared according to species: for example, in the case of *Arundinella hirta*, 96% in the control, 96% in fresh leaf leachates, 90% in fallen leaf leachates, and 80% in leachate of soil; in *Achyranthes japonica*, 70%, 44%, 36%, and 68%, respectively.

In seedling growth the mean dry weight of plant species tested against the control was 24% in the leachates of fresh leaves, 53% in fallen leaves, and 59% in soil.

It is evident that the inhibition of germination or seedling growth occurs most strongly in the leachates of fresh leaves. According to the results obtained from eight species tested, the longer the leaching period, the more the inhibition of their germination in both inside and outside species. However, the outside species were more inhibited than the inside species by pine leachate. These results are similar to those of Newman and Rovira (1975). When plants receive the leachates of other species, their growth is retarded, while their growth is stimulated if they receive their own leachates.

The amount of dry weight growth in species effected by leachates showed an exponential decrease as the leaching time was prolonged. Therefore, GII can be used as an index expressing the degree of dry weight growth inhibited by the leachates of various concentrations. The following formula interpreting the above-mentioned phenomenon satisifed, as a whole, in all inside species of the undergrowth investigated in the red pine forest (Figure 4).

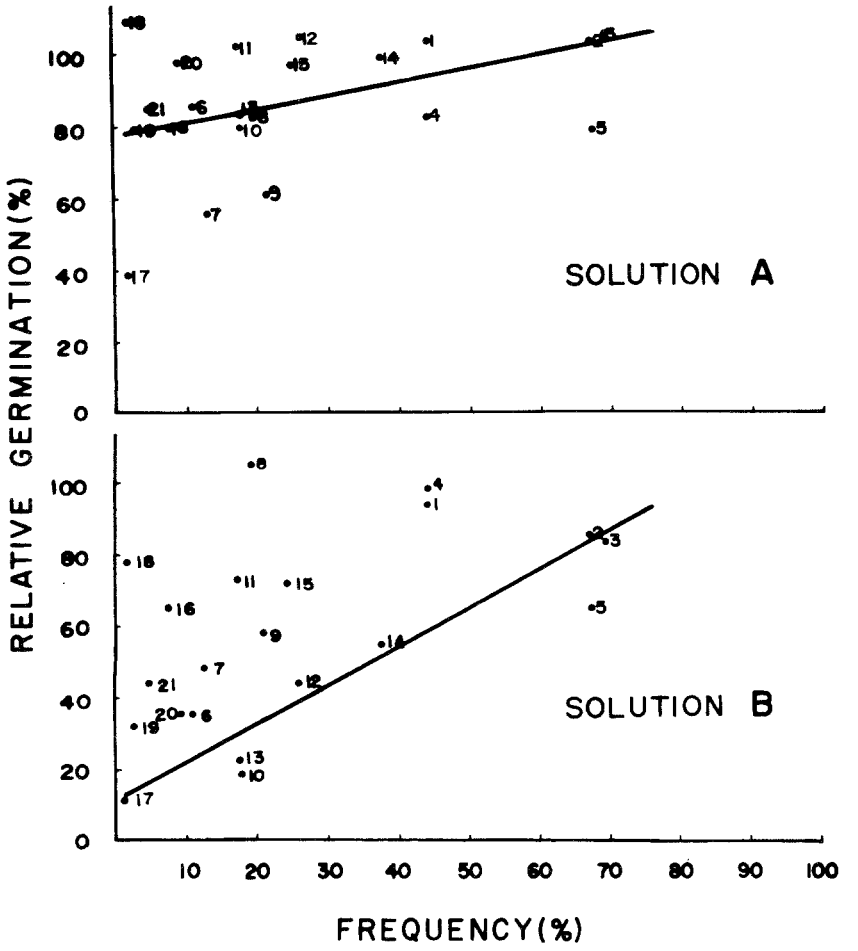

FIG. 3. Relationship between the frequency of different species of the undergrowth in pine forest and their relative germination percentage in solutions A and B. (Key to solutions A and B as in Table 2.)

$$GII = \exp(-KDt)$$

where Dt = leaching time (concentration of leachate) and t = time after leaching started. Therefore K is a constant in the species shown in Figure 4.

Isolation and Identification of Chemical Substances and Bioassay. Twelve substances were isolated in acid and neutral fractions by thin-layer chromatography. Three substances were detected by paper chromatography in the ether fraction of fresh leaves at *Rf* 0.63, 0.78, 0.89 in BAW, and 0.75, 0.78, 0.83 in 6% AA, respectively. However, they failed to identify inhibitory

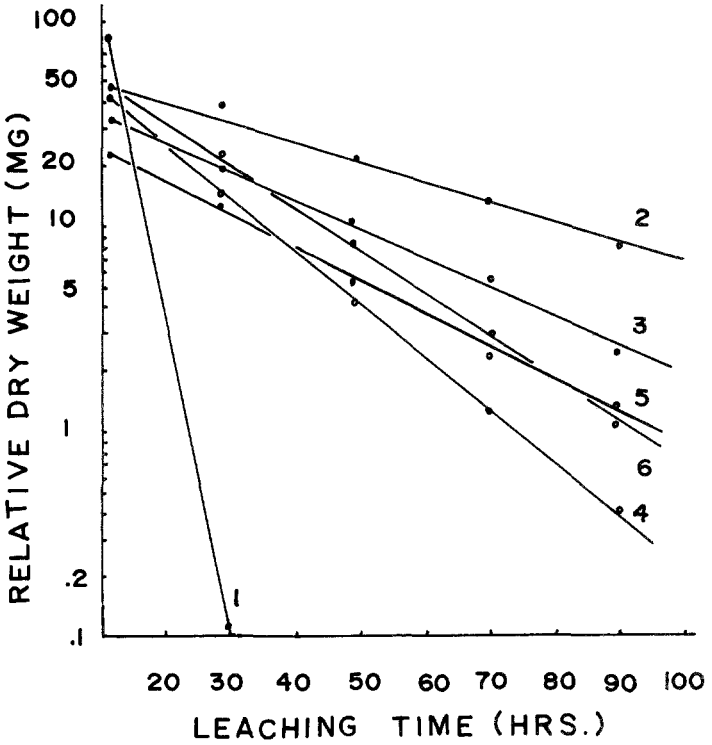

FIG. 4. Dry weights of different species grown in pine leaf leachates; 1 = *Erigeron annuus*; 2 = *Arundinella hirta*; 3 = *Achyranthes japonica*; 4 = *Plantago asiatica*; 5 = *Celosia argentea*; 6 = *Leonurus sibiricus*.

substances. Bioassays within the range of *Rf* values of TLC, showed *Achyranthes japonica* was inhibited at *Rf* 0.60–0.70 and 0.80–0.90 in the acid fraction and at *Rf* 0.65–0.85 in the neutral fraction. Most fractions especially suppressed germination at *Rf* 0.80–0.85. On the other hand, germination was stimulated slightly by substances with *Rf* 0.25–0.30 and 0.50–0.55 in the acid fraction and *Rf* 0.25–0.35 in the neutral fraction.

Eleven phenolic acids were detected on the gas chromatograms. Benzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid were significant in different materials of pine at different soaking times (Table 3, Figures 5 and 6).

It has been reported by many researchers that phytotoxic substances affecting plant-plant interactions are contained in rain-drip or dew (Halligan, 1976; Gliessman and Muller, 1978; Mubarak and Hussain, 1978). Benzoic acid was found by gas chromatography in the analysis of pine rain which penetrated through the crown of pine trees.

TABLE 3. IDENTIFICATION OF PHENOLIC ACIDS FROM PINE LEAVES BY GAS CHROMATOGRAPHY[a]

No.	Phenolic Acids	Relative retention time	Fresh leaves			Fallen leaves		
			W1	W24	P2	W1	W24	P2
1	Benzoic (non-phenolic)	0.28	+++	+++	+++	+	+	++
2	Salicylic	0.75		+++			+	
3	Cinnamic	0.78	+	+			+	
4	*p*-Hydroxybenzoic	1.0		+++	+	+	+	+
5	Gentisic	1.38		+				
6	Protocatechuic	1.48		+	+	+	+	+
7	Syringic	1.63		+		+	+	
8	*p*-Coumaric	1.70		+++			+	+
9	Gallic	1.80						+
10	Ferulic	2.03		+				
11	Caffeic	2.15		+				
12	Vanillic	1.33			+	+	++	+

[a]W1, W24 mean soaked in water for 1 hr and 24 hr, P2 means soaked in pyrophosphate solution for 2 weeks. Quantitative comparison was made by the order +++ > ++ > +.

DISCUSSION

According to Yim and Kira (1975), the investigated pine forest is neither climatic climax nor topographic or edaphic climax. Mt. Obong is in the *Quercus mongolica* zone (cool temperate, central zone) and the surveyed area of Jeonra Bugdo province in the *Carpinus* zone (cool temperate, southern zone).

On the other hand, Toyohara (1982) recognized five successional stages, A, B, C, D and E (variante level), by floristic composition of the undergrowth in the pine forest of lowlands in South Korea. He suggested seven species as differential ones: *Albizzia julibrissin, Viola mandshurica, Pinus densiflora, Quercus serrata* in stage A; and *Eupatorium lindleyanum*, *Quercus acutissima*, *Quercus aliena* in stage B. In our field survey, species in high frequency were *Carex lanceolata, Quercus serrata, Quercus acutissima, Symplocos chinensis* for. *pilosa, Indigofera kirilowii, Smilax china, Cocculus trilobus, Rhododendron mucronulatum, Lespedeza bicolor*. Generally, high-frequency species were less or not inhibited by pine chemical substances in their germination or seedling growth, while low-frequency species were greatly inhibited. Therefore it seems that the floristic composition of pine forest undergrowth is greatly influenced by allelopathic effects, although such allelopathic effects may be changed by other factors.

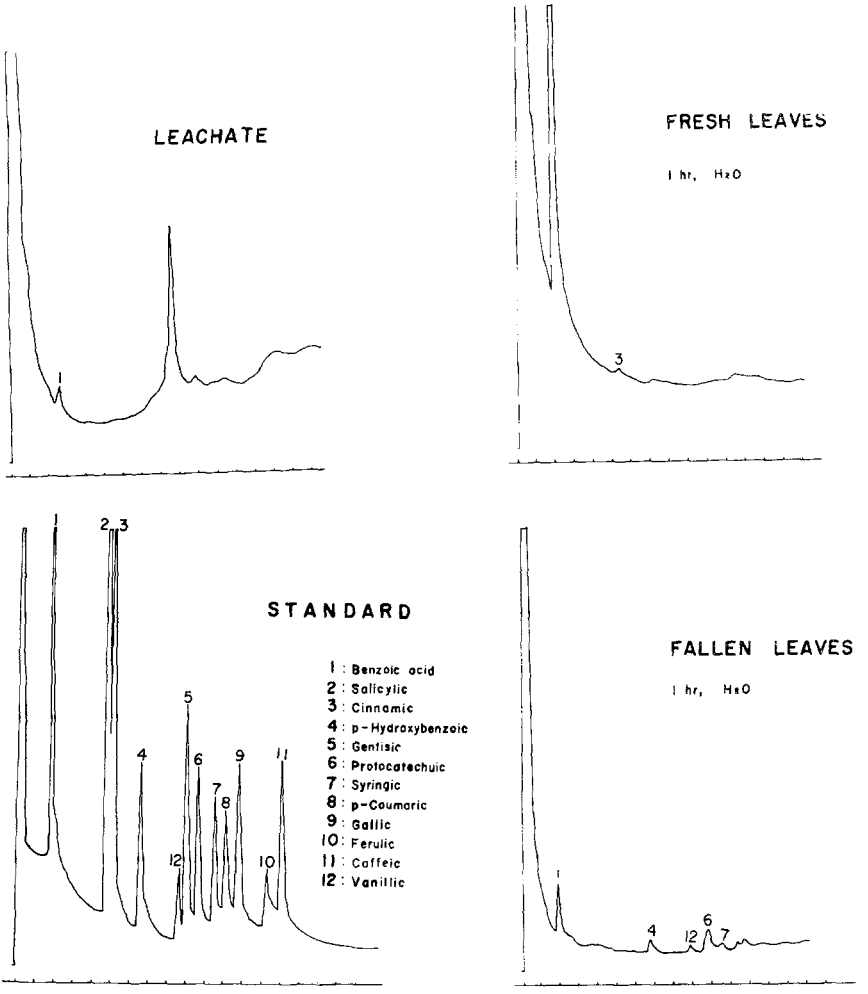

FIG. 5. Gas chromatographs of phenolic acids from the leachates of pine trees and leaves extracted by 1 hr.

Considering the fact that an approximately linear relationship exists between the increasing degree of frequency of inside species and increasing RGP of their seeds by pine extracts, the allelopathic effects of pine toxic substances on the floristic composition of the undergrowth in pine forest is obvious.

The gradual changes in GII of the inside species by the extracts or leachates of fresh and fallen leaves, roots, soil, and pine rain may be considered attributable to the degree of dilution of running pine toxic substances along with the lapse of time.

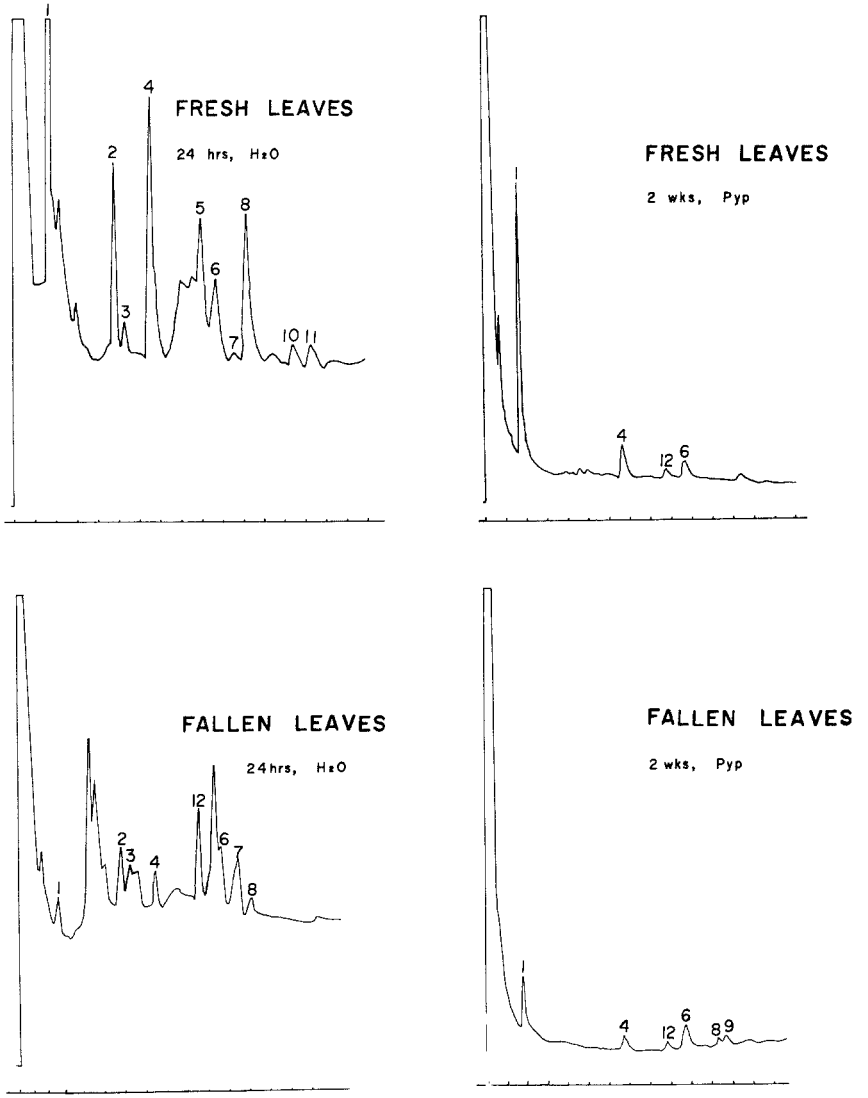

FIG. 6. Gas chromatograms of phenolic acids from pine leaves extracted for 24 hrs and for 2 weeks. Pyp, 0.1 M pyrophosphate solution.

The suggested role of *p*-coumaric acid (Lee and Monsi, 1963) as an important phytotoxic substance on the undergrowth of red pine forest has not yet been proven. If the results of recent studies by many researchers (Kuwatsuka and Shindo, 1973; Li, 1974; Lodhi, 1975; Ballester et al., 1977; Carballeria, 1980) could be applied to pine materials, phenolic acids should be mentioned.

It is noticeable that benzoic acid, in addition to 11 phenolic acids detected by gas chromatography in this study, was found in all pine materials and dominated other phenolic acids in chromatograms. This seems to agree with the opinions of many investigators on the role of benzoic acid in seedling growth inhibition (Dedonder and Van Sumere, 1971), uptake of inorganic phosphate (Glass, 1974), an important phytotoxin (De Bell, 1970), and as the most important toxic substance in temperate ecosystems (Horsley, 1977).

It seems apparent that some of the phenolic acids and benzoic acid, identified by gas chromatography, act as the factors affecting the germination and vegetative growth or the floristic composition of undergrowth in the pine forest. Benzoic acid is considered to be one of the most important of the acids in this allelopathic role.

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ALLELOPATHIC EFFECTS OF LITTER ON THE GROWTH AND COLONIZATION OF MYCORRHIZAL FUNGI

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Abstract—In laboratory studies, water-soluble extracts of the litter of four shrub and three conifer species had variable effects on the growth of four species of ectomycorrhizal fungi. In general, low concentrations (parts per thousand) stimulated fungal growth; while, high concentrations (parts per hundred and parts per ten) either stimulated growth, inhibited growth, or had no effect, depending on both fungal and litter species. In greenhouse studies, litter applied to the surface of a sand–soil mixture reduced the formation of *Rhizopogon* sp. on Douglas-fir seedlings. Allelochemicals in the litter may inhibit seedling growth and suppress fungal growth and root colonization in the field, thus explaining the failures of reforestation by conifer species in disturbed sites.

Key Words—Allelopathy, allelochemicals, ectomycorrhizal fungi, reforestation, *Thizopogon vinicolor, Pisolithus tinctorius, Laccaris laccata, Cenococcum geophilum, Pseudostuga menziesii.*

INTRODUCTION

Brush species quickly establish in clear-cut openings following logging in the Pacific Northwest. Successful reforestation by conifer species is reduced through competition by the brush for nutrients, water, and light. In addition, potential allelochemicals produced by the brush may inhibit seedling growth or the growth of ectomycorrhizal fungi which aid conifers in nutrient uptake and confer protection against root diseases (DeBell, 1970; Fisher, 1980; Marx, 1969; Stewart, 1975).

Allelopathic interactions may occur throughout the life of a stand, but are most commonly observed during reforestation or regeneration (Fisher,

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1980). Robinson (1972) found fewer mycorrhizal roots in the second rotation of *Pinus patula* in South Africa and suggested that this was due to toxic substances in pine litter. Alvarez et al. (1979) counted more mycorrhizal root tips in mineral soils without organic layers. Schoeneberger and Perry (1982) showed that litter leachates from a Douglas-fir forest in Oregon reduced mycorrhizal formation on Douglas-fir but not on western hemlock. Litter from a second nearby Douglas-fir forest had no effect.

Whitehead et al. (1981) state that water-soluble extracts from roots and surface litter leach into the soil and influence plant growth and microbial activity. Handley (1963) extracted water-soluble substances from *Calluna* (heather) humus which inhibited the development of mycorrhizal hymenomycetes. Extracts of prairie soils and grass roots reduced the respiration of excised mycorrhizal roots (Persidsky et al., 1965). Olsen et al. (1971) reported inhibition of mycorrhizal fungi in pure culture with additions of water-soluble extracts from the leaves of *Populus tremula*. However, Tan and Nopamornbodi (1979) observed increased fungi growth with additions of several fulvic acids and soil extracts, suggesting species-specific interactions with different litter components.

Evidence from Handley (1963), Hillis et al. (1968), and Persidsky et al. (1965) demonstrates that organic toxins originating in forest floor litter affect mycorrhizal formation. However, it is not certain if the fungus is directly inhibited, or if the allelochemicals interfere with the ability of the mycorrhizal fungus to colonize a seedling root. We initiated laboratory and greenhouse studies to determine the effect of water-soluble litter substances upon in vitro fungal growth and upon in vivo mycorrhizal formation on tree seedlings.

METHODS AND MATERIALS

During summer and fall of 1979, before the first rains, fresh litter of the following species was collected from forests and shrub fields of central and southern Oregon: *Ceanothus velutinus* Dougl. (snowbrush), *Lithocarpus densiflorus* Rehd. (tanoak), *Arbutus menziesii* Pursh. (madrone), *Pteridium aquilinum* (L.) Kuhn (bracken fern), *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir), and *Tsuga heterophylla* (Rafh.) Sarg. (western hemlock). Litter from *Pinus cembroides* v. *edulis* (Engelm.) Voss (pinyon pine) was collected from northern Arizona in the spring of 1980.

Laboratory Study. The objective of the laboratory study was to determine if water-soluble extractants from litter were involved in inhibition of fungal growth.

Air dried litter was ground through a 20-mesh Wiley mill and 100-g aliquots mixed with 1000 ml distilled deionized water and allowed to imbibe at

room temperature for 24 hr. Litter was then gravity- and vacuum-filtered, the supernatant frozen and freeze-dried. An average of 1 g extract per 100 g litter resulted and established a base level of parts per hundred (PPH) for pure culture studies. Freeze-dried extracts were added to MN liquid media (Melin, 1963) in concentrations of parts per ten (PPt), parts per hundred (PPH), and parts per thousand (PPT); the liquid was Millipore-filtered through 0.2-μm filters and inoculated with a plug of fresh, pure cultures of a mycorrhizal fungal symbiont of Douglas-fir. Four ectomycorrhizal fungal symbionts were tested: *Rhizopogon vinicolor* Smith, *Pisolithus tinctorius* (Pres.) Coker and Couch, *Laccaria laccata* (Scop. ex Fr.) Berk. and Br., and *Cenococcum geophilum* Fr. Three replicate flasks were prepared for each concentration of extract, for each litter, and for each fungal species. Two control treatments were included: an MN liquid broth without litter additions and an MN liquid with glucose additions at concentrations of PPt, PPH, and PPT to monitor a carbohydrate response. We will refer to these as control and glucose-control, respectively. Fungi were grown in 300-ml flasks under constant agitation for two months at room temperature. Upon harvesting, fungal hyphae were Millipore-filtered and dried at 50°C until constant weight.

Greenhouse Study. The objective of the greenhouse study was to determine if litter was soil active and inhibitory to conifer seedling growth and mycorrhizal formation.

Douglas-fir seeds were surface-sterilized in 30% H_2O_2 for 1 hr, rinsed in sterile distilled water and stratified in sterile Bacto Agar for 30 days at 5°C. Sterile seeds were either sown in nonsterile or steam pasteurized southern Oregon undisturbed forest soil amended with 10 g ground litter of one of the shrub or conifer species. In addition, two control treatments were included: forest soil without litter to determine indigenous mycorrhizal types and steam pasteurized soil without litter amendments to monitor greenhouse contamination levels. Ten tubes were prepared for each treatment. Seedlings were grown in 1:1 soil–sterile peat vermiculite mixture in Ray Leach tubes (150 ml) for five months under 16 hr light (11,000 lx) with water added as a fine mist on alternate days.

At harvest, the seedlings were carefully removed from the tubes, rinsed in water, shoot and root systems measured, and the number of ectomycorrhizal and nonmycorrhizal root tips counted under magnification of 3–10×. Different types of mycorrhizae were described and quantified. Shoot and root tissues were dried at 50°C until constant weight.

Data were analyzed by analysis of variance. However, the laboratory experiment controls (no leachate added) and the pinyon-pine leachate treatments had missing cells, and so could not be included in the complete ANOVA. Glucose-controls are included (Table 1).

TABLE 1. ANALYSIS OF VARIANCE OF EFFECTS OF LITTER LEACHATE TYPE AND CONCENTRATION ON DRY WEIGHT OF MYCORRHIZAL FUNGI IN VITRO[a]

Source	Degrees of freedom	Mean square	F	Significance
Fungal species (F)	3	8675.03	17.89	$P < 0.001$
Litter type (L)	5	1460.49	3.01	$P < 0.005$
F × L	15	1421.97	2.93	$P < 0.005$
Leachate concentration (C)	2	9582.08	19.76	$P < 0.001$
F × C	6	4486.01	9.25	$P < 0.005$
L × C	10	1935.93	3.99	$P < 0.005$
F × L × C	30	621.07	NS	
Error	153	434.89		

[a]Because of empty cells, controls (no leachate added) and pinyon pine were not included in ANOVA. Glucose-controls are included (see Methods section).

RESULTS

Laboratory Study. Water-soluble extracts of the shrubs and conifers affected the growth of the fungi in pure culture (Figure 1); however, there were significant interactions between fungal and litter species, fungal species and extract concentration, and litter species and extract concentration (Table 1).

The growth of *Cenococcum geophilum* was stimulated by all litter extracts at all concentrations. This was apparently not an energy-related phenomenon, as additions of glucose did not stimulate growth. Response of other fungal species depended on litter type and concentration. *Pisolitus tinctorius* was most sensitive to shrub litter: tanoak and madrone were inhibitory as parts per ten (PPt) concentration, while bracken fern was inhibitory at parts per hundred (PPH) and parts per thousand (PPT). At PPT and PPH, conifer (Douglas-fir plus hemlock) and madrone extracts were stimulatory. The growth of *Rhizopogon vinicolor* was not stimulated by any extract: bracken fern, tanoak, conifer, madrone, and pinyon pine were highly inhibitory at PPt and conifer was inhibitory at PPH as well. Pinyon pine reduced *R. vinicolor* growth at all concentrations. *Laccaria laccata* was the least sensitive to the litter extracts of the fungal species tested. Conifer extracts were stimulatory at all concentrations and tanoak at PPT. *Ceanothus* (snowbrush) and madrone inhibited growth at PPt. There was no significant response to pinyon pine or bracken fern extracts at any concentration.

Greenhouse Study. Four mycorrhizal types formed on seedlings grown in nonsterile forest soil: *Rhizopogon* sp., *Cenococcum* sp., *Thelephora* sp. (a probable greenhouse contaminant), and an unidentified brown type. *Rhizopogon* sp. and the brown type predominated on Douglas-fir seedlings, comprising 40 and 54% of mycorrhizae formed in control soils, respectively.

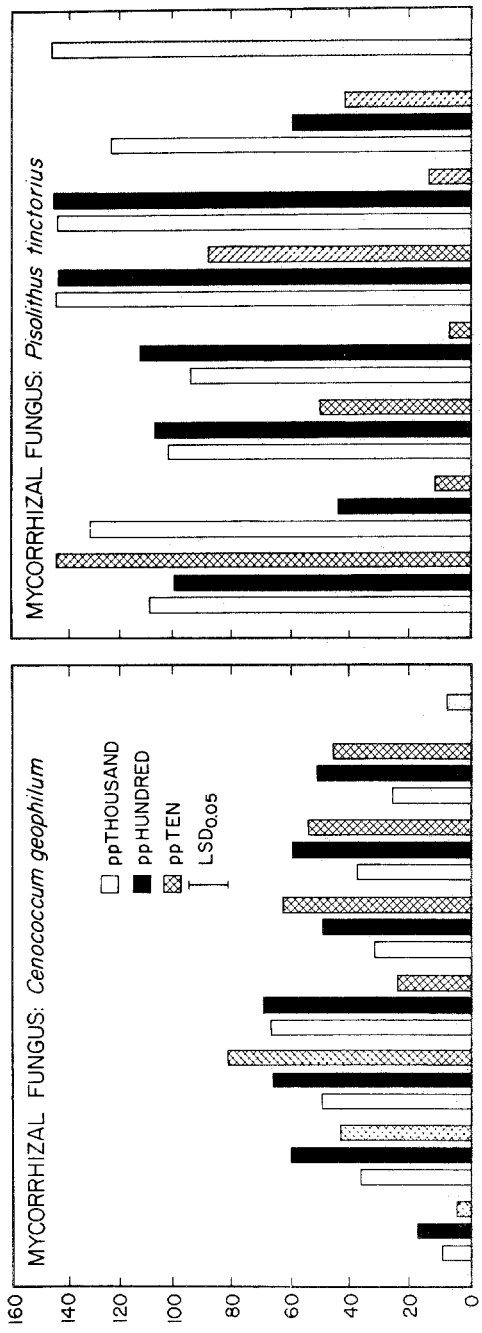
Litter leachates had a significant effect on both mycorrhizal formation and growth of Douglas-fir. Madrone, pinyon pine, and snowbrush leachate all reduced the percent mycorrhizae relative to control (Figure 2). This effect resulted from a reduction of *Rhizopogon* root tips, which was observed with all litter types except conifer (Figure 3). Relative to controls, the average number of brown mycorrhizae was lower in some litter treatments and higher in others; however, these differences were not significantly different at the 5% level.

The effect of litter leachate on Douglas-fir seedling growth was quite different. Roots were larger in the tanoak and conifer litter treatments than in controls (Figure 2), and both tops and roots were larger in the bracken fern treatment.

DISCUSSION

Ectomycorrhizal fungi are sensitive to substances exuded from plant roots or leached from dead plant material (Rice, 1979). Olsen et al. (1971) found that at high concentrations aqueous extracts of aspen leaves strongly inhibited growth of several species of mycorrhizal fungi, but at low concentrations (5%) fungal growth was stimulated. Our experiments show that the interaction between fungal species, litter type, and leachate concentration cannot be generalized. Parts per thousand of leachate from each litter we tested stimulated the growth of at least one fungal species. Growth stimulation at low concentrations may be due to an increase in ions, amino acids, as well as a carbohydrate release (Nykvist, 1963). Stimulation of *Rhizopogon vinicolor* appeared to be primarily an effect of added energy, as its growth was increased by glucose as well as litter leachates. This was not true, however, for the other fungal species.

Parts-per-ten of all litter extracts inhibited the growth of *R. vinicolor* and *Pisolithus tinctorius* and stimulated the growth of *Cenococcum geophilum* (relative to glucose controls). *Laccaria laccata* was stimulated by high concentration of some litters and inhibited by others. It is debatable whether concentrations as high as ten parts per million have ecological relevance. It is not uncommon, however, to find mycorrhizae in intimate contact with litter or soil organic material, and it seems reasonable to postulate the fungal hyphae may experience very high leachate concentrations at those interfaces. The marked effect of leachates on formation of *Rhizopogon* sp. on Douglas-fir, which we saw in out greenhouse study, argues that our laboratory results may well have relevance to field conditions. In previous work at this laboratory about 70% more *Rhizopogon* sp. tips formed on greenhouse ponderosa pine seedlings in pinyon pine litter treatments than on control seedlings; however, this difference was not statistically significant. Why litter


MYCORRHIZAL FUNGUS: Cenococcum geophilum
MYCORRHIZAL FUNGUS: Pisolithus tinctorius
ppTHOUSAND
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160
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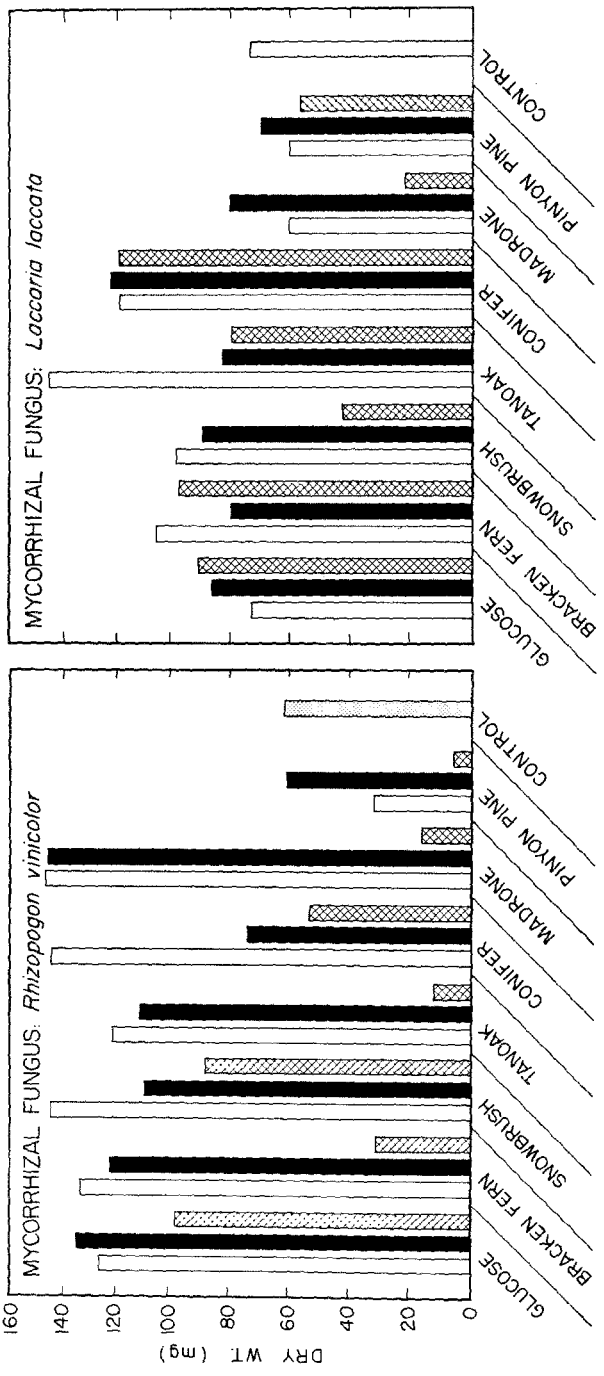

FIG. 1. In vitro growth of four species of mycorrhizal fungi as affected by type and concentration of litter leachate.

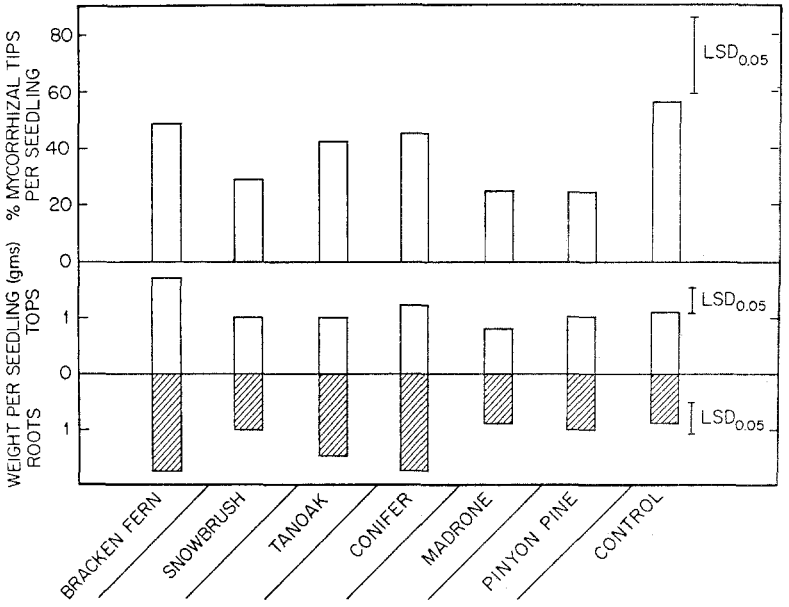

FIG. 2. Effect of litter leachate on mycorrhizal formation and weight of Douglas-fir seedlings.

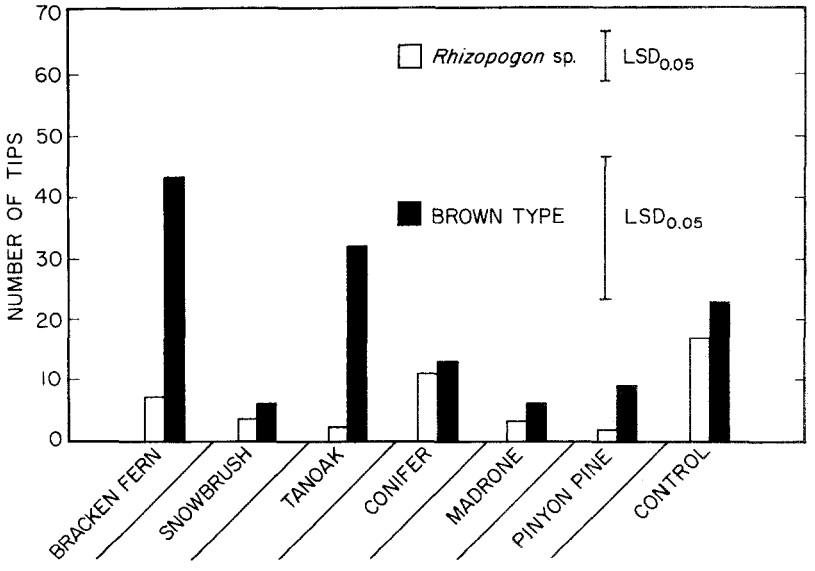

FIG. 3. Effect of litter leachate on Douglas-fir mycorrhizal types.

leachates may affect the formation of *Rhizopogon* sp. differently on ponderosa pine and Douglas-fir seedlings is unclear and should be researched further.

Root growth of Douglas-fir seedlings, as measured by dry weight, was not inhibited by snowbrush, pinyon pine, and madrone litter. However, the reduction in mycorrhizal tips caused by these litter types may be a more relevant measure of impact on the seedling. The importance of mycorrhizae in nutrient uptake is well known (e.g., Voight, 1971). Mycorrhizae also protect seedlings from root diseases (Marx, 1969) and have been shown to increase drought resistance of Douglas-fir (Parke, 1982). Therefore allelopathic effects on mycorrhizae may have direct, negative effects on seedling performance. McClure et al. (1978) found that low concentrations of ferulic acid, leached from leaves and roots, interfered with phosphorus uptake in soybean. Bevege (1968) observed that soil leachates reduced total plant growth and caused necrosis and chlorosis in conifer seedlings, a response possibly due to nutrient deficiency or imbalance.

In summary, our results suggest that ectomycorrhizal fungi are affected by allelochemicals in forest floor litter. Growth may be stimulated or inhibited and the ability of the fungus to colonize a conifer root may be similarly affected, depending upon the host, fungus, and litter type. In addition, seedling physiology may be influenced by allelochemicals in the soil, an important question not addressed in our work. Some cases of regeneration failure may be a result of seedling mortality due to interactions of allelochemicals with seedling roots and mycorrhizal fungi. Knowledge of host, fungus, and litter species may be an important aid in managing organic residues.

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EFFECTS OF ALLELOPATHIC SUBSTANCES PRODUCED BY ASPARAGUS ON INCIDENCE AND SEVERITY OF ASPARAGUS DECLINE DUE TO *Fusarium* CROWN ROT

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Abstract—The effects of toxic components isolated from asparagus tissue on *Fusarium* spp. and other soil microorganisms and their effects on the susceptibility of asparagus to *Fusarium* crown rot was investigated to determine what role allelopathic substances may play in the asparagus ecosystem and in asparagus crown rot decline. Dried sterilized asparagus crown and root tissues were incorporated into pots of 3-month-old asparagus seedlings with and without *Fusarium* inoculum. Root tissue alone and treatments in which crown and root tissues were combined with the *Fusarium* inoculum showed significant reduction of plant growth over nontreated controls. Root and crown tissues were partitioned with polar and nonpolar solvents and bioassayed on pregerminated asparagus and cress seeds. Inhibition of radicle growth was confined to the polar fractions. Further separation with paper chromatography gave several fractions that were inhibitory to radish, cress, tomato seed germination as well as inhibitory to growth of pregerminated asparagus seeds. Crude extracts from roots and crown residues were bioassayed on many different fungal isolates on Petri plates and were found to inhibit the growth of oomycetous fungi. Extracts from the roots were found to be more active than extracts from other portions of the asparagus plants.

Key Words—Asparagus, allelopathy, *Fusarium oxysporum, Fusarium moniliforme, Fusarium*, asparagus decline, *Fusarium* crown rot, germination inhibitors, allelopathic interaction with fungi.

INTRODUCTION

Asparagus (*Asparagus officinalis* L.) is a high-value vegetable crop grown on sandy soils not suitable for many other crops. Decline of asparagus plantings

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due to *Fusarium* crown rot is a serious problem in Michigan (Lacy, 1979) and other asparagus-producing areas of the world (Endo and Burkholder, 1971; Grogan and Kimble, 1959; Johnson et al., 1979; Molot and Simone, 1965; VanBakel and Kerstens, 1970). In a 1978 survey of Michigan asparagus fields, an average of 11.3% of the ferns exhibited symptoms due to crown rot (A. Putnam, unpublished report). In severe cases, as few as 55% of the crowns survived when replanted into fields where asparagus was previously grown (M. Lacy, personal communication). The two species of *Fusarium* involved in asparagus decline, *F. moniliforme* (Sheld.) emend. Snyd. & Hans. and *F. oxysporum* (Schlecht.) emend. Snyd. & Hans. f. sp. *asparagii* Cohen, are soil-borne fungi that, once introduced, survive in the soil for long periods of time. Since most soils in asparagus growing areas are already *Fusarium*-infested, the disease cannot be readily avoided. It has been shown that asparagus storage roots quickly "wall-off" *Fusarium* infections and wounds which are potential *Fusarium* infection sites (Endo, personal communication). Stressed storage roots take longer to form wound periderm that nonstressed storage roots, which may give the fungal pathogen an opportunity to cause further infection (Endo, personal communication). Therefore, growers that employ known management practices which reduce plant stress are most successful in minimizing disease damage due to *Fusarium*.

Putnam et al. (1982) confirmed previous reports of possible allelopathy and autotoxicity in asparagus (Laufer and Garrison, 1977; Shafer and Garrison, 1980a, 1980b). Rice (1974) defined allelopathy as any direct or indirect harmful effect of one organism, which may include a microorganism, on another through the production of chemical compounds. Putnam et al. (1982) found that soil amended with living or dead tissues of asparagus and asparagus root extracts inhibited growth of tomato (*Lycopersicon esculentum* Mill.), barnyard grass (*Echinochloa crus-galli* (L.) Beauv.), and one-year old asparagus crowns. In addition, asparagus root extracts caused extensive mortaility among populations of isolated asparagus cells and were very phytotoxic to callus tissue. Although isolated chemical components of the asparagus root tissue may be responsible for phytotoxic activity, none have been identified. Putnam et al. (1982) found that the phytotoxic components of the asparagus plant tissue were water-soluble and chloroform-insoluble. Kitahara et al. (1972) identified one such compound from asparagus shoots as asparagusic acid and found it was inhibitory to germination of a number of different seeds. Rohde and Jenkins (1958) isolated and purified one compound from asparagus that was active against the stubby root nematode (*Trichodorus christiei*) (Allen, 1957), but which was not antifungal or antibacterial.

Allelopathic substances have been implicated in the promotion of infection by root rotting organisms, specifically in the predisposition of plants to disease (Cochrane, 1948; Patrick et al., 1964; Tossoun and Patrick, 1963).

Kommedahl and Ohman (1960) reported that quackgrass rhizomes produce a toxin that predisposes alfalfa seedlings to infection by root rot pathogens. Toxic products of decomposing plant residues greatly enhanced the pathogenesis of *Fusarium solani* f. *phaseoli* on beans (Toussan and Patrick, 1963). Following a study of the peach tree decline and replant problem, Chandler and Daniell (1974) postulated the toxins from dead peach roots may predispose newly planted peach trees to bacterial canker and thus contribute to short life of the trees.

Since both asparagus tissue and extracts are toxic to root systems (Putnam et al., 1982), this stress on the roots could increase *Fusarium* crown and root rot incidence and severity. Asparagus toxins may also affect soil microorganisms, leading to an ecological imbalance of the naturally occurring soil microflora. If certain microorganisms are inhibited by the toxins and *Fusarium* spp. are not, the toxins may indirectly influence the incidence and severity of Fusarium crown and root rot by favoring the predominance of *Fusarium* sp. in the rhizosphere of the asparagus plant.

The objectives of this study were (1) to investigate the effect of asparagus plant tissue on the susceptibility of asparagus to *Fusarium* root and crown rot, (2) to attempt to isolate and purify chemical species involved in these interactions, and (3) determine the effects of crude extracts on *Fusarium* spp. and other soil microorganisms.

METHODS AND MATERIALS

Preparation of Inoculum. Millet seeds *Setaria italica* (L.) Beauv. (250 g) and distilled water (100 ml) were placed in a 1-liter flask, autoclaved 1 hr, shaken to loosen the seed from sides of the flask, then left to cool overnight on the lab bench. The millet seeds were reautoclaved the next day for 1 hr, allowed to cool, then inoculated with a 4-mm plug of actively growing mycelium of *F. moniliforme* (FM) or *F. oxysporum* f. sp. *asparagii* (FO). The cultures were grown at 26° C, and each flask was shaken daily to facilitate mycelial spread throughout the millet seeds. The cultures to be used as inoculum were harvested after 2 weeks, allowed to air dry, and then stored in paper bags at 26° C until ready for use in soil amendment studies.

Preparation of Asparagus Plant Material. A commercial asparagus field in Oceana County, Michigan, was excavated and Martha Washington asparagus plants were collected, washed, and separated into living roots and rhizomes. All dead tissue was discarded. The roots and rhizomes were oven dried (50° C) and ground in a Wiley mill. Asparagus ferns were clipped from actively growing plants in the greenhouse and prepared in a similar manner. All dried plant tissue were sterilized with propylene oxide (Tuite, 1969). Ten grams of sterilized plant material were tested for *Fusarium* sp. contami-

nation by planting on Komada's medium (Komada, 1975). Dried tissues were stored in plastic bags at −20°C until used in soil amendment studies or chemical isolation experiments.

Interaction of Fusarium *spp. and Dried Asparagus Plant Tissues on Asparagus Seedlings.* Asparagus seedlings, UC 147, 8 weeks old, were grown in soil (sand–greenhouse soil, 2:1) containing the following: (1) FM, (2) FM and root tissue, (3) FM and rhizome tissue, (4) FO, (5) FO and root tissue, (6) FO and rhizome tissue, (7) both *Fusarium* isolates together, (8) both isolates and root tissue, (9) both isolates and rhizome tissue, (10) root tissue, (11) rhizome tissue, and (12) a sterilized millet seed control. In a second experiment, dried fern tissues were used in place of root or rhizome tissue. All other treatments were the same as in the first experiment. In each experiment infested millet seed inoculum was incorporated into soil at 8 g millet seed inoculum/4-in. pot. When both isolates were incorporated, the inoculum was applied at 4 g each/4-in. pot. Dried plant tissues were incorporated into soil at 50 g/4-in. pot (8% of soil weight/4-in. pot).

The inoculated seedlings were placed at random on a greenhouse bench and watered daily. After 8 weeks, the plants were harvested and evaluated visually for root and rhizome rot separately using a scale of 0–5. The ferns were removed and the crowns dried and weighed. The data were subjected to analysis of variance and Duncan's multiple-range test.

Isolation of Germination Inhibitors from Asparagus Root Residue. In order to determine what chemical components of the plant tissues were active in allelopathic interactions, the following isolation procedure was developed. Dried asparagus root tissue (50 g) was added to 500 ml of 50% methanol and ground in a Waring blender for 1 min. The slurry was stirred overnight at 1°C, then filtered through four layers of cheesecloth and centrifuged at 6000g for 15 min to remove particulates. The supernatant was decanted and the methanol–water fraction was precipitated with acetone (4:1 acetone–sample) overnight at 1°C. The precipitate was discarded and the liquid concentrated to 1/4 volume. This concentrate was extracted with chloroform ($CHCl_3$) three times (1:1). The $CHCl_3$ was discarded and the polar fraction extracted three times with two volumes of water-saturated butanol. The butanol extracts were combined and concentrated to dryness. The extract was resuspended in 20 ml methanol, and 5 ml were removed and concentrated to 2 ml under N_2. The concentrate was applied to a Sephadex G-25 column (2 × 20 cm) prepared according to Rouser and Fleischer (1967) and eluted as follows: 100 ml of $CHCl_3$–methanol–acetic acid–H_2O (19:1:4:0.6) (fraction 1), 100 ml of Methanol–H_2O (1:1) (fraction 2), and 100 ml of 10% acetic acid in 50% methanol (fraction 3). Each fraction was concentrated under reduced pressure, dissolved in a small volume of methanol and further separated into individual components by preparative thin-layer chromatography. Aliquots were removed and spotted on ITLC-SA polysilicic acid gel impregnated

glass-fiber sheets (Gelman Sciences Inc., Ann Arbor, Michigan) and the chromatograms developed with isopropyl alcohol–NH_4OH (58%) (100:7). The chromatograms were viewed under short- and long-wave UV light. The fluorescent spots were marked and R_f values were calculated. Like spots or areas of equal R_f values were removed, combined, and eluted with 100 ml of methanol, concentrated to dryness with a flash evaporator, dissolved in 1 ml of MeOH, and bioassayed for germination inhibition on cress seed (*Lepidium sativum*, curly cress).

Bioassay for Germination Inhibition by Asparagus Root Extracts. In order to determine which fraction of the asparagus root tissue extract was responsible for allelopathic interactions, each extraction step was bioassayed for radicle elongation on pregerminated asparagus and germination inhibition cress seeds. After experiments had established that those fractions which inhibited asparagus also inhibited cress germination, cress seeds were used in further bioassays. Seeds of several other plant species were also bioassayed with the most active fraction after solvent extraction.

Because asparagus seeds exhibit wide variability in germination rates, they were pregerminated before use in bioassay studies. Asparagus seeds were surface sterilized with 0.5% sodium hypochlorite and 2 drops Tween 20 for 15 min, rinsed three times with DW, incubated for 10–15 min in DW, and placed on premoistened Whatman No. 1 filter paper in 9-cm Petri plates and sealed with Parafilm. The plates were placed in the dark at 26°C until radicle emergence was visible.

Asparagus extract was diluted in a tenfold series, 1 ml of each dilution was added to Whatman No. 1 filter paper in 9-cm Petri dishes and allowed to air dry. Asparagus seedlings with radicles of equal length (10 seeds/plate) were placed on the filter paper which was then dampened with glass distilled water (GDW). The plates were sealed with Parafilm and placed in the dark at 26° C. Five replicate plates were prepared for a total of 50 seeds per test. If the extract was dissolved in solvents or GDW, solvent only and GDW only controls were used. Germination was considered inhibited if the radicle did not elongate.

For cress seed assays, similar dilutions were done with the extract; the extract was spotted on Whatman 540 filter paper, using 2.5 ml/2.1-cm disks with 4 disks/plate. Disks were dried, wetted with 2.5 ml GDW, and 10 seeds were placed on each disk. Appropriate solvent controls or GDW controls were also spotted in a similar manner. The plates were sealed with Parafilm, placed on the lab bench, and evaluated after 4 days. Seeds were considered germinated if radicle emergence was complete. A dilution series was not performed on compounds eluted from ITLC plates.

The last step of the solvent extraction (butanol fraction) was also bioassayed using lettuce (*Lactuca sativa*), radish (*Raphanus sativus* L.), barnyard grass, Tendergreen mustard (*Brassica campestris*), National

Pickling cucumber (*Cucumis sativus*), tomato, alfalfa (*Medicago sativum* L.), and asparagus seeds. Bioassays were conducted as before on Whatman No. 1 filter paper using 100 or 50 seeds/test. No dilution series was performed in this test due to scarcity of extract material.

Inhibition of Fungal Growth by Root Extract. Preliminary experiments were conducted with *Pythium ultimum, Rhizoctonia solani, F. oxysporum* f. sp. *asparagii* (FO), and *F. moniliforme* (FM) to determine if any of these soil-borne organisms would exhibit growth inhibition in the presence of a known amount of asparagus root extract. A 4-mm plug of agar containing the fungal isolate to be assayed was placed in the middle of a potato-dextrose agar (PDA) plate and 2.5 g of autoclaved or nonautoclaved dried asparagus root tissue was sprinkled in a localized area on the side of the petri plate. Plates were viewed 5 days later for inhibition of fungal growth.

In another test asparagus root extract prepared by adding 20 g dried asparagus root tissue to 200 ml DW was placed in dialysis tubing (3500 mol wt cutoff) and dialyzed overnight against 2300 ml DW. The bag and its contents were discarded. The dialyzed fraction was concentrated to 200 ml final volume (equivalent to 1 ml/0.1 g dry wt) under reduced pressure at 50°C, then filter sterilized. A 4-mm plug of agar containing an actively growing culture of *P. ultimum* was placed in 50-ml potato-dextrose broth (PDB) and immediately treated with one of the following levels of root extract: 0, 2.5, 5.0, or 10 ml. The cultures were incubated for 10 days on a reciprocal shaker, then harvested by filtering through preweighed, glass-fiber filter paper (Whatman GF/C 7.0 cm), dried, and weighed. Any contaminated cultures were discarded. There were 4 replica flasks/treatment. The data were subjected to an analysis of variance and Duncan's multiple-range test.

RESULTS

Interaction of Fusarium *spp. and Dried Asparagus Plant Tissues on Asparagus Seedlings.* In the first experiment the dry weights of seedlings treated with root tissue alone or with root tissue and *Fusarium* spp. were significantly lower ($P = 0.01$) than dry weights of controls (Table 1). Dry weight of seedlings was not significantly reduced by rhizome tissue unless FM or FO were present. Neither FM nor FO alone significantly decreased dry weight unless the root or rhizome tissue was present. However, there was a significant difference between root rot rating of seedlings treated with FO or FM and controls. Root rot was greatest in treatments when root tissue was combined with FM or FO (Figure 1).

In the second experiment, there was no significant reduction in dry weight or root rot in the treatments with fern tissue alone (Table 2). Seedling dry weight was significantly reduced when fern tissue was added in combina-

TABLE 1. DRY WEIGHT AND ROOT ROT RATING FOR ASPARAGUS SEEDLINGS TREATED WITH COMBINATIONS OF *Fusarium* SP. AND DRIED ASPARAGUS TISSUE

Treatment	Dry weight (g)[a]	Root rot rating[b]
FM[c] + root tissue	0.24 a[e]	4.8 f[f]
FO[d] + root tissue	0.36 ab	4.6 f
FM + FO + root tissue	0.53 ab	3.8 e
Root tissue	0.66 ab	3.8 e
FO + rhizome tissue	0.95 abc	3.2 d
FM + rhizome tissue	1.41 bc	2.8 d
FM + FO	1.94 c	1.3 b
FM + FO + rhizome tissue	2.03 d	3.3 de
FM	2.07 d	2.2 c
Rhizome tissue	2.42 d	1.0 b
FO	2.54 d	1.8 c
Control	2.85 d	0.0 a

[a]Dry weight = mean of 6 repetitions/treatment.
[b]Root rot rating scale: 0 = no root rot or rhizome discoloration, 1 = 25% root rot or few red or pink streaks in the rhizome, 2 = 50% root rot or prominent streaking in rhizome tissues, 3 = 75% root rot or death of 25% of the rhizome, 4 = greater than 75% root rot or death of 50% of the rhizome, 5 = death of the plant.
[c]FM = *F. moniliforme.*
[d]FO = *F. oxysporum* f. sp. *asparagii.*
[e]Means without a letter in common are significant at $P = 0.05$ confidence level for Duncan's multiple-range test.
[f]Means without a letter in common are significant at $P = 0.05$ confidence level for Duncan's multiple-range test.

tion with FM or FO. Root rot ratings were not significantly different when fern tissues alone was added. However, there was significantly greater root rot in treatments containing fern tissue in combination with either FO or FM (Table 2).

Isolation of Germination Inhibitors from Asparagus Root Extract. The three different fractions from the Sephadex column were separated into individual components by thin-layer chromatography (TLC) (Table 3). Fraction 1 contained five components, fraction 2 contained eight components, and fraction 3 contained six components, as determined by fluorescence under UV light.

Bioassay of Germination Inhibitors from Asparagus Root Extracts. To determine which components of the solvent extraction contained germination inhibitors, all steps of the solvent extraction were assayed on cress and pregerminated asparagus seed. The chloroform fraction exhibited no germination inhibition and the water fraction inhibited germination only when used undiluted. However, the butanol fraction exhibited 100% germination

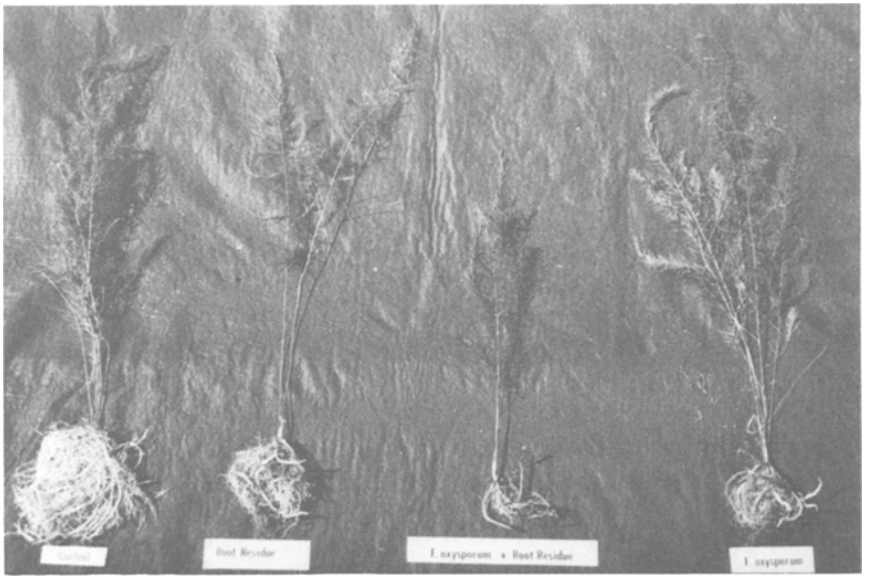

FIG. 1. Decrease in root dry weight and increase in root rot caused by a combination of *Fusarium oxysporum* f. sp. *asparagus* and asparagus dried tissue. From the left: control; root tissue alone; FO plus root tissue; and FO alone.

inhibition even after a 10^{-2} dilution and was chosen for further chemical isolation experiments.

Bioassay after column elution demonstrated that all fractions contained some germination inhibition of cress and asparagus. Therefore, components of each column fraction were separated by ITLC. Individual components visible under UV light were all bioassayed on cress seeds (Table 3). Fraction 1 contained one component (R_f 0.83) that resulted in 90% inhibition of germination compared to controls. The only component to inhibit germination in fraction 2 was found at the origin, while fraction 3 contained two components (R_f 0.85 and 0.49), each of which inhibited germination by 45%.

Bioassay of the butanol solvent extract on eight different plant species (Table 4) resulted in inhibition of germination on all plant species tested. Alfalfa seeds were able to break through seed coats, but elongation of the radicle did not take place.

Inhibition of Fungal Growth by Root Extracts. Preliminary experiments indicated that dried asparagus tissue had no visible effect on the growth of *Rhizoctonia solani*, FO or FM. However, *P. ultimum* hyphae would not grow within a 1.5-cm area around autoclaved or nonautoclaved asparagus root tissue on PDA after 5 days. When *P. ultimum* was grown in PDB in the presence of varying levels of asparagus root extract for 10 days, all levels of extract significantly inhibited fungal growth (Table 5).

TABLE 2. DRY WEIGHT AND ROOT ROT RATING FOR ASPARAGUS SEEDLINGS TREATED WITH COMBINATIONS OF *Fusarium* SPP. AND DRIED ASPARATUS FERN TISSUE

Treatment	Dry weight (g)[a]	Root rot rating[b]
FM[c] + fern tissue	0.03 a[e]	3.3 f[f]
FM + FO[d] + fern tissue	0.15 ab	2.3 de
FM + FO	0.22 abc	4.3 g
FO + fern tissue	0.42 cd	1.6 cd
FO	0.51 de	2.7 ef
FM	0.65 e	1.0 bc
fern tissue	1.03 f	0.3 ab
Control	1.12 f	0.0 a

[a] Dry weight = mean of 6 repetitions/treatment.
[b] Root rot rating scale: 0 = no root rot or rhizome discoloration, 1 = 25% root rot or few red or pink streaks in the rhizome, 2 = 50% root rot or prominent streaking in rhizome tissues, 3 = 75% root rot or death of 25% of the rhizome, 4 = greater than 75% root rot or death of 50% of the rhizome, 5 = death of the plant.
[c] FM = *F. moniliforme*.
[d] FO = *F. oxysporum* f. sp. *asparagii*.
[e] Means without a letter in common are significant at $P = 0.05$ confidence level for Duncan's multiple-range test.
[f] Means without a letter in common are significant at $P = 0.05$ confidence level for Duncan's multiple-range test.

DISCUSSION

This study presents evidence that an interaction of *Fusarium moniliforme* and *F. oxysporum* f. sp. *asparagii* and allelopathic asparagus tissues enhances severity of root rot and decreases dry weight in asparagus seedlings. Asparagus root tissue alone was more toxic to asparagus seedlings than either

TABLE 3. COMPONENTS ISOLATED FROM DRIED ASPARAGUS ROOT TISSUE WHICH INHIBIT CRESS SEED GERMINATION

Fraction[a]	Separate fluorescent components[b]	Number of inhibitory components	R_f of inhibitory components	Cress seed germination[c]
1	5	1	0.83	6/60
2	8	1	origin	19/40
3	6	2	0.85	22/40
			0.49	22/40

[a] Each fraction represents one elution from Sephadex G-25 column.
[b] Components separated by TLC using isopropyl alcohol–NH_4OH (58%) (100:7) as mobile phase.
[c] Number of seeds that germinated out of the total number of seeds tested.

TABLE 4. GERMINATION INHIBITION OF DIFFERENT SEEDS BY BUTANOL FRACTION OBTAINED FROM ASPARAGUS

Indicator species	Asparagus extract[a]	Control[b]
Lettuce	0/100[c]	100/100[c]
Radish	0/100	86/100
Barnyard grass	0/60	52/60
Alfalfa	0/100	96/100
Tendergreen mustard	0/100	90/100
Cucumber	0/50	40/50
Tomato	0/100	95/100
Asparagus[d]	0/50	50/50

[a] 1 ml of the butanol fraction dissolved in MeOH was used in each assay.
[b] 1 ml of methanol.
[c] Number of seeds that germinated out of the total number of seeds tested.
[d] Asparagus seeds were pregerminated.

rhizome or fern tissue. Neither rhizome nor fern tissue caused a significant dry weight decrease, whereas root tissue did.

There were no apparent differences in the ability of either FM or FO to cause root rot. Both significantly increased the severity of seedling root rot but neither significantly decreased the dry weight of the asparagus root mass in one experiment and only slightly decreased the dry weight in the other, at the inoculum levels used.

However, in combinations of FM or FO and asparagus tissue, there was a dramatic increase in root rot and an equally dramatic decrease in seedling dry weight. The FM or FO/root tissue combinations were the most toxic to the seedlings but both FM and FO in combination with rhizome or fern tissue were also toxic to tested seedlings.

TABLE 5. WEIGHT OF *Pythium ultimum* HYPHAE GROWN IN PDB IN PRESENCE OF VARIOUS LEVELS OF ASPARAGUS ROOT EXTRACT

Extract (ml)[a]	Weight (g)[b]
0.0	0.14 b[c]
2.5	0.02 a
5.0	0.03 a
10.0	0.03 a

[a] Amount of asparagus root extract/50 ml PDB.
[b] Weight of fungal tissue after 10 days growth in PDB. Each treatment is the mean of 4 replica flasks.
[c] Means without a letter in common are significant at $P = 0.05$ confidence levels for Duncan's multiple-range test.

The means by which this interaction took place was not investigated in this study. The dry weight decrease indicated that either root growth was severely restricted or roots rotted off more quickly in FM or FO/asparagus tissue combination. We postulate that either more sites on the asparagus roots were successfully colonized by FM or FO in the presence of asparagus tissue or that FM or FO could spread more quickly in the roots once fungal penetration took place.

Asparagus seedlings were mildly to severely inhibited by four components that were isolated from root tissue. Identification of these chemical species is now being carried out in our laboratory. The inhibition of asparagus seedlings by these components suggests that asparagus tissue in the soil may affect replanting of asparagus seedlings into fields where asparagus has grown for long periods of time. Asparagus seedling and crown death have been observed to be greater in fields formerly planted to asparagus than in fields never before planted to that crop.

Dried asparagus tissue did not adversely affect the growth of either of the *Fusarium* spp. pathogenic to asparagus. However, dried asparagus tissue and asparagus root extracts were toxic to another fungus tested. Toxic effects by asparagus tissue on this fungus and other soil microorganisms may favor the predominance of *Fusarium* sp. in the rhizosphere of the asparagus plant. A number of microorganisms commonly found in the rhizosphere of the asparagus plant are being collected and assayed against dried asparagus tissue and extracts.

As root rot by *Fusarium* sp. is considered to be one of the major causes in asparagus decline, presence and buildup of asparagus tissue in the soil may accelerate the asparagus decline process. Currently the levels of asparagus tissue in soils of asparagus fields of varying ages are being investigated.

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EFFECTS OF JUGLONE CONCENTRATION ON GROWTH IN VITRO OF *Frankia* ArI3 AND *Rhizobium japonicum* STRAIN 71

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Abstract—Nitrogen-fixing nurse crops and cocrops of plant species nodulated by *Frankia* and *Rhizobium* have been used to promote the growth of black walnut trees. Although walnut is known to inhibit the growth of certain associated plants due to its allelopathic derivative juglone (5-hydroxy-1,4-napthoquinone), juglone inhibition of symbiotic, nitrogen-fixing soil microorganisms had not been investigated. This research revealed that a concentration of 10^{-3} M juglone absolutely inhibited the growth in vitro of a *Frankia* isolate from root nodules of red alder and of *Rhizobium japonicum* strain 71. Lesser concentrations of juglone inhibited the growth of these bacteria relative to the controls. The more-rapidly growing *Rhizobium* strain exhibited slight growth at 10^{-4} M juglone concentration, whereas *Frankia* growth was completely inhibited. Considering both the susceptibility of the host plant and nitrogen-fixing endophyte to the allelochemical juglone, caution should be exercised in selecting nitrogen-fixing plants as nurse crops for black walnut.

Key Words—Allelopathy, black walnut, *Juglans nigra* L., nitrogen fixation, soil microorganisms, *Frankia*, *Rhizobium*.

INTRODUCTION

Trees and shrubs nodulated by nitrogen-fixing *Frankia* and *Rhizobium* bacteria have been planted with black walnut (*Juglans nigra* L.) in order to help promote walnut growth. However, little is known about the effects of walnut's allelochemical juglone (5-hydroxy-1,4-napthoquinone) on nitrogen-fixing microorganisms.

Black walnut interplanted with autumn-olive (*Elaeagnus umbellata* Thunb.) is a successful mixture, resulting in more rapid growth of walnut

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compared to walnut planted alone (Funk et al., 1979). Autumn-olive forms root nodules symbiotically with nitrogen-fixing actinomycetes of the genus *Frankia*, eventually increasing the amount of soil nitrogen available to the walnuts.

Black walnut multicropping regimes have been found to be an economical land use alternative in the midwestern United States (Garrett, 1980). The regimes range from those just providing protection for the walnut seedlings and grazing throughout the walnut rotation, to more complex systems consisting of growing black walnut at a wide spacing and intercropping with soybeans and winter wheat for the first ten years, followed by five years of fescue hay and seed production and then grazing for the remainder of the walnut rotation.

Juglone has been found to inhibit growing of a variety of plant species at concentrations ranging from 10^{-3} to 10^{-5} M (Rietveld, 1983). European black alder [*Alnus glutinosa* (L.) Gaertn.] showed heavy mortality apparently due to juglone after 7–11 years when used as a nurse crop for black walnut (Rietveld and Schlesinger, 1982). Toxic effects ascribed to juglone have also been found to persist in nature even after the walnuts were removed (Gabriel, 1975). Kentucky bluegrass (*Poa pratensis* L.), however, is not inhibited by juglone (Brooks, 1951), and it is likely that other plants and organisms exhibit varying degrees of resistance to juglone.

Although black walnut seems to inhibit the growth of certain associated plants due to juglone, allelopathy in forest ecosystems due to the effect of juglone and other allelochemicals on nitrogen-fixing or other soil microorganisms has not been extensively investigated. In a preliminary study, it was found that as juglone concentration increased from 10^{-6} to 10^{-3} M, the packed-cell volume of *Frankia* cultures measured after 14 weeks of growth decreased exponentially (Dawson et al., 1981). Researchers have found that extracts and leachates from the grass *Aristida adscensionis* (Gray) Nash inhibit the growth of both *Rhizobium* and *Azotobacter* (Murthy and Nagodra, 1977; Murthy and Ravindra, 1975). Moleski (1976) investigated the effects of tannins from *Quercus stellata* Wangenh. and *Q. marilandica* Muenchh. on microbial populations, and found that numbers of *Nitrosomonas* and *Nitrobacter* increased in a clear-cut area compared to the unaltered oak forest. In a central hardwood bottom-land forest, digallic, ellagic, caffeic, and ferulic acids were identified by Lodhi (1978) under the cover of hackberry (*Celtis occidentalis* L.), white oak (*Q. alba* L.), red oak (*Q. rubra* L.), and sycamore (*Platanus occidentalis* L.). These same compounds were previously found to be inhibitive to nitrifying bacteria (Rice and Pancholy, 1973; 1974).

The purpose of this study was to determine the effect of juglone concentration on the growth of two nitrogen-fixing soil microorganisms that form nodules with actinorhizal (*Frankia* nodulated) and leguminous plants.

The two isolates tested for in vitro response to varying levels of juglone were *Frankia* isolate ArI3 (isolated from root nodules of *Alnus rubra* Bond.; Berry and Torrey, 1979) and *Rhizobium japonicum* strain 71 that forms root nodules on soybeans.

METHODS AND MATERIALS

The nutrient medium for *Rhizobium* consisted of the following, at pH 6.6, in grams per liter: K_2HPO_4, 1.0; NH_4Cl, 0.32; $CaCl_2 \cdot 2H_2O$, 0.013; $MgSO_4 \cdot 7H_2O$, 0.18; Na_2SO_4, 2.5; $FeCl_3 \cdot 6H_2O$, 0.004, mannitol, 5.0; L arabinose, 0.5; and yeast extract, 0.5. Juglone[1] was added to nutrient solutions immediately after autoclaving to generate treatment solutions of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} molarity. By adding juglone to hot media immediately after autoclaving, recapping the flasks tightly with aluminum foil, and mixing the solution on a stir plate, asepsis of nutrient media and complete dissolution of juglone were achieved. Four milliliters of treatment solutions were poured aseptically into 25 × 150-mm culture tubes, then inoculated with 0.1 ml of inoculum via Pasteur pipets. To prepare this inoculum *Rhizobium japonicum* strain 71 was grown in 50 ml of the nutrient medium for one week, then diluted by adding 150 ml of sterile distilled water. Forty-two tubes per treatment, including uninoculated, sterile controls, were prepared along with uninoculated, sterile blanks as reference standards. Isolates were grown in shaken culture at 25° C.

Changes in absorbance (growth) at 560 nm were measured with a Bausch and Lomb spectrophotometer. Sampling was done at 12-hr intervals during the logarithmic phase of *Rhizobium* growth, otherwise the sampling interval was 24 hr. At each sampling time, the contents of three tubes per treatment were poured into 10-ml quartz tubes for spectrophotometry and then discarded.

The *Frankia* trial used a yeast extract medium containing, at pH 6.5, in grams per liter: yeast extract, 5.0; dextrose, 10.0; casamino acids (acid hydrolyzed), 5.0; and vitamin B_{12}, 0.0016. Eight-milliliter aliquots of *Frankia* medium were poured into culture tubes (18 × 150 mm) and then autoclaved. Juglone solutions prepared in the manner previously described in sterile, distilled water were added in 2-ml quantities per tube, resulting in liquid cultures with juglone concentrations of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} molarities. Controls were prepared by adding 2 ml of sterile, distilled water to additional culture tubes. Isolates were grown in the dark at 25° C. There were 15 *Frankia* cultures in each of the five treatments. Three cultures from each treatment were destructively sampled at 6-day intervals. Growth of individual *Frankia* colonies was measured as packed cell volume by centrifuging each colony at

[1]Sigma Chemical Co., grade II, approximately 90%; a purity correction was made when preparing solutions.

TABLE 1. MEAN GROWTH OF *Frankia* ArI3 IN VARIOUS CONCENTRATIONS OF JUGLONE

Juglone concentration (M)	Packed cell volume (cc) after inoculation				
	6 days	12 days	18 days	24 days	30 days
0	0.0053a[a]	0.0063a	0.0127a	0.0207a	0.0260a
10^{-6}	0.0057a	0.0050ab	0.0110ab	0.0173ab	0.0237a
10^{-5}	0.0047ab	0.0040b	0.0083abc	0.0150b	0.0200a
10^{-4}	0.0027b	0.0027b	0.0030c	0.0030c	0.0050b
10^{-3}	0.0023b	0.0035b	0.0043bc	0.0043c	0.0043b

[a]Means in a column followed by the same letter are not significantly different ($\alpha = 0.05$).

2000 rpm for 15 min in a Bauer-Schenk sedimentation tube (Owens-Illinois, Inc., Toledo, Ohio).

RESULTS AND DISCUSSION

In the *Frankia* trial, the mean packed cell volumes of the 10^{-4} and 10^{-3} M treatments were significantly less than means for all other treatments after day 24 (Table 1). These higher concentrations absolutely inhibited *Frankia*

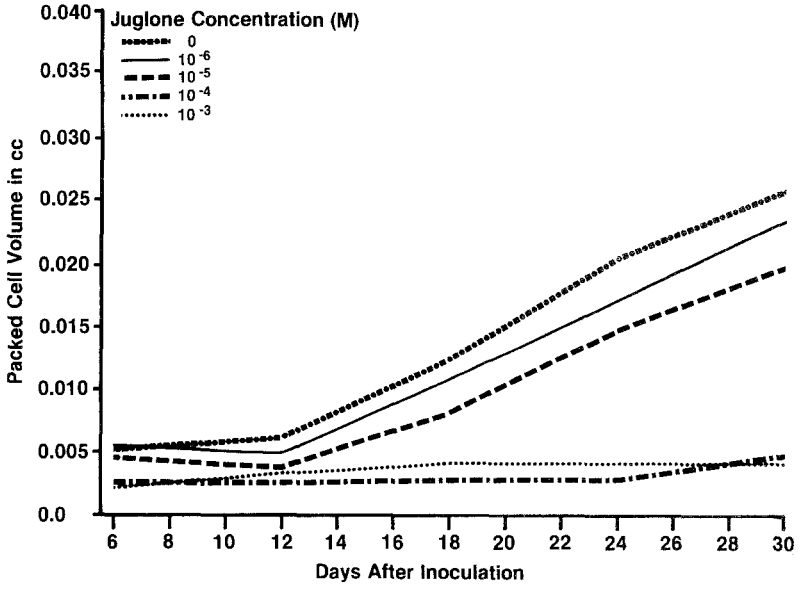

FIG. 1. Growth of *Frankia* ArI3 at differing juglone concentrations.

growth over a 30-day period and were not significantly different from each other (Figures 1 and 2).

In the *Rhizobium* trial, the controls had a significantly greater mean absorbance value than the others beginning 5 days after inoculation. After day 6 the 10^{-6} and 10^{-5} M treatments became significantly different from each other. Throughout the trial the 10^{-4} and 10^{-3} M treatments were significantly less than the others and showed no observable growth until the last sample time. At this time the 10^{-4} M treatment exhibited some growth after the controls and low-concentration juglone treatments had completed the logarithmic phase of their growth curves (Table 2, Figure 3).

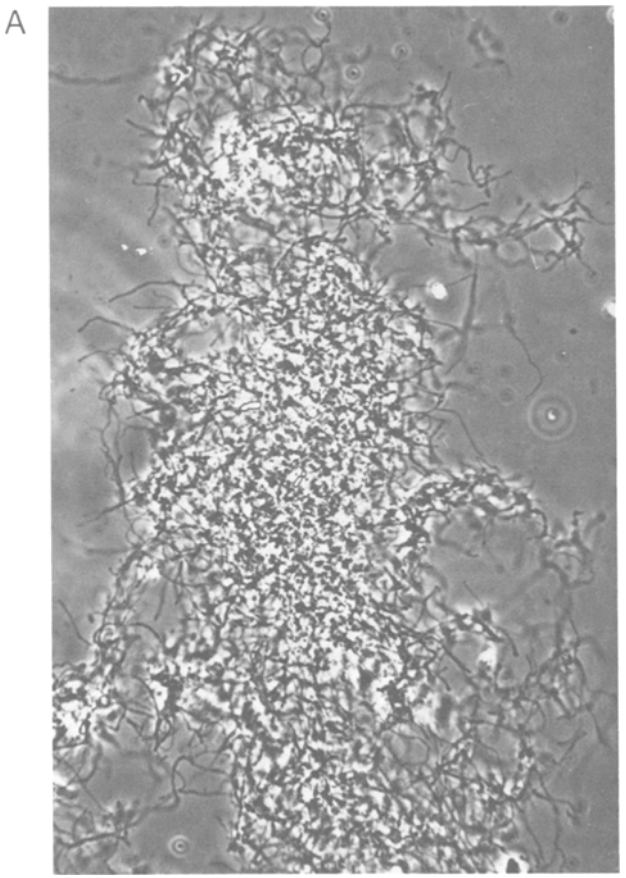

FIG. 2. Contrast between (A) the necrotic colony of *Frankia* ArI3 grown in 10^{-3} M juglone and (B) the healthy colony of *Frankia* ArI3 grown with no juglone (100×).

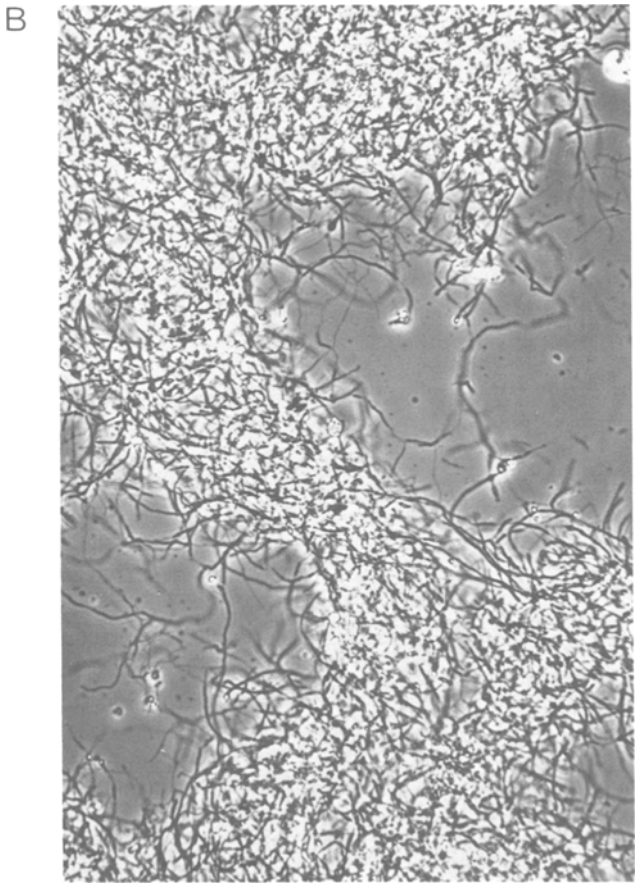

FIG. 2. Continued.

Since juglone has been shown to inhibit both nitrogen-fixing microorganisms and plants in controlled experiments at 10^{-3} and 10^{-4} concentrations (Rietveld, 1983), and plant mortality occurs around walnut in nature (Rietveld, 1982), we postulate that 10^{-3} and 10^{-4} juglone concentrations may occur in soil solution. Juglone may also persist in soil after black walnut is harvested (Gabriel, 1975). If juglone occurs in inhibitory quantities in soil solution, then some nitrogen-fixation and growth inhibition might occur when soybeans are grown with walnut. This would be because they are a host plant for the nitrogen-fixing, nodule-forming bacterium *Rhizobium japonicum* for which we have demonstrated growth inhibition by juglone. For the same reason, juglone might also be an important factor limiting productivity of walnut plantations using *Frankia*-nodulated autumn-olive or alders as

TABLE 2. MEAN GROWTH OF *Rhizobium japonicum* STRAIN 71 IN VARIOUS CONCENTRATIONS OF JUGLONE

Juglone concentration (M)	Absorbance at 560 nm after inoculation												
	1 day	2 days	3 days	4 days	4.5 days	5 days	5.5 days	6 days	6.5 days	7 days	7.5 days	8 days	9 days	10 days
0	0.0100a[a]	0.0223a	0.0720a	0.1887a	0.3343a	0.4907a	0.6650a	0.7733a	0.9400a	0.9883a	1.1767a	1.2267a	1.2667a	1.2400a
10^{-6}	0.0087ab	0.0227a	0.0537b	0.1433b	0.2887ab	0.4000b	0.4560b	0.6767b	0.8267b	0.9233b	1.1000b	1.1900a	1.2133b	1.1933b
10^{-5}	0.0063b	0.0157b	0.0567ab	0.0963c	0.2653b	0.3900b	0.4450b	0.6400b	0.7633c	0.7817c	1.0380c	1.1733a	1.2067b	1.1867b
10^{-4}	0.0000c	0.0000c	0.0000c	0.0000d	0.0023c	0.0013c	0.0070c	0.0047c	0.0087d	0.0053d	0.0073d	0.0040b	0.0263c	0.1300c
10^{-3}	0.0000c	0.0000c	0.0000c	0.0000d	0.0000c	0.0000c	0.0000c	0.0000c	0.0000d	0.0000d	0.0000b	0.0000b	0.0000c	0.0000d

[a] Means in a column followed by the same letter are not significantly different ($\alpha = 0.05$).

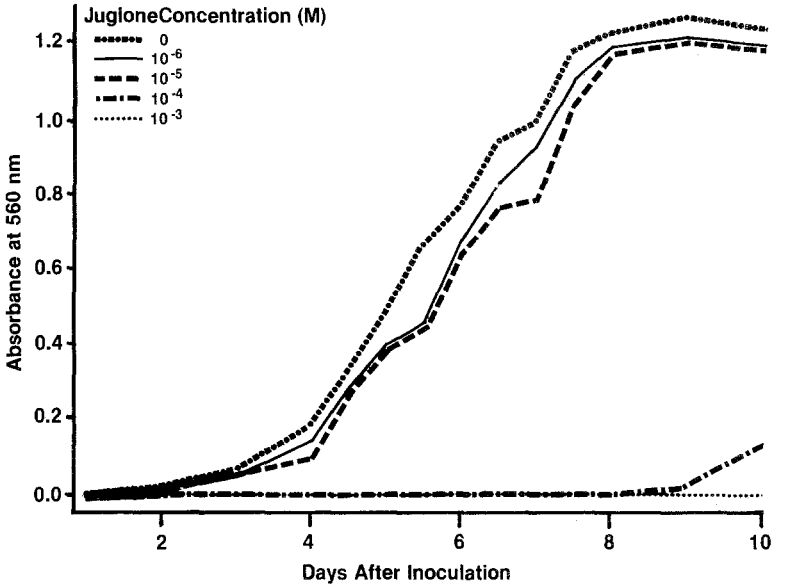

FIG. 3. Growth of *Rhizobium japonicum* strain 71 at differing juglone concentrations.

nurse crops or cocrops, particularly in second rotations. Further research will be necessary to establish the ecological importance of juglone toxicity to these soil microorganisms.

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ALLELOPATHIC SUBSTANCES IN ECOSYSTEMS: Effectiveness of Sterile Soil Components in Altering Recovery of Ferulic Acid[1]

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Abstract—Recovery studies were conducted with ferulic acid, a common allelopathic agent, using various soils and soil components. Ferulic acid was placed into sterile soil components (gibbsite, geothite, Georgia kaolin, and Utah bentonite), and different sterile soil materials (from different horizons in the same profile) varying in mineralogy and in organic matter content. The initial concentration of ferulic acid added to the soil materials was 1000 μg/g (5.149 mmol/g). The pH of the soil materials was adjusted and maintained at approximately 4.5 or 7.5. Samples were extracted with 0.03 M EDTA at days, 1, 4, 7, 10, and 13 after addition of ferulic acid. Concentrations of ferulic acid in the extracts were determined with a high performance liquid chromatograph. No breakdown products were detected. Models were developed to describe the recovery of ferulic acid from each soil material and soil component over time. Organic matter was the most active soil component involved in the irreversible retention of ferulic acid. The inorganic soil components were much less active than organic matter but appeared to be similar to each other in activity. Irreversible retention of ferulic acid by soil and soil components was greatest as pH 7.5.

Key Words—Ferulic acid, allelopathy, soils, gibbsite, geothite, Georgia kaolin, Utah bentonite, organic matter.

INTRODUCTION

Plants release a variety of organic compounds into the environment by way of leaf leachates, root exudates, and through decomposition of litter. Certain

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classes of these compounds, predominately phenolic acids, have been shown to be allelopathic to seed germination and seedling growth (Rice, 1974, 1979). It has been demonstrated that phenolic compounds are released from living plants and plant litter into the environment, but little information is available on the active fraction of these compounds in soils. The active fraction is defined as that portion of a substance available in soil which can modify plant growth.

When phenolic acids are released into the soil environment a number of processes can occur that reduce their concentrations in the soil solution and thus modify the active fraction. The compounds in the soil solution may interact directly with plants. If the concentrations are appropriate, plant growth is modified. Microorganisms may also modify the phenolic acid pool via decomposition (Turner and Rice, 1975; Martin and Haider, 1979), thereby depleting the active soil solution fraction of these compounds or transforming them into new compounds (Haider and Martin, 1975) which could exert, depending on concentration, a greater, lesser, or the same allelopathic effect on plant growth.

Phenolic acids are also known to polymerize in the presence of microorganisms (Martin et al., 1972, 1974; Martin and Haider, 1976; Haider et al., 1977) and through the activity of small quantities of polyphenoloxidases or peroxidase enzymes present in the soil (Skujins, 1967). Once these compounds polymerize, they appear to be very stable and resistant to microbial degradation (Martin et al., 1967, 1978; Haider and Martin, 1967; Martin and Haider, 1971, 1976). This would also remove potential allelopathic compounds from soil solution and render them, for the most part, unavailable to plants.

Aside from biological means, plant phenolic acids can be depleted from the soil solution by abiotic or physical processes. Phenolic molecules adsorbed by either organic or inorganic soil particles (Huang et al., 1977) may be reversible bound and therefore have the potential to reenter the soil solution. This type of retention is due to electrostatic bonding or the formation of organometallic complexes (Hamaker and Thompson, 1972). However, some adsorbed molecules may be irreversibly retained under natural conditions. The irreversibly bound phenolic molecules may be: (1) incorporated into humic substances (Felbeck, 1971; Haider and Martin, 1967; Wang et al., 1971); (2) involved in ligand exchange on a hydroxide surface, in which an anion actually penetrates the coordination sphere of a metal ion, e.g., iron or aluminum, thereby being incorporated into the surface hydroxyl layer (Greeland, 1971); and/or (3) polymerized due to the catalytic properties of certain clay minerals (charge transfer theory) (Theng, 1974; Wang et al., 1978).

Several extractants have been used to determine the active concentration of phenolic acids in soil systems. The most frequently used extractants are

water, ethylenediaminetetraacetic acid (EDTA), and sodium hydroxide. The use of Na_2EDTA at pH 7.5 in this study is based on the very strong chelating ability of this reagent (stability constants of 8.7–16.1 for common soil polyvalent cations). If the phenolic acid is complexed in soil systems through exchange cations or bonded as an anion to positively charged sites, it would be displaced into the extracting solution (Martin and Haider, 1976; Kaminsky and Muller, 1977). Neutral water extractions (Shindo and Kuwatauka, 1976) are meaningful in terms of soil solution concentrations, but reversibly bound fractions are not released from soil particles. Therefore extractions of this type may only partially address the bioavailability of these compounds in soils. Alkaline extractions, using NaOH or EtOH at pH 11, partially solubilize the organic material present in soils, thereby introducing phenolic compounds that would not normally be present in the soil solution. This can result in an overestimation of available phenolic acids in soils. Maintaining the pH of the extracting solution at neutral or slightly acid conditions is very important in minimizing the dispersion of humified organic matter which occurs with alkaline extractions.

Martin and Haider (1976) found that [^{14}C] ferulic acid readily linked into humic acid-like model polymers and constituted 10–20% of the dry weight in their experiments. They also observed that when [^{14}C]ferulic acid was linked to model humic acid-like polymers, all the carbons (with the exception of methoxyl carbon) were stable. At the end of a 12-week period, 93–97% of the ring-labeled ^{14}C was still bound in the humic acid-type polymers.

In another experiment (Haider et al., 1977) in which ferulic acid was incorporated into model humic acid-like polymers and allowed to decompose for 28 weeks, only 6–8% of the ferulic acid carbons evolved as CO_2. This appears to indicate that the phenolic acid compounds are incorporated into humic substances, perhaps undergoing some side-chain transformations or modifications while retaining the ring structure. Once they become bound to the humic acid fraction, they are very stable, tightly bound, and not readily available to the soil solution. Upon degradation of the humic-type polymers by microorganisms or enzymes and subsequent release of simple phenolic acids into the soil solution, these compounds would then be extractable with the Na_2EDTA at pH 7.5. Alkaline soil extractions (Chou and Muller, 1972; Guenzi and McCalla, 1966; Lodhi, 1976; Rice, 1974, 1979) are thought to extract the total amount of the phenolic acids including the fraction forming part of the humified organic matter. Therefore it seems reasonable that a neutral extraction procedure, using water and a chelating agent (Na_2EDTA at pH 7.5) which displaces reversibly bound phenolic acids, estimates more closely the natural availability (active fraction) of phenolic acids in soil systems.

The work discussed in this paper is part of a larger project whose objective is to investigate the effects of soil factors (clays, oxides, organic

matter, pH, nutrients, and microbes), in modifying the plant availability of phytotoxins. We observed in preliminary experiments that phenolic acids added to our sterile soil controls were somehow irreversibly lost to the extraction procedures utilizing Na_2EDTA at pH 7.5 and that the loss increased with time. The objective of this research was to determine the importance of the physical factors and, more specifically, to determine the effectiveness of sterile soil components (clay minerals, Fe and Al hydroxides, and organic matter), at different pH values, in altering the bioavailability of ferulic acid (3-methoxy-4-hydroxycinnamic acid), a commonly identified phytotoxin found in soils (Geunzi and McCalla, 1966; Rasmussen and Rice, 1971; Wilson and Rice, 1968; Lodhi, 1978).

METHODS AND MATERIALS

Source and Preparation of Soil Components and Soils. Kaolinite (Georgia kaolin) was obtained from Theile Kaolin Company, Sanderville, Georgia. Montmorillonite (Utah bentonite) was obtained from the Department of Soil Science and Biometeorology, Utah State University. Both minerals were treated by dispersion and sedimentation (Jackson, 1969) yielding fractions less than 2 μm in effective particle diameter.

Reagent grade $Al(OH)_3$ was obtained from Baker Chemical Co. and was found by X-ray diffraction and chemical analysis to be gibbsite. Goethite (α-FeOOH) was prepared according to Golden (1978).

Cecil (clayey, kaolinitic, thermic Typic Hapludults) and White Store (fine, mixed, thermic Vertic Hapludalfs) soils were sampled in the Piedmont, and Portsmouth (fine loamy, mixed, thermic Typic Umbraquualts) soil was sampled in the coastal plain of North Carolina. Soil materials were air-dried and pass through a 0.250-mm sieve. Material not passing through the sieve without grinding was rejected.

All soils and components were autoclaved two times (121°C at 1.5 kg cm^2 for 15 min) with the second treatment coming 3 days after the first (Lopes and Wollum, 1976). One-tenth-gram samples of soil material in 15-ml tubes were suspended in 0.5 ml of sterile deionized H_2O for 48 hr prior to pH adjustment. With the exception of Utah bentonite, the pH adjustment was made with dilute NaOH and HCl. A portion of the Utah bentonite was saturated with Al^{3+} by leaching with 1.0 N $AlCl_3$. After removal of excess $AlCl_3$, the pH of the suspended clay was 5.0. Another portion was saturated with Ca^{2+} (1.0 N $CaCl_2$), resulting in a pH of 7.9. Aseptic conditions were maintained throughout the study. Samples were found to be sterile by plate count at the end of the 13-day period.

Analysis of Soil Components. Qualtitative mineralogical analyses of soil clays were determined (Table 1) by X-ray diffraction using CuK radiation. Standard treatments were employed (Jackson, 1969).

TABLE 1. MINERALOGY OF SOILS FROM MOST ABUNDANT (*****) TO LEAST ABUNDANT (*) AND TRACE AMOUNTS (+)[a]

	Kaolinite	Smectite	Hydroxy-interlayered vermiculite	Geothite	Gibbsite	Mica	Quartz	Surface area (m^2/g)
Cecil A_1	*****		***	*	**	***		4.0
Cecil A_2	*****		***[b]	*	**	**	**	4.0
Cecil B_1	*****		*	+	+	+	+	33.7
Portsmouth A_1	****	*****[c]				****	***	2.4
Portsmouth B_1[d]	****	*****[c]				****	***	8.2
White Store A_1	**	*****[e]		*		**	**	4.0
White Store A_2	**	*****[e]		*		**	*	9.5
White Store B_1	**	*****		*		**		35.8

[a]Qualtitative comparisons can only be made within soils and not between soils.
[b]Vermiculite/hydroxy-interlayered vermiculite.
[c]Smectite/hydroxy-interlayered smectite.
[d]Contains lepidocrocite (**).
[e]Some hydroxy-interlayering of smectite.

Organic carbon was quantitatively determined by acid dichromate digestion (Peech et al., 1947; Allison, 1965). Particle-size analysis was performed by the pipet method (Day, 1965). Surface area of soils (<60 mesh) and soil components was determined by N_2 adsorption using a Quantasorb Surface Area Analyzer (Quantachrome Corporation, Syosset, New York). Samples were outgassed at 110°C.

Addition and Recovery of Ferulic Acid. Adsorbent (0.1 g) and sterile deionized H_2O (0.5 ml) were mixed in 15-ml tubes and the pH adjusted with NaOH or HCl. One milliliter of ferulic acid (100 μg) was added to the pH-adjusted samples, and the tubes were subsequently capped. The pH of the ferulic acid solution was preadjusted to that of the substrate pH. All samples were mixed initially and daily thereafter using a vortex mixer. The pH of the soils was monitored daily and adjusted when necessary using 0.05 or 0.1 N HCl or NaOH.

Samples were extracted on days 1, 4, 7, 10, and 13, by adding 14 ml of Na_2EDTA (0.03 M at pH 7.5), mixing for 30 minutes, and centrifuging at 14,500*g* for 10 min. The supernatant solution was then passed through a 0.2 μm membrane filter. Ferulic acid was determined quantitatively with a Waters high-performance liquid chromatograph at 313 nm using a model 440 absorbance detector with a μBondapak C_{18} column. Gradient elutions, to produce separation of ferulic acid and its common breakdown products (caffeic, vanillic, protocatechuic, and gallic acids), were achieved with a model 660 solvent programer. The initial concentration was 90% of solvent A (10% methanol, 1% ethyl acetate, and 2% acetic acid) and 10% of solvent B (80% methanol, 18% water, 2% acetic acid). Final concentrations were 10% solvent A, 90% solvent B programed at 2.67% change per minute. A flow rate of 2.5 ml/min was used. The recovery of ferulic acid in the absence of soil material was approximately 99 ± 0.66 (mean ± standard error) %. The data were not adjusted for this loss.

Statistical Analysis. Data were analyzed using the Statistical Analysis System (SAS) programs for analysis of variance, linear and multiple regressions (Helwig and Council, 1979). First-, second-, and third-order polynomial equations were fitted to the data. Final model selection criteria were significance level ($\leq$0.05) and R^2 values.

RESULTS AND DISCUSSION

Recovery of Ferulic Acid from Soil Components. The recovery of ferulic acid in the EDTA solution was significantly reduced over time and varied with pH (Figure 1). The decline in recovery at pH 7.5 was linear for all components except gibbsite which exhibited an initial linear decline and then appeared to level off after day 10. At pH 4.5 the change in recovery over time for all

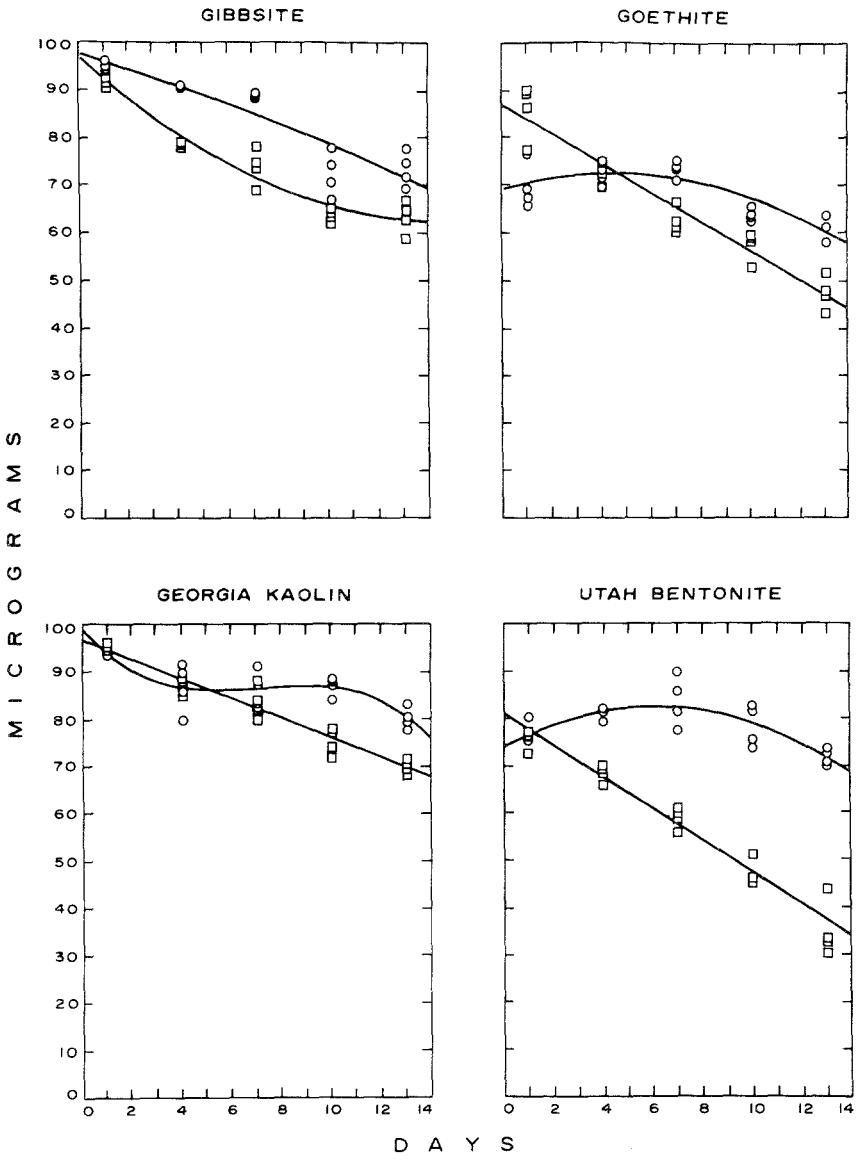


FIG. 1. Recovery of ferulic acid from soil components at pH 4.5 (○) and 7.5 (□) over time. The initial concentration of ferulic acid added to each soil sample was 1000 μg/g.

components, except Georgia kaolin, was described by a second-order polynomial. For Georgia kaolin, the decline in recovery was described by a third-order polynomial. There is no apparent explanation for the initial increase in recovery between day 1 and 7 for the goethite and Utah bentonite systems at pH 4.5. The mean difference in recovery at day 13, between pH 4.5 and 7.5, was approximately 10% for all soil components except Utah bentonite which had a mean difference of 36.65%.

Recovery data can be misleading when making comparisons between soil components since surface areas of the various components differ. The external surface area (N_2) of gibbsite, geothite, Georgia kaolin, and Utah bentonite was <1, 96, 24, and 97 m^2/g, respectively. The order of the soil components at day 13 from greatest to least when the results are expressed as grams of ferulic acid retained per square meter of adsorbent (pH 4.5) was gibbsite (266), Georgia kaolin (8.2), goethite (4.1), and Utah bentonite (2.9). At pH 7.5 the ranking was gibbsite (267.5), Georgia kaolin (12.4), Utah bentonite (6.7), and goethite (5.5). This ranking assumes that the entire N_2 surface of an adsorbent is active in retaining the ferulic acid, when, in fact, the active fraction may vary for each component. If account is taken of the fact that different amounts of ferulic acid were added per adsorption site, i.e., per unit area of surface for each adsorbent, the ranking changes. The amount of ferulic acid added per unit weight was 1000 $\mu g/g$ of adsorbent, which translates to 1000, 41.7, 10.4, and 10.3 g/m^2, respectively, for gibbsite, kaolinite, goethite, and Utah bentonite. The amount retained per unit area was directly related to the amount added per unit area, regardless of the adsorbent used. Thus, differences among the adsorbents appear to be related more to differences in specific surface and in amounts of ferulic acid added per unit area than to differences in retention mechanisms, although this cannot be fully evaluated with the present data.

The substantial difference in retention of ferulic acid by Utah bentonite between pH 5.0 and 7.9 may relate to the Ca^{2+} ion saturation. At pH 7.9 ferulic acid, in its anionic form, may conceivably be bonded through calcium via a cation bridge to the clay particles (Edwards and Bremmer, 1967). Under acid conditions, most of the ferulic acid would be nonionized. Ferulic acid molecules retained by bonding through a calcium bridge should be recovered by extraction with Na_2EDTA at pH 7.5. However, adsorption through an exchange ion will bring the organic molecules into closer proximinity to each other and to the clay surface, thus encouraging polymerization. Adsorption could then be considered as an intermediate step leading to irreversible retention. This would also explain the time dependence of the loss of ferulic acid. We have also observed (unpublished data) that Ca^{2+}-saturated soil material from a Cecil A_p horizon retained considerably more ferulic acid against extraction with water, NaOAC, or Na_2EDTA than the same material

TABLE 2. PARTIAL REGRESSION COEFFICIENTS AND R^2 VALUES FOR RECOVERY OF FERULIC ACID FROM SOIL COMPONENTS AND SOIL MATERIALS AT pH 4.5 AND 7.5[a]

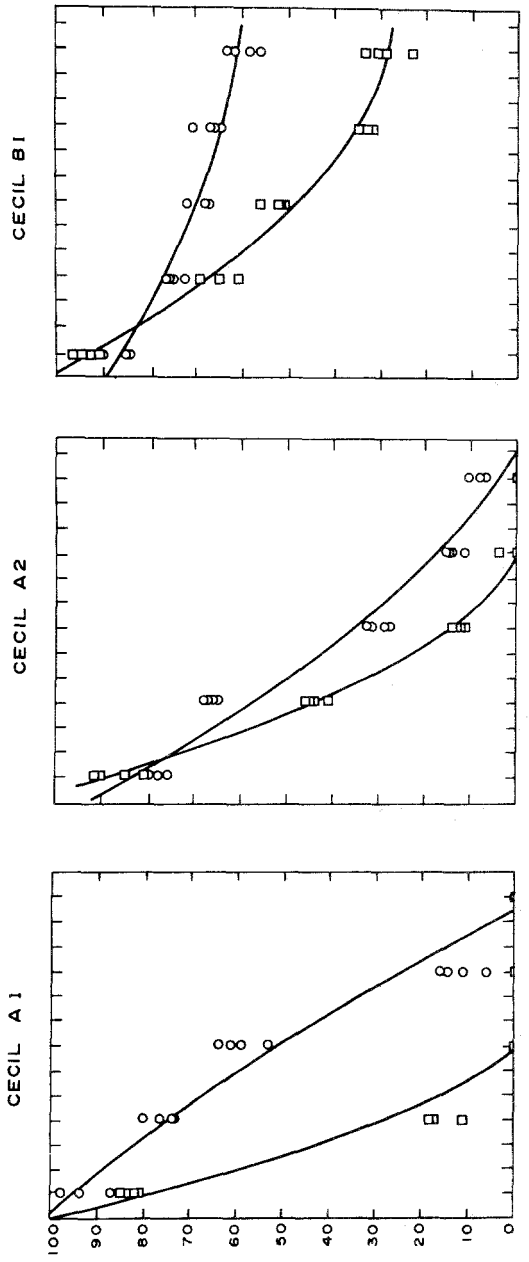
Soil	pH	Intercept	Linear	Quadratic	Cubic	R^2
Gibbsite	4.5	97.14	−1.57	−0.03		0.82
Gibbsite	7.5	96.17	−4.68	0.16		0.94
Goethite	4.5	68.75	1.48	−0.17		0.69
Goethite	7.5	86.34	−3.08			0.93
Georgia kaolin	4.5	98.84	−6.00	0.91	−0.04	0.69
Georgia kaolin	7.5	96.61	−2.05			0.94
Utah bentonite	4.5	73.90	2.78	−0.23		0.62
Utah bentonite	7.5	80.78	−3.38			0.95
Cecil A_1	4.5	101.34	−6.04	−0.16		0.95
Cecil A_1	7.5	99.29	−22.42	1.16		0.95
Cecil A_2	4.5	92.15	−9.78	0.23		0.96
Cecil A_2	7.5	105.09	−18.75	0.82		0.99
Cecil B_1	4.5	89.75	−3.61	0.11		0.91
Cecil B_1	7.5	102.43	−10.14	0.34		0.97
Portsmouth A_1	4.5	118.09	−24.12	3.45	−0.16	0.94
Portsmouth A_1	7.5	112.95	−26.14	1.38		0.94
Portsmouth B_1	4.5	97.01	−4.93			0.99
Portsmouth B_1	7.5	89.85	−13.08	0.47		0.98
White Store A_1	4.5	82.23	−12.08	0.44		0.98
White Store A_1	7.5	99.41	−19.62	0.93		0.99
White Store A_2	4.5	95.40	−5.64			0.99
White Store A_2	7.5	106.97	−17.54	0.71		0.99
White Store B_1	4.5	100.76	−7.19	0.32		0.90
White Store B_1	7.5	105.29	−17.02	2.27	−0.09	0.95

[a]The initial concentration of ferulic acid added to the soil samples was 1000 μg/g. All soil material models were significant at the 0.001 level. All soil component models were significant at <0.003. $N = 4$.

saturated with K^+. While the mechanism of retention has not been specifically identified, it is apparent that the exchange cation has an effect.

Recovery of Ferulic Acid from Soils. The recovery of ferulic acid from the soil materials decreased significantly over time and varied with pH. Partial regression coefficients and R^2 values of models and recovery data for soils at pH 4.5 and 7.5 on which models were based are given in Table 2 and in Figures 2 and 3. The irreversible disappearance of ferulic acid was more rapid at pH 7.5 than at pH 4.5, similar to the results obtained for the soil components.

Surface areas for the various soil materials were determined by the N_2 adsorption technique (Table 1). This method measures the external surface area of the mineral fraction which represents the total surface area if no expansive minerals, e.g., montmorillonite and vermiculite, are present.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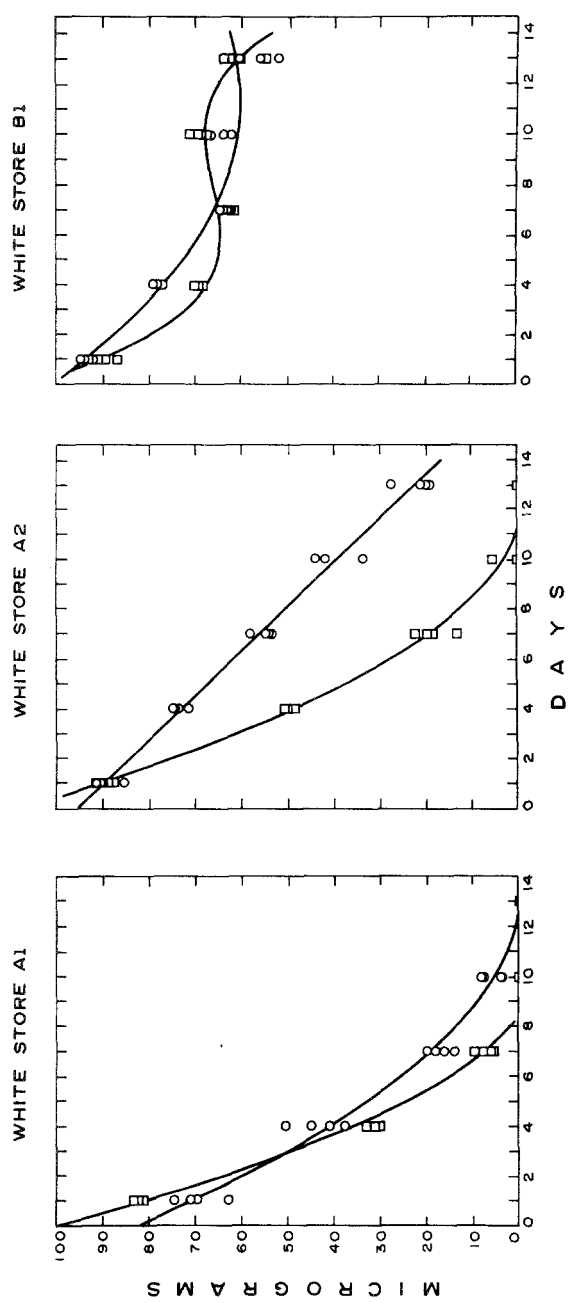

FIG. 2. The recovery of ferulic acid from soil materials taken from different horizons within the same Cecil and White Store profile (pH 4.5 = ○ , pH 7.5 = □). The initial concentration of ferulic acid added to the soil samples was 1000 μg/g.

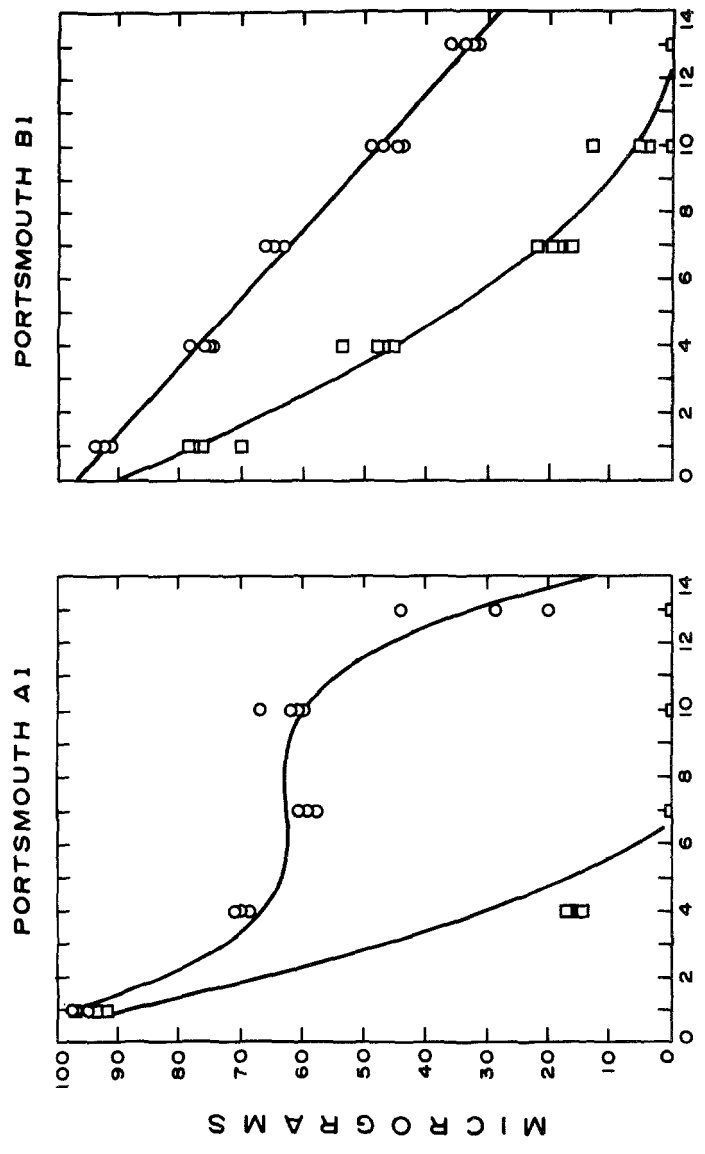

FIG. 3. The recovery of ferulic acid from soil materials taken from different horizons within the same Portsmouth soil profile (pH 4.5 = ○, pH 7.5 = □). The initial concentration of ferulic acid added to the soil samples was 1000 μg/g.

However, it measures only a small fraction of the total or hydratable surface of humic material. Hence, N_2 surface area is not an effective measure of the potential of a soil to retain ferulic acid unless organic matter is not present. The increase in surface area from the A to B horizons of the soil materials (Table 1) reflects the increase in clay (Table 3). Quantitative clay mineralogical analyses were not available for the soil materials used in this study, but equations were derived for organic matter content versus recovery of ferulic acid. Lines described by these equations are presented in Figure 4.

Less ferulic acid was recovered from topsoils than from subsoils, suggesting that organic matter, which decreased with soil depth (Table 3), was the most active component in the irreversible retention of ferulic acid. Regardless of the amount of organic matter, a greater loss of ferulic acid occurred at pH 7.5 than at 4.5.

Martin and Haider (1979) reported that ring-labeled [^{14}C]ferulic acid was highly stable in alkaline soil (very little release of $^{14}CO_2$). In acid soil carbon loss was over 80% within 12 weeks at all concentrations for both ring [^{14}C]benzoic and ferulic acids. They concluded that in the acid soil material essentially none of the ring structures were stabilized in organic complexes, and microorganisms apparently utilized the free monomeric benzoic and ferulic acid molecules. Our work differs from that of Martin and Haider in that no microorganisms were present to degrade the free ferulic acid molecules and, therefore, the stabilization process (irreversible retention) was allowed to proceed uninterrupted for the 13-day period. Although the loss of ferulic acid was generally greater in the alkaline than in the acid systems the difference ranged from essentially none in the case of White Store B_1 horizon (Figure 2), 10–20% in Cecil A_2 and White Store A_2 horizons, to as much as 60–70% difference in Cecil A_1 and Portsmouth A_1 horizons. Comparison between the

TABLE 3. PARTICLE-SIZE ANALYSIS, ORGANIC MATTER CONTENT, AND pH OF SOIL MATERIALS

	Particle-size analysis			Organic matter (%)	pH
	% Sand	% Silt	% Clay		
Cecil A_1	66	29	5	3.7	6.2
Cecil A_2	66	24	9	1.5	6.0
Cecil B_1	17	23	60	0.2	5.4
Portsmouth A_1	61	28	11	3.9	6.6
Portsmouth B_1	45	36	20	0.07	4.85
Whitestore A_1	45	47	9	2.4	5.2
Whitestore A_2	37	45	18	0.67	4.95
Whitestore B_1	12	38	49	0.34	4.65

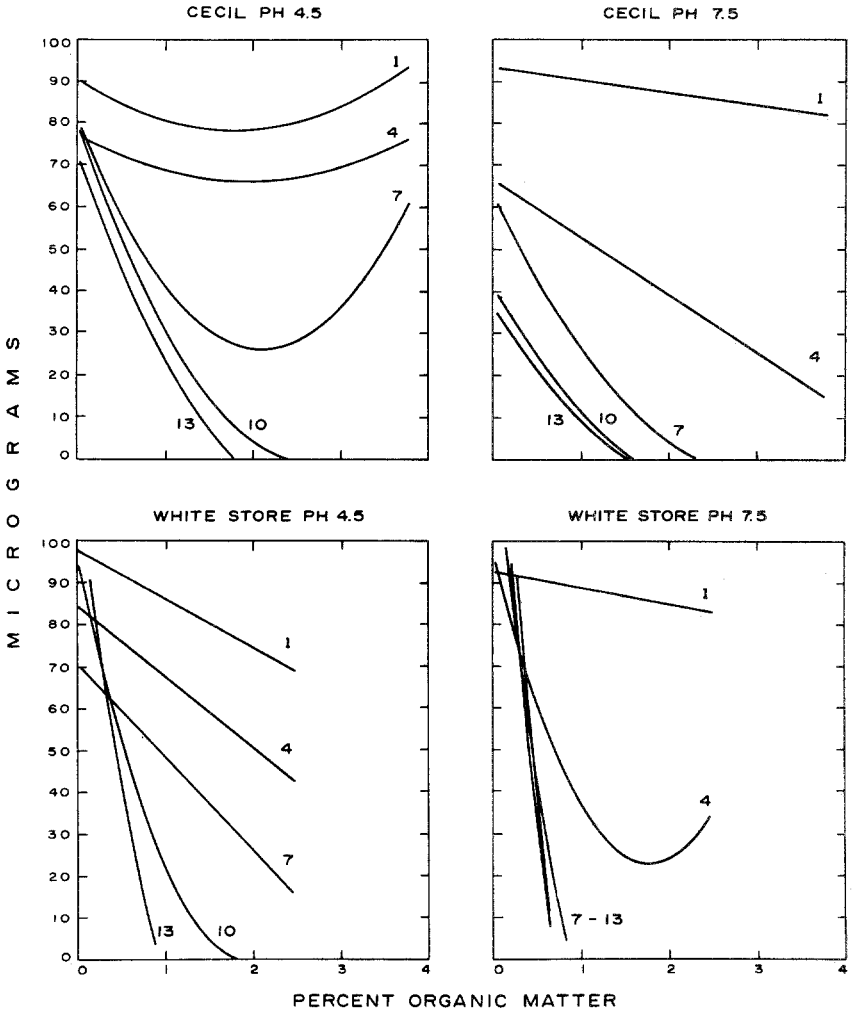

FIG. 4. Relationships of the recovery of ferulic acid to organic matter content of materials from Cecil and White Store soils at pH 4.5 and 7.5 at various time intervals. The initial concentration of ferulic acid added was 1000 μg/g. Percent organic matter of Cecil A_1, A_2, and B_1 was 3.7, 1.5 and 0.2, respectively. Percent organic matter of White Store A_1, A_2, and B_1 was 2.4, 0.67, and 0.34, respectively. Numbers in figures represent days.

pure soil components and the soil materials indicates that organic matter is much more effective than the inorganic fraction in the irreversible retention of ferulic acid.

Martin and Haider (1976) concluded that the stabilization of the simple phenolic compounds used in their experiments was due to the presence of phenolases and perioxidases in the soil and/or microbial metabolism and incorporation of these units into melanins of phenolic polymers (autoxidative polymerization). Since our work was conducted in sterile systems, the involvement of enzymes in degradation and/or polymerization is unlikely. In addition, very little degradation of ferulic acid was observed in pure solution, i.e., in the absence of soil material, and no breakdown products were found in any of the soil extracts. Therefore, we conclude that the irreversible retention of ferulic acid, whether a stabilization by polymerization or bonding to soil particles, may occur in an abiotic environment. Whatever the mechanism(s) involved in the disappearance of ferulic acid, it is evident that the majority of the loss is not instantaneous when the compound is added to the sterile soil materials and that it is greatly enhanced in the presence of organic matter, especially at slightly alkaline conditions. Our research clearly indicates the importance of abiotic processes in the irreversible retention of ferulic acid in soils. It seems logical to assume that these physical processes are operating simultaneously with biological factors in the soil environment in reducing the concentrations of plant phenolic compounds that can interact with the biota. The extent of the reduction in availability by physical factors in a nonsterile soil system is not known at this time.

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SEASONAL CHANGES OF JUGLONE POTENTIAL IN LEAVES OF BLACK WALNUT (*Juglans nigra* L.)[1]

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Abstract—The literature concerning the extraction and quantification of juglone (5-hydroxy-1,4-naphthoquinone) frequently fails to differentiate between juglone and its immediate precursors. The proposal that the term "juglone potential" be used to define the total concentration of juglone under an oxidative environment in walnut tissues is made. The juglone potential of a given tissue will more accurately reflect the allelopathic agent's presence than will juglone concentration of the tissue alone. The term juglone potential accepts the multiple chemical nature of juglone found in walnut tissue and gives a precise way for quantification of allelopathic action. The objective of this study was to measure seasonal changes in black walnut juglone potentials at various locations in the crown. Juglone potentials were measured weekly in leaves at the top, middle, and bottom of the tree crowns. The results showed a linear decrease in juglone potential over the growing season. The results also showed no significant difference in leaf juglone potentials among the three crown positions. If reanalyzed without the middle crown position juglone potentials were significantly greater in the lower crown positions when compared with the higher crown position. Potential use of juglone to manipulate species composition and decrease interference in plant communities is discussed.

Key Words—Allelopathy, *Juglans nigra*, juglone, 5-hydroxy-1,4-naphthoquinone, physioecology, kolines, phytotoxins, juglone potential.

INTRODUCTION

The biological system of a tree incorporates the economics of nutrients, water, light, and metabolites to control health and growth. Additionally, the tree

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must adapt to the presence of other organisms and to the site. The problem of health and growth then becomes one of space, competition for physiological inputs, and the chemical coadaptation to other organisms and to the site.

The chemical coadaptation to other organisms and the site is termed allelopathy (Rice, 1974, 1979). One of the most noticeable allelopathic effects in silviculture occurs in black walnut (*Juglans nigra* L.) associations. As early as 77 AD, the *Juglans* genus was cited as having a poisonous effect on other plants. Black walnut was first mentioned as having an inhibitory effect on other vegetation by Hoy and Stickney (1881). Numerous authors have since reported inhibition of plant growth in close association with black walnut. The active agent in growth inhibition was suggested (Massey, 1925), then confirmed (Davis, 1928), to be juglone (5-hydroxy-1,4-naphthoquinone). Juglone's effect on plant tissue is to inhibit hydrogen ion transfer in respiration (Perry, 1967) and inhibit oxygen uptake at two loci. Low concentrations ($<3\ \mu$M) of juglone in the plant bypass steps in the electron transport system. Higher concentrations of juglone increasingly inhibited intermediates of oxidative phosphorylation (Koppe, 1972).

Roles of Juglone. The physiological significance of juglone in the tree is probably of a protective nature. Juglone's immediate precursors (Figure 1) are found in high concentrations in bud scales, flowers, fruit exocarps, and in the phloem. Juglone's precursors are concentrated in tissues that are preparing to undergo a rapid growth phase and seem to be translocated from older tissue to younger tissue. Upon oxidative conditions, such as when tissue is damaged, juglone is formed (Daglish, 1950b).

Juglone is found only in minute amounts in walnut tissue because of its toxicity (Gries, 1943, Langhans et al., 1978). Juglone is compartmentalized in

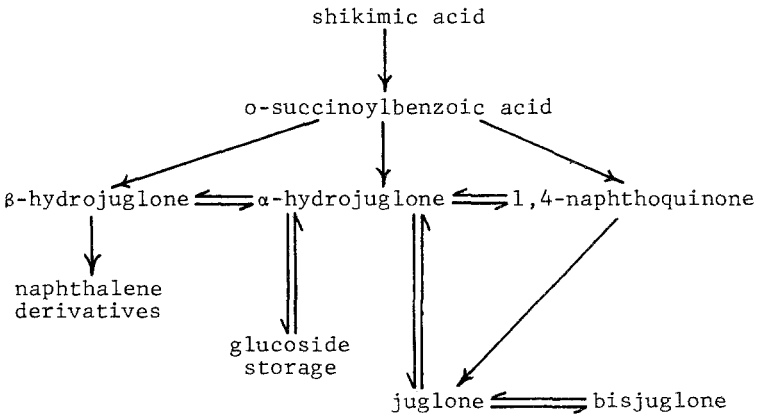

FIG. 1. Metabolic synthesis "net" leading to juglone and close chemical associates in *Juglans* species. (Muller and Leistner, 1978, 1976; Pardhasaradhi and Babu, 1978).

its reduced forms, principally α-hydrojuglone (1,4,5-trihydroxynaphthalene) (Hedin et al., 1980) and in a bound state with glucose (Daglish, 1950a, Langhans et al., 1978). Hydrojuglone is colorless and generally nontoxic, but is immediately converted to juglone by oxidizing agents. Upon continual contact with oxidative conditions, juglone is decomposed (Gries, 1943). Plant injury can cause a conversion of hydrojuglone to juglone in the affected region by providing an oxidative environment (Hedin et al., 1980).

The ecological significance of juglone requires definition and much further study. Many arguments remain in approaching the synecology of black walnut and the role of juglone. One problem in viewing juglone within and between plants is in the active versus storage form of the substance. Juglone is the active form but is found in low concentrations in walnut tissue. Juglone precursors are found in relatively large concentrations in tissue, but are ecologically inactive. The metabolic "net" of juglone synthesis is given in Figure 1. The chemical compounds involved in juglone metabolism and their equilibrium states suggest that measurement of juglone concentration in tissue may not be an accurate method of gauging the ecological significance of juglone to a particular site. Instead, the total amount of potentially active material must be assessed. Because juglone precursors move toward juglone in an oxidative environment, determination of juglone availability in the walnut tissue under oxidative conditions will provide a relative ecological input level for this allelopathic agent. Instead of determining only juglone concentration in tissue, a "juglone potential" for black walnut tissue should be developed. Juglone precursor concentrations become meaningful only in respect to the additional juglone that would occur if the tissue is put under oxidative conditions, as in leaf fall or injury. The term "juglone potential" accepts the multiple chemical nature of juglone in the plant tissue and the amount of ecologically active substance potentially available for addition to a site.

The purpose of this study was to measure the relative levels of juglone potential in the leaves at various positions in the crown over the growing season. This information should allow for a better description of the synthesis, transport, and elimination of juglone in black walnut over time, as well as for a better ecological definition of juglone.

METHODS AND MATERIALS

Four trees, systematically located in a black walnut phantation near Ames, Iowa, were sampled. The plantation was established by the USDA Forest Service in 1964 from wild seed. The sample trees were approximately 5 in. in diameter at 4.5 ft above the ground and 23 ft tall, with a spacing of 12 ft between trees. All sample trees were not closer than six rows from the plantation edge, and none appeared phenotypically atypical relative to other plantation trees.

Leaves in the sample trees were divided into three relative positions: high, medium, and low. High leaves were selected as being in the highest 33% of the live crown and exposed to direct sunlight for a substantial portion of the day. Medium leaves were in the middle 33% of the live crown and received occasional direct sunlight. Low leaves were in the lowest 33% of the crown and received little direct sunlight. Leaves were sampled systematically within each crown position.

Trees were sampled once a week at the three crown positions. Sampling began 2 weeks after bud-burst when the leaves had expanded enough to provide an adequate sample. Sampling ceased 12 weeks later when lower leaves on the sample trees senesced and fell. Each sample was composed of 10–25 g (fresh weight) of whole leaves. The leaves were stored in the field in high-humidity crispers away from light. The crispers were returned to the laboratory and stored in a refrigerator at 4° C until extraction could be completed. A 4-g subsample was taken from each sample and oven-dried at 65° C to determine moisture content.

The juglone extraction technique followed that of Hedin et al. (1979). The accuracy and precision of the extraction technique was determined by repeatedly extracting the same leaf sample until no measurable juglone could be extracted. The technique was found to remove 81% of the total extractable juglone in the leaf tissue after one extraction. After the fourth extraction, no measurable juglone could be removed. Because results were to be developed on a relative basis, all samples of leaf material were extracted once.

Each sample was ground in a blender for 45 sec with 75 ml of 2:1 chloroform–methanol mixture. The blender container sides were then brushed down, and the sample was ground for additional 30 sec. The homogenate then was filtered. An aliquot of 1 ml was taken from the filtrate with a micropipet and lined out on a thin-layer chromotography plate with a silica gel medium. The carrier solution used in chromotography was a 1:2 mixture of methylene chloride and hexane.

After 10 min in the chromotography chamber, six distinct visible bands were resolved. Each band was resuspended in 10 ml of the original extraction mixture and filtered, and the absorbance peak or peaks were determined for each band over the range of 340–700 nm by using a spectrophotometer. Only one band showed the single absorbance peak at 420 nm characteristic of juglone in chloroform–methanol. As an additional test, analytical-grade juglone (99.8% purity from Aldrich Chemical Co., 940 West Saint Paul Avenue, Milwaukee, Wisconsin) was lined onto a chromotography plate parallel to the extracted leaf filtrate and resolved by using the same procedure. The analytical juglone was carried on the chromotography plate to the same reference point as the extracted juglone band and was of similar color. In addition, the spectral absorbance curve of the juglone band from the extract and that of analytical juglone were identical. All evidence indicated that

juglone was being extracted and isolated from black walnut leaf material. All samples then were extracted by using this laboratory procedure.

The juglone band from each sample was removed from the chromotography plate and resuspended in 10 ml of the original chloroform–methanol extraction mixture. The solution then was filtered to remove the chromotography medium. The absorbancy of the sample was determined at 420 nm in a spectrophotometer. The absorbance of the sample was compared with the absorbance of analytical juglone at known concentrations to determine the amount of juglone present in the sample.

The standard absorbance curve developed by using analytical juglone provided a basis for quantification of extracted juglone. The absorbance of analytical juglone was taken at 13 concentrations ranging from 0.48 ppm to 54.88 ppm. The regression curve was defined by: juglone ppm = −(absorbance) + (0.0033/0.0219); (r = 0.999). The data were analyzed as a splitplot design after Steel and Torrie (1980).

RESULTS AND DISCUSSION

Juglone potential in black walnut leaves decreased linearly over the growing season (Table 1 and Figure 2). Juglone potential differences between the three leaf positions were nonsignificant at the 95% probability level. If the medium leaf position was removed from the analysis, juglone potentials were significantly greater in the lower leaves than in the higher leaf position (Table 2 and Figure 3). The expected role of sunlight, which directs the synthesis process of juglone, showed an inversely proportional relationship with height in the live crown. Less photon-induced decay, less efficient photosynthesis, or dark reaction cycles may increase the juglone potential of the leaves in the lower crown.

TABLE 1. ANOVA TABLE FOR JUGLONE POTENTIAL (OVEN-DRY BASIS) IN BLACK WALNUT (*Juglans nigra* L.) LEAVES AT THREE CROWN POSITIONS OVER THE GROWING SEASON[a]

Source	*df*	MS	Pr > F
Tree	3	0.0254	0.0001
Date	12	0.0146	0.0001
Lin	(1)	(0.1502)	(0.0001)
Lof	(11)	(0.0023)	(0.8906)
Error a	36	0.0048	
Position	2	0.0058	0.0684
Date*position	24	0.0016	0.7723
Error b	76	0.0021	

[a] MS = mean square; Pr > F = probability of a greater F-value; Lin = linear effect; Lof = lack-of-fit.

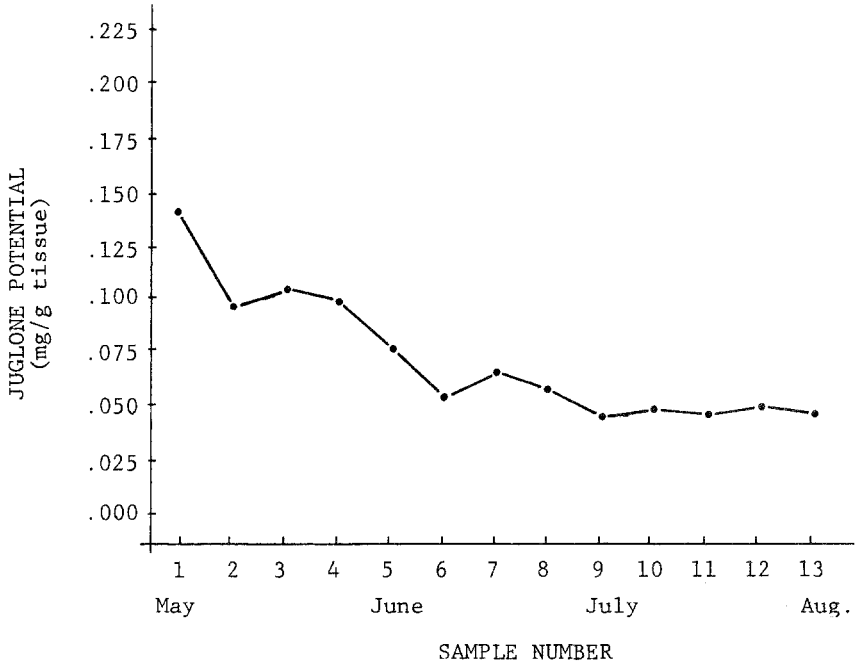

FIG. 2. Graph of juglone potential (oven-dry basis) in black walnut (*Juglans nigra* L.) leaves over the growing season.

Juglone concentrations usually are reported on either a green-weight or oven-dry-weight basis. Analysis of these data indicate that green-weight basis is not as precise in the measurement of juglone concentrations, or of juglone potential, in walnut leaves as oven-dry-weight basis (Table 3). Use of green-weight basis can lead to misinterpretation of data because of moisture-content differences.

TABLE 2. ANOVA TABLE FOR JUGLONE POTENTIAL (OVEN-DRY BASIS) IN BLACK WALNUT (*Juglans nigra* L.) LEAVES AT HIGH AND LOW CROWN POSITIONS OVER THE GROWING SEASON

Source	*df*	MS	Pr > F
Tree	3	0.0241	0.0001
Date	12	0.0070	0.0001
Position	1	0.0082	0.0139
Date*position	12	0.0017	0.1990
Error	37	0.0012	

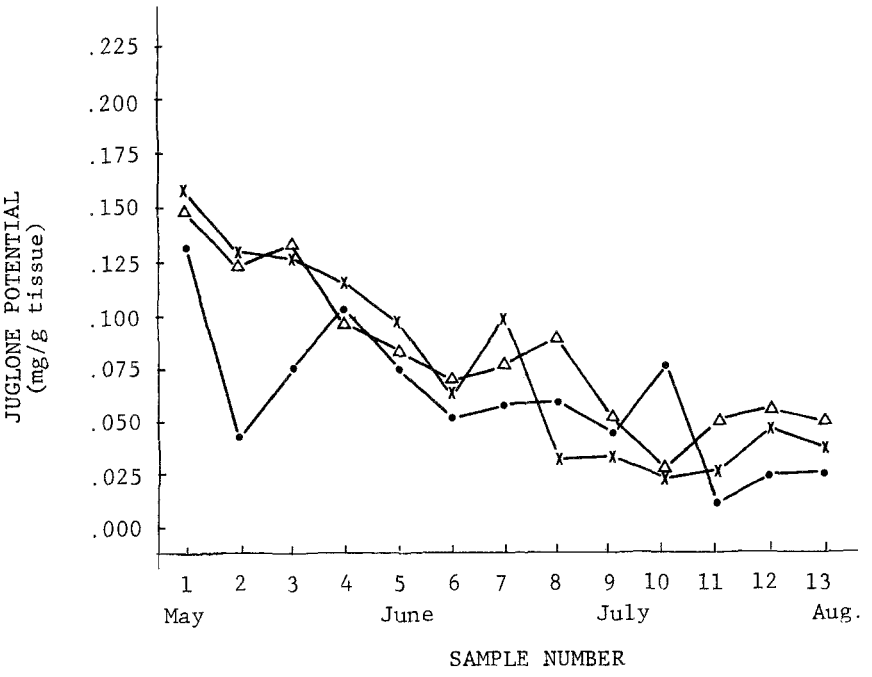

FIG 3. Graph of juglone potential (oven-dry basis) in black walnut (*Juglans nigra* L.) leaves at three positions in the crown over the growing season. Leaves in high (Δ), medium (x), and low (●) crown positions.

TABLE 3. ANOVA TABLE FOR JUGLONE POTENTIAL (GREEN-WEIGHT BASIS) IN BLACK WALNUT (*Juglans nigra* L.) LEAVES AT THREE CROWN POSITIONS OVER THE GROWING SEASON

Source	*df*	MS	Pr > F
Tree	3	0.0022	0.0001
Date	12	0.0005	0.0002
Lin	(1)	(0.0051)	(0.0003)
Lof	(11)	(0.0001)	(0.9703)
Error a	36	0.0003	
Position	2	0.0006	0.0151
Date*position	24	0.0001	0.6515
Error b	76	0.0001	

These data tentatively suggest that fall or winter harvesting would add the least amount of juglone to a site because of its natural breakdown and/or containment in the leaf. If walnut is intercropped with another species where continual high juglone potentials might be a problem, walnut harvesting after leaf fall will minimize juglone influx on the site. Alternatively, if reestablishment of walnut is desired, harvesting in early summer might maximize juglone influx to the site and help walnut seedlings interfere with other species better. Harvesting will always provide a large pulse of juglone to a site due to slash and root decay.

Integrating this study with the literature on walnuts and juglone, and practical experience in walnut silvics, suggest several areas for practical use and further investigation. The areas are herbicides, vectors of affect, species adaptation, and microflora populations with each area realted to the toxic effect of juglone on other organisms. From this synthesis of information, some conclusions can be drawn regarding the ecological significant of juglone.

Juglone has a great herbicidal potential because of its vegetation-controlling characteristics. In a plantation suitation, young walnuts usually require herbicide assistance to interfere with forbs and grasses. Pretreatment and periodic application of juglone to walnut sites might provide control of specific species and assist in establishing walnut seedlings. In an urban setting, Kentucky bluegrass lawns might be maintained relatively weed free because of this species' affinity for sites with high juglone potentials (Brooks, 1951). The use of naturally occurring toxins as herbicides is ideal because of the specificity of action, presence of decomposition processes in the soil providing a short active life and, in some cases, ease of production. Instead of producing a near-total elimination of unwanted species on the site, natural ecological processes can be magnified by chemically stressing unwanted species, thereby promoting crop-species growth. This is the action of juglone on a forested site.

The vectors of the toxic effect of black walnut on other vegetation are the fruit, leaves, and roots. The leakage from the root symplast of hydrojuglone and its oxidation to juglone produces a walnut rhizosphere that is highly antagonistic to some plant root systems. Close association with walnut roots by juglone-sensitive species probably causes respiration interference, changes in membrane permeability, and inhibition of elongation. The wilting symptom found in some juglone-sensitive plants may involve the slowed movement of water across the root to the vascular tissue and/or the disruption of hormonal control mechanisms. Walnut leaf litter decomposes quickly, producing an organic layer with a yearly influx of juglone. Walnut fruit, which have large juglone potentials, may substantially alter a site chemically upon abscission.

Rain drip from the crown may further chemically antagonize other species by polluting rainfall with minute amounts of juglone. Although juglone is only slightly soluble in water, the toxicity symptoms appear at low concentrations. Those species that grow in the presence of walnut roots still

have difficulty surviving under the drip of the tree, suggesting that rhizosphere adaptation systems of walnut-associated species depend upon the avoidance of toxin or biological negation of its effects. Biological negation might be a possibility because of the tree species found associated with walnut (Brooks, 1951); a majority of those are themselves allelopathic. Species with strong chemical interference components seem to grow well together because of the maintenance of a specific rhizosphere environment around their roots.

Macroflora speciation and distribution in the walnut stand has been shown to be greatly affected by juglone. More study is needed concerning microflora dynamics in the root zone. The evolutionary pressure on species associated with walnut to adapt to the presence of juglone needs further definition. Mycorrhizal fungi and rhizosphere-inhabiting microbes may be highly adapted to walnut trees and the presence of juglone (Khan, 1966). In the forest, as each tree species chemically alters the site to increase the potential for interference, the sphere of chemical influence around the tree will affect the species present and their population dynamics. As the tree varies in its chemical nature over its range, so will the macrobial and microbial populations found associated with it. Out of ecological necessity, the greater the chemical coadaptation component of phenotype a tree species possesses, the more specialized will be the species associated with it.

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STRESS MODIFICATION OF ALLELOPATHY OF *Helianthus annuus* L. DEBRIS ON SEEDLING BIOMASS PRODUCTION OF *Amaranthus retroflexus* L.[1]

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Abstract—When added to soil, *Helianthus annuus* debris from plants grown under various nutrient stresses in the greenhouse and field had a significant negative effect on *Amaranthus retroflexus* seedling dry weight production. The regression models for dry weight versus total phenolic compounds and the variation in N, P, and K contents of the *Helianthus* debris were significant. It was possible to partially simulate debris inhibition of *A. retroflexus* seedling dry weight production when chlorogenic acid alone was added to the soil instead of *Helianthus* debris. The inhibition of *Amaranthus* growth by *Helianthus* debris and chlorogenic acid was not evident when nutrient solution was applied to the soil.

Key Words—*Helianthus annuus, Amaranthus retroflexus*, allelopathy, chlorogenic acid, seedling growth, plant debris, stress.

INTRODUCTION

It has been shown that the addition of *Helianthus annuus* L. debris, obtained from plants grown under different nutrient regimes in the greenhouse and field, significantly inhibited seed germination of *Amaranthus retroflexus* L. (Hall et al., 1982). Seed germination was found to be correlated more closely with total phenolics added to the soil than with any other variable measured. This observation confirmed a long-held postulate that environmentally

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induced changes of phenolic compounds in plants can modify allelopathic interactions (Rice, 1974). The present report is an extension of the germination study by Hall et al. (1982) The primary objectives of this research were: (1) to determine how plant debris obtained from plants grown under different nutrient regimes affected seedling dry matter production; (2) to determine if any modifications of seedling dry weight could be correlated with the total phenolic compounds and N, P, and K contents of the debris; and (3) to determine how the nutrient status of the soil influenced seedling dry weight in the presence of plant debris or chlorogenic acid, a phenolic compound found in *Helianthus* debris.

METHODS AND MATERIALS

Debris Bioassay. Lyophilized whole plant material from field and greenhouse-grown *H. annuus* plants was coarsely ground with a Waring blender and added to 11-cm (500-cc) plastic pots containing a 2:1 soil-sand mixture with the following initial conditions: pH = 5.6, NO_3^- = 0 mg/dm^3, NH_4^+ = 28 mg/dm^3, P = 46 mg/dm^3, extractable K = 53 mE/100 cc, and 1.0% organic matter content. The phenolic and nutrient contents of the debris types used are presented in Table 1. Debris was obtained from plants grown in

TABLE 1. PHENOLIC AND NUTRIENT CONTENT OF *H. annuus* DEBRIS USED IN DEBRIS-BIOASSAY STUDY

Debris number and source	Phenolic content (mg/g)	Percent (per g)						Amount (μg/g)				
		N	P	K	Ca	Mg	Na	Fe	Mn	Zn	Cu	B
Greenhouse[a]												
1. Full-strength[b]	32.48	2.87	0.36	1.90	1.65	0.44	0.11	410	50	47	5	56
2. $\frac{1}{2}$	46.44	2.06	0.24	1.55	1.20	0.36	0.12	460	67	47	7	46
3. $\frac{1}{4}$	40.36	2.16	0.20	1.60	1.15	0.35	0.10	415	96	58	7	49
Field[a]												
4. Full-strength[b]	52.01	2.35	0.25	4.50	1.30	0.44	0.21	420	78	71	9	10
5. $\frac{1}{2}$	68.46	1.88	0.20	4.25	1.20	0.42	0.17	420	98	86	10	49
6. $\frac{1}{4}$	79.23	1.34	0.15	2.90	1.25	0.40	0.14	405	140	88	10	56
7. Field[c]	18.92	0.74	0.07	3.92	1.18	0.36	0.06	344	56	58	12	43

[a] Debris treatments from each nutrient-density study were combined within each of the nutrient treatments to produce the debris used in the debris-bioassay study. Whole lyophilized tissues from the various experiments were stored at 20C in the dark until bioassay was run. Maximum storage for tissue was one year.

[b] Nutrient treatments (Hoagland's solution; Hoagland and Arnon, 1938) under which the *H. annuus* plants were grown in their respective experiments.

[c] Plants from local garden. Low phenol content of this tissue was due to poor condition of plants at time of harvest.

sand culture supplied with various strengths of Hoaglad's solution (Hoagland and Arnon, 1938). For complete details on how debris was generated, see Hall et al. (1982). Two levels of debris, 2.86 and 7.14 mg debris/g of soil were thoroughly mixed into the upper half of the soil–sand mixture. These levels were equivalent to 2 and 5 g debris/700 g of soil. In addition a control soil was included to which no debris was added. Half the pots received 30 ml of full-strength Hoagland's solution (Hoagland and Arnon, 1938) weekly while the others received only deionized water. All bioassays were carried out in the greenhouse, and all pots were supplemented with deionized water as needed. In June 1979, thirty *A. retroflexus* seeds, which had received a cold treatment of 10° C for a month, were sown per pot and covered lightly with the soil mixture. Seedlings were thinned to 5 per pot after 14 days and plants were harvested 4 weeks later. Average daily light intensity for the greenhouse was 2552 W/m^2. Each treatment (7 debris types × 2 debris levels × 2 nutrient levels) was replicated 5 times.

Chlorogenic Acid Bioassay. Four different chlorogenic acid concentrations (100, 200, 400, 800 g/g soil) were incorporated into the upper half of the soil–sand mixture in 11-cm (500-cc) plastic pots. One series of pots received no chlorogenic acid and served as controls. The initial conditions of the soil–sand mixture were: pH = 5.4, NO_3^- = 10 mg/dm^3, NH_4^+ = 22 mg/dm^3, P = 9 mg/dm^3, extractable K = 0.08 mE/100 cc, and 1.4% organic matter. Half the above pots also received 30 ml of an NPK nutrient solution at the onset of the study. Thirty milliliters of the solution supplied 50.4 mg N in the form of NO_3^-, 140.7 mg K as KNO_3, and 5.3 mg P as HPO_3. This NPK solution was designed to mimic the amounts of N, P, and K in the *H. annuus* debris (2.86 mg) used in the previous bioassay. All pots were placed in the greenhouse and received deionized water as needed. Twenty-five seeds of *A. retroflexus*, which had received a cold treatment of 10° C for a month, were sown in each pot in March 1979. Seedlings were thinned to 5 per pot after 14 days and plants were harvested 5 weeks later. There were 5 replicates for each treatment (5 chlorogenic acid concentrations × 2 nutrient levels).

Soil Analyses. Soil analyses were performed by the North Carolina Department of Agriculture Soil Testing Laboratory. Soil samples were extracted with 0.05 N HCl and 0.025 N H_2SO_4 to determine available amounts of NO_3^-, NH_4^+, P, and K. Soil pH in water (1 : 1, v/v) and percent organic matter were determined by standard procedures.

Plant Nutrient Analyses. Dried plant material was ground in a mill (40 mesh) before plant analysis was performed. Nutrient analyses were performed by the North Carolina Department of Agriculture, Agronomic Division Plant Analysis Section. Total nitrogen was determined by a distillation method using Devarda's alloy after the tissue was wet ashed (Bremner and Kenney, 1964). All other elements were determined on dry-ashed plant samples. Phosphorus was determined using the ammonium molybdate–

ascorbic acid colorimetric method. Ca, Mg, Na, Fe, Mn, Zn, and Cu were determined by atomic absorption spectrophotometry. Potassium and Na were determined by a flame photometer using lithium as an internal standard. Boron was determined by a modified curcumin method (Grinstead and Snider, 1967).

Plant Phenolic Analyses. The polyvinylpyrrolidone (PVP) method was used to determined total phenolic compounds in plant tissue (Andersen and Todd, 1968). Total phenolic compounds are expressed as chlorogenic acid equivalents by this method. Lyophilized, ground (40 mesh) plant material was used for all determinations.

Data Analysis. Data were analyzed using the Statistical Analysis System (SAS) programs for analysis of variance, least significant difference (LSD), linear regression, and multiple regression (Barr et al., 1976). Regressions were based on means of replicated observations at each level of the independent variable.

RESULTS AND DISCUSSION

Debris Bioassay. Total phenolics alone did not account for a significant amount of the variability found in *A. retroflexus* plant weight (Table 2) unless N, P, and K contents of the debris were included in the model:

$$\text{Plant weight (mg)} = 121.37 - 0.19\ (\text{phenol}) - 2.81\ (\text{N}) + 26.10\ (\text{P}) + 0.21\ (\text{K})$$

$$(r^2 = 0.89, \quad \alpha = 0.0001)$$

where phenol represents total phenolics, N represents total nitrogen, P represents total phosphorus, and K represents total potassium (all units in μg/g soil) added to the soil by *H. annuus* debris. Total N added to the soil by the debris was correlated negatively with plant dry weight, whereas P and K were positively correlated. The negative correlation of N with dry weight may have been due to: (1) a nitrogenous inhibitor, (2) nitrogen in a predominantly unavailable form that was closely correlated with phenolic content, (3) rapid microbial utilization of nitrogen with an associated release of inhibitors or conversion of nontoxic substances to more toxic constituents, and/or (4) competition for N between the plants and soil microbes (Harper, 1977). The relationship between inhibition of seed germination and debris phenolic content was stronger than the dry weight/phenolic relationships since up to 86% of the model variation in germination could be accounted for by the concentration of phenolic compounds in the debris (Hall et al., 1982). For the dry weight/phenolic relationships, total phenolics alone did not account for a significant amount of the variability found in *A. retroflexus* plant weight unless, N, P, and K content of the debris were included.

TABLE 2. *Amaranthus retroflexus* PLANT WEIGHT AND INTERNAL NPK LEVELS FROM PLANTS GROWN WITH *Helianthus annuus* DEBRIS FROM VARIOUS SOURCES (NO ADDITIONAL NUTRIENT SOLUTION ADDED)

Debris			Percent (± SD)		
Source[a]	Amount (mg/g soil)	Dry weight (mg ± SD)[b]	N	P	K
1	2.86	153.95 ± 15.08	1.84 ± 0.12	0.36 ± 0.02	2.28 ± 0.08
	7.14	194.79 ± 67.50	1.74 ± 0.14	0.44 ± 0.02	4.10 ± 0.01
2	2.86	117.53 ± 16.60	1.88 ± 0.02	0.36 ± 0.01	3.42 ± 0.18
	7.14	123.56 ± 26.10	2.19 ± 0.48	0.48 ± 0.03	3.78 ± 0.08
3	2.86	86.96 ± 35.03	1.90 ± 0.19	0.36 ± 0.04	3.30 ± 0.01
	7.14	36.47 ± 8.63	2.82 ± 0.68	0.38 ± 0.10	4.25 ± 0.55
4	2.86	121.34 ± 9.34	2.18 ± 0.14	0.40 ± 0.01	4.70 ± 0.05
	7.14	116.63 ± 16.59	1.80 ± 0.01	0.44 ± 0.04	4.55 ± 0.10
5	2.86	116.23 ± 23.16	1.48 ± 0.28	0.34 ± 0.04	3.48 ± 0.62
	7.14	82.09 ± 17.81	1.50 ± 0.32	0.49 ± 0.03	3.35 ± 0.10
6	2.86	111.24 ± 25.29	1.40 ± 0.01	0.26 ± 0.02	2.83 ± 0.33
	7.14	62.50 ± 6.07	1.65 ± 0.01	0.45 ± 0.02	3.80 ± 0.18
7	2.86	76.37 ± 13.34	2.16 ± 0.08	0.35 ± 0.04	3.68 ± 0.02
	7.14	64.65 ± 22.67	1.81 ± 0.17	0.28 ± 0.01	3.88 ± 0.12
Soil control		134.87 ± 14.09	1.64 ± 0.04	0.20 ± 0.01	1.98 ± 0.12

[a] Debris source numbers correspond to those in Table 1.
[b] $N = 5$.

Amaranthus internal percent N and K contents were not affected by different debris treatments. Percent N ranged from 1.40 to 2.82% ($\bar{X}$ = 1.85% ± 0.088 SE), and percent K ranged from 1.98 to 4.70% ($\bar{X}$ = 3.46 ± 0.208 SE). Percent P content was significantly correlated with *H. annuus* debris total phenolics:

$$\% \text{ P} = 0.273 + 0.0004 \text{ (phenol)}$$

$$(r^2 = 0.61, \qquad \alpha = 0.0004)$$

where phenol represents total phenolics (μg/g soil) added to the soil by *H. annuus* debris. The positive correlation of % P and total phenolics added to soil may simply be associated with growth reductions of *A. retroflexus*. That is, as growth was inhibited by *Helianthus* debris, P concentrations per gram of tissue tended to increase.

The addition of Hoagland's solution to the soil once a week resulted in a significant increase in total plant weight of *A retroflexus* seedlings compared to the seedlings grown in soils with debris but without nutrient solution (Table 3). However, within the nutrient treatment, there were no significant differences between the different debris treatments (range 179.38–452.33 mg;

TABLE 3. *Amaranthus retroflexus* PLANT WEIGHT AND INTERNAL NPK LEVELS FROM PLANTS GROWN WITH *Helianthus annuus* DEBRIS FROM VARIOUS SOURCES (NUTRIENT SOLUTION ADDED)

Debris			Percent (± SD)		
Source[a]	Amount (mg/g soil)	Dry weight (mg ± SD)[b]	N	P	K
1	2.86	396.46 ± 135.89	1.62 ± 0.05	0.28 ± 0.01	2.95 ± 0.01
	7.14	452.33 ± 72.87	1.86 ± 0.18	0.30 ± 0.02	3.32 ± 0.22
2	2.86	283.76 ± 25.26	1.71 ± 0.07	0.28 ± 0.01	3.42 ± 0.08
	7.14	295.12 ± 37.05	1.66 ± 0.05	0.30 ± 0.02	3.65 ± 0.01
3	2.86	245.36 ± 22.91	2.11 ± 0.07	0.26 ± 0.02	3.10 ± 0.01
	7.14	213.00 ± 38.77	2.15 ± 0.15	0.38 ± 0.04	4.40 ± 0.40
4	2.86	292.59 ± 33.19	1.83 ± 0.01	0.28 ± 0.01	4.00 ± 0.05
	7.14	284.87 ± 44.98	1.62 ± 0.07	0.32 ± 0.01	3.78 ± 0.12
5	2.86	249.72 ± 45.17	1.86 ± 0.04	0.28 ± 0.04	3.00 ± 0.15
	7.14	183.64 ± 29.68	1.88 ± 0.04	0.36 ± 0.02	3.60 ± 0.15
6	2.86	179.38 ± 43.78	1.74 ± 0.20	0.30 ± 0.02	3.45 ± 0.10
	7.14	202.18 ± 55.07	1.69 ± 0.14	0.36 ± 0.01	3.50 ± 0.02
7	2.86	187.46 ± 41.19	2.14 ± 0.22	0.24 ± 0.01	3.35 ± 0.05
	7.14	196.62 ± 39.57	1.59 ± 0.04	0.23 ± 0.01	3.40 ± 0.05
Soil control		248.72 ± 42.97	2.00 ± 0.13	0.19 ± 0.01	2.58 ± 0.28

[a] Debris source numbers correspond to those in Table 1.
[b] $N = 5$.

$\overline{X}$ = 260.02 mg ± 19.02 SE). A reduction in inhibition was also observed for seed germination when nutrient solution was added to the soil (Hall et al., 1982). Some possible reasons for this may be that phenolics could react with additional ions added to the soil and in the process produce nontoxic precipitates, and/or that nutrients added could stimulate microbial detoxification of phenolic compounds.

A retroflexus percent N content was not correlated with debris total phenolics in the soil and ranged from 1.59 to 2.15% ($\overline{X}$ = 1.84% ± 0.048 SE, Table 3). Percent P was positively correlated with total phenolics:

$$\% \text{ P} = 0.223 + 0.0003 \text{ (phenol)}$$

$$(r^2 = 0.74, \quad \alpha = 0.0001)$$

where phenol is in the same units as above. Percent K was also positively correlated with debris total phenolics in the soil although not highly:

$$\% \text{ K} = 3.03 + 0.0016 \text{ (phenol)}$$

$$(r^2 = 0.32, \quad \alpha = 0.0230)$$

where phenol is also in the same units as above. These observations weakened the hypothesis that the relationship between total phenolics added to the soil and seedling nutrient content was primarily a result of reduced growth as suggested earlier.

Some initial bioassay experiments were conducted which utilized debris from field-grown *Nicotiana tabacum* L. leaves. The preliminary results demonstrated a similar relationship between debris phenolics and *A. retroflexus* dry-weight production as was found previously when *H. annuus* debris was used. The *N. tabacum* leaf debris also contained large amounts of chlorogenic acid (Hall, unpublished data). *N. tabacum* debris total phenolics added to the soil accounted for more than 63% of the variation in *A. retroflexus* plant weight. The addition of a nutrient solution did not modify the reduction of plant dry weight, thus indicating *N. tabacum* leaf debris acted differently than *H. annuus* plant debris in this regard.

Chlorogenic Acid Bioassay. Since the most abundant phenolic compounds present in *H. annuus* were the chlorogenic acids and they had been implicated previously as allelopathic agents (Rice, 1974; Wilson and Rice, 1968), chlorogenic acid was substituted for *H. annuus* debris in the soil. *Amaranthus retroflexus* total plant biomass was significantly reduced by increasing levels of chlorogenic acid added to the soil (Table 4). This relationship is represented by the equation:

$$\text{Plant weight (mg)} = 131.95 - 0.10\ (\text{CLA})$$

$$(r^2 = 0.89, \qquad \alpha = 0.0164)$$

where CLA represents the total amount (μg/g soil) of chlorogenic acid added to the soil. However, chlorogenic acid did not inhibit seed germination (Hall et al., 1982). *A. retroflexus* plant percent N content was positively correlated with increasing levels of chlorogenic acid in the soil:

$$\%\ \text{N} = 0.99 + 0.0008\ (\text{CLA})$$

$$(r^2 = 0.98, \qquad \alpha = 0.0020)$$

where CLA is in the same units as above. Percent P content of *A. retroflexus* was inversely correlated with increasing chlorogenic acid:

$$\%\ \text{P} = 0.56 - 0.0002\ (\text{CLA})$$

$$(r^2 = 0.73, \qquad \alpha = 0.0375)$$

where CLA is also in the same units as above. Percent K content was not significantly modified by increasing chlorogenic acid levels and ranged from 2.10 to 2.88% ($\overline{X} = 2.42\% \pm 0.14$ SE). The positive correlation between % N and chlorogenic acid added to the soil may be related to growth effects, as suggested earlier for the debris bioassays. The negative correlation of % P with

TABLE 4. *Amaranthus retroflexus* PLANT WEIGHT AND INTERNAL NPK LEVELS GROWN IN PRESENCE OF INCREASING LEVELS OF CHLOROGENIC ACID

Chlorogenic acid (μg/g soil)	No nutrient solution added				Nutrient solution added			
	Dry weight (mg ± SD)[a]	Percent (± SD)			Dry weight (mg ± SD)	Percent (± SD)		
		N	P	K		N	P	K
0	134.82 ± 30.58	1.05 ± 0.06	0.56 ± 0.01	2.45 ± 0.15	497.45 ± 126.76	1.84 ± 0.19	0.38 ± 0.02	3.12 ± 0.28
100	132.80 ± 25.12	1.05 ± 0.14	0.52 ± 0.12	2.55 ± 0.60	645.58 ± 140.93	1.50 ± 0.10	0.32 ± 0.02	3.00 ± 0.10
200	93.09 ± 10.67	1.09 ± 0.18	0.58 ± 0.04	2.88 ± 0.25	563.90 ± 180.08	1.79 ± 0.53	0.34 ± 0.01	2.75 ± 0.50
400	95.81 ± 18.46	1.32 ± 0.06	0.43 ± 0.02	2.10 ± 0.05	476.12 ± 224.31	1.60 ± 0.12	0.30 ± 0.02	2.45 ± 0.10
800	53.71 ± 16.69	1.63 ± 0.05	0.40 ± 0.02	2.15 ± 0.20	596.76 ± 170.63	1.67 ± 0.35	0.33 ± 0.01	2.62 ± 0.48

[a] $N = 5$.

chlorogenic acid added to the soil suggested that chlorogenic acid inhibited the uptake of P. This appeared to be similar to what Glass (1975) observed for P uptake in the presence of ferulic acid or hydroxybenzoic acid.

The relationships between *A. retroflexus* dry weight, plant nutrient content, and chlorogenic acid concentrations in the soil were lost when a NPK solution was added to the soil containing the various levels of chlorogenic acid. The levels of NPK in the solution added to the soil were roughly equivalent to the NPK added by the 2.86-g debris treatment. Plant dry weights ranged from 476.12 to 645.58 mg ($\overline{X}$ = 555.96 ± 22.21 SE, Table 4). Stowe and Osborn (1980) observed that phenolic toxicity in nutrient solutions depended intimately on nutrient concentration of the solution. Their work led them to suggest that allelopathy with phenolics as chemical inhibitors seemed most likely to occur in nutrient-poor soils. Furthermore, the present results of the debris and chlorogenic acid bioassays demonstrated that nutrients in debris act differently in modifying phenolic effects on seedling growth than do nutrients added to the soil as a solution. Thus, it would appear that form and availability are important. *A retroflexus* internal NPK concentrations were not significantly modified by increasing chlorogenic acid levels (Table 4).

As with our seed germination study, debris from *H. annuus* or *N. tabacum* was inhibiting. Inhibition of dry weight was correlated significantly with the total phenol content in the tobacco debris and total phenol content and nutrient status (NPK) of the sunflower debris. It was possible to simulate debris inhibition of *A. retroflexus* seedling biomass production in part by adding pure chlorogenic acid to the soil. In this regard seedling dry matter production differed from germination, since chlorogenic acid, at the concentrations used here, did not inhibit seed germination. The addition of nutrient solutions to the soil containing *H. annuus* debris or chlorogenic acid resulted in the elimination of the inhibition found when nutrient solution was not added. A similar loss in inhibition was observed for seed germination. This suggested that the nutrient status of the soil may have an important influence on allelopathic mechanisms. Finally, the data here indicate that nutrients in debris as opposed to nutrients in soil (added in solution form) act differently in modifying allelopathic interactions.

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ALLELOPATHIC POTENTIAL OF SORGHUM (*Sorghum bicolor*): Isolation of Seed Germination Inhibitors[1]

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Abstract—The major inhibitory components obtained after fractionation of an aqueous extract of field-grown sorghum (*Sorghum bicolor* cv. Bird-a-boo) herbage were quantified in terms of biological potency by indexing three aspects of cumulative cress (*Lepidium sativum* cv. Curlycress) seed germination. The inhibitory potential expressed in the crude aqueous extract reflected a complex interaction of numerous individual components of diverse chemical compositions and potencies. Some of these inhibitory components included chemical classes not previously associated with herbage phytotoxicity.

Key Words—Allelopathy, *Sorghum bicolor*, germination inhibitors, *Lepidium sativum*, cress, Richards' function, germination index, bioassay, plant residues, sorghum herbage.

INTRODUCTION

Aqueous extracts of the herbage or roots of several members of the genus *Sorghum* are inhibitory to the germination and seedling growth of a number of plant species (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1962, 1967; Lawrence and Kilcher, 1961; Lehle and Putnam, 1982; Megie et al., 1966). Much of the inhibition obtained has been attributed to the content of phenolic acids, dhurrin, and *p*-hydroxybenzaldehyde. Chlorogenic acid, *p*-coumaric acid, and *p*-hydroxybenzaldehyde were reported to be the principle

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inhibitors isolated from the herbage and rhizomes of *S. halepense* (L.) Pers. (Abdul-Wahab and Rice, 1967). Guenzi and McCalla (1966) detected a number of phytotoxic phenolic acids in hydrolysates of *S. vulgare* (L.) Pers. residues including ferulic, *p*-coumaric, syringic, vanillic, and *p*-hydroxybenzoic acids.

Dhurrin, a cyanogenic glucoside of high mammalian toxicity, is present in all plant parts and at all growth stages of many members of the genus sorghum (Martin et al., 1938). Injury or death of living sorghum tissue results in a rapid enzymatic hydrolysis of dhurrin to HCN and *p*-hydroxybenzaldehyde through the action of glucosidases and oxynitrilases (Conn and Akazawa, 1958; Dunstan and Henry, 1902). While both compounds are phytotoxic and HCN released from dhurrin has been implicated as the principle phytotoxin in *S. halepense* rhizome exudates (Kovacs, 1972), the rapid dissipation of HCN suggests that *p*-hydroxybenzaldehyde plays a greater role in contributing to the phytotoxicity of dried sorghum residues (Abdul-Wahab and Rice, 1967).

Despite the isolation and identification of these inhibitory compounds, there has been an absence of direct evidence that the quantities present were sufficient to account for the observed levels of inhibition (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1966). Since there is little indication that the isolation procedures previously employed were chosen solely on the basis of biological activity, significant components of inhibition expressed in the initial extracts may have been overlooked or discarded. In addition, the lack of quantitative determinations of biological activity during previous isolation procedures makes it difficult to establish if any alterations in biological activity occurred simply as a result of the procedures utilized. These problems could have been largely avoided with the use of systematic determinations of biological activity at increasingly sophisticated levels of chemical purification.

The objective of the present study was to establish the major inhibitory components present in sorghum herbage previously reported to contain water-soluble, plant-growth inhibitors (Lehle and Putnam, 1982). Our approach consisted of quantitative determination of inhibitory potential of isolated fractions obtained from a crude aqueous extract of dried sorghum herbage using cumulative seed germination as the bioassay response. The significance of inhibition contributed by all isolated fractions relative to that expressed by the crude aqueous extract was assessed both in terms of specific inhibitory activity and quantity of dry residue recovered.

METHODS AND MATERIALS

Plant Material. Sorghum (*Sorghum bicolor* cv. Bird-a-boo; Taylor Evans Seed Co., Tulia, Texas) was seeded July 16, 1979, and August 4, 1980,

at East Lansing, Michigan, on a Spinks sandy loam soil. Seed were drilled both years at a rate of 81 kg/hectare in 17.7-cm rows. Herbage (cut at the soil surface) was harvested in 1979 and 1980 on August 27 and October 16, respectively. After drying at about 50° C for several days and grinding to pass a 40-mesh screen on a Wiley mill, herbage samples were stored frozen at −20° C until used.

Cress (*Lepidium sativum* cv. Curlycress) seed (Burpee Seed Co., Warminster, Pennsylvania) were dusted with 0.95 mg thiram [bis(dimethylthiocarbamoyl)disulfide] and 0.95 mg captan {*cis*-*N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboxiimide}/g seed, prior to use.

Herbage Extraction and Purification. Water used throughout extraction procedures was deionized and double-distilled. All fractions were concentrated or freed of organic solvents by rotary flash evaporation at 55–60° C. Procedures utilized during extraction and purification are summarized in Figure 1.

A crude aqueous extract was prepared by soaking 2.0 kg ground herbage material in 12.0 liters of H_2O in a glass container overnight at 10° C. The container was drained from the bottom through glass wool and an additional 10.0 liters of H_2O added to the container. The process of soaking, draining, and adding of H_2O was repeated until the effluent specific conductivity was <200 μmhos/cm. Leachates obtained by this procedure for a total of 10.0 kg

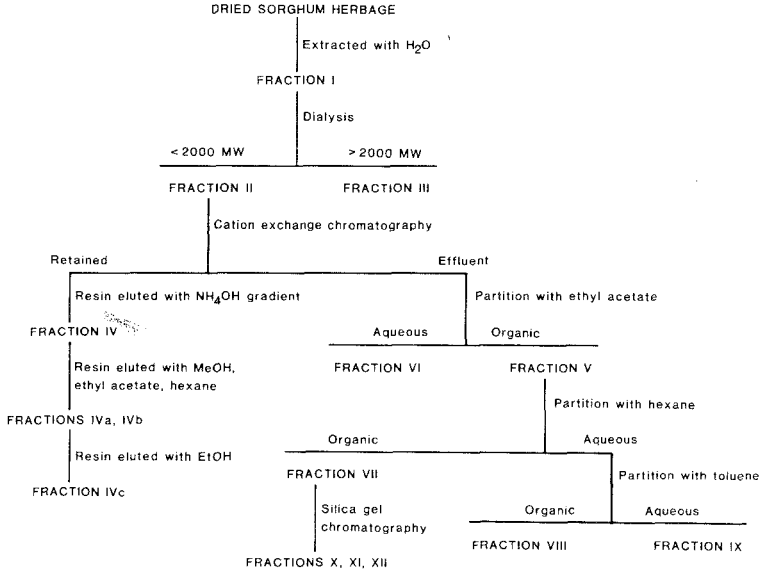

FIG. 1. Summary of procedures utilized for the separation of inhibitory components from an aqueous extract of Bird-a-boo sorghum herbage.

(2.0 kg, 1979 harvest; 8.0 kg, 1980 harvest) of ground herbage were combined (about 150 liters) and concentrated to a final volume of 3.918 liters (pH 6.1) containing 2014.63 g of dissolved residue (I).

One hundred milliliters of the crude aqueous extract (I) was added to a 4.5- (flat width) × 55-cm length of cellulose dialysis tubing (Spectra Por 6, 2000 mol wt cutoff; Spectrum Medical Ind., Inc., 60916 Terminal Annex, Los Angeles, California) and dialyzed with agitation at 10°C for about 24 hr against 10.0 liters of H_2O along with three other similarly prepared tubes. The nondialyzable portions were concentrated to a volume <400 ml and again dialyzed against 10.0 liters of H_2O as just described. Dialyzable and nondialyzable fractions obtained by this procedure from a total of 3868 ml of the crude aqueous extract (I) were combined and concentrated to final volumes of 2495 and 3400 ml containing 1342.3 (II) and 379.4 (III) g of dissolved residues, respectively.

A large portion (2445 ml) of the dialyzable fraction (II) was eluted with about 45 liters of H_2O through a 5.6-kg (dry weight) column of cation exchange resin (Dowex 50W-X8, 20–50 mesh, H^+ form; Bio-Rad Laboratories, 220 Maple Ave, Rockville Centre, New York) at about 10°C and the effluent concentrated to a final volume of 1407 ml. Materials retained on the column were eluted with a 0.0–4.0 N NH_4OH linear gradient followed by 36-liter washes of 4.0 N NH_4OH and H_2O. The combined gradient effluent and washes were concentrated twice more to remove traces of NH_4OH. The final syrup (IV, 265.23 g dry weight) was brought to a volume of 1968 ml (pH 8.6). The resin column was then eluted with about 2.0 liters each of MeOH, ethyl acetate, and *n*-hexane and the effluents were combined and concentrated to a yellow oil, which upon standing yielded 0.9 g of white crystals (IVa) which were collected by filtration. The remaining oil (IVb, 21.9 g) gave a positive test for alcohol using the Jones oxidation procedure (Fieser and Williamson, 1975). The resin column was finally eluted with about 8.0 liters of 95% EtOH, which upon concentration yielded 3.5 g (IVc) of clear orange syrup.

The concentrated cation exchange column effluent (1407 ml, pH 3.8) was partitioned four times against 1.0-liter portions of ethyl acetate. The pooled organic phases yielded 196.71 g of orange acidic residue (V) after solvent flash evaporation. The aqueous phase was concentrated to a volume of 852 ml containing 547.15 g (VI) of dissolved residues. A 15% solution (10.4 g/67.0 ml H_2O) of the orange acidic residue (V) was partitioned against four 100-ml portions each of hexane and toluene. After flash evaporation of the solvents, the pooled hexane and toluene phases yielded 0.3173 (VII) and 1.2312 (VIII) g of yellow oily residue. The remaining aqueous phase was frozen in liquid N_2 and after lyophilization yielded 7.97 g (IX) of orange residue.

In a separate experiment, 410.4 mg of the hexane-soluble residue (VII), obtained as just described, was chromatographed (acetone–hexane–MeOH;

80:15:5) through a 40-g (dry weight), 2- × 18-cm silica gel (silica gel G 60, 63–200 μm; MCB Mfg. Chemists, Inc., 209 Highland Ave, Cincinnati, Ohio) column; sixty 8.0-ml fractions were collected. A 10-μl sample was taken from selected tubes, dissolved in 2.0 ml MeOH, and absorbance measured at 254 nm using MeOH as a reference. Collected fractions were separated into three groups (X, tubes 1–8; XI, tubes 9–20; and XII, tubes 21–60); weights of the residues after solvent removal were 93.9 (X), 269.9 (XI), and 20.1 (XII) mg.

Cress Germination Bioassay. The inhibitory effects of all obtained fractions on cumulative cress seed germination were quantified by an automated procedure described previously (Lehle et al., 1982). Treatments consisted of 29 contiguously grouped 6- × 50-mm stoppered glass tubes containing a single cress (*Lepidium sativum* cv. Curlycress) seed (Burpee) and 25 μl of either 10 mM MES [2-(*N*-morpholino) ethanesulfonic acid] buffer (Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri), pH 6.1, or similarly buffered test solution in the dark at 25° C. Seed were designated as having germinated if growth extended 7.0 mm above the tube bottoms. Experimental design was completely randomized with two replications. Richards' functions were fitted to raw cumulative seed germination data for each replicate using methods described previously (Lehle and Putnam, 1982). A custom Fortran IV program, NONLIN3, was utilized instead of the nonlinear subprogram of SPSS (Robinson, 1977) for regression sum of squares minimization according to Marquardt's method. Germination statistics derived from the Richards' function parameters included the final cumulative germination percentage (A), the onset of germination ($t_{0.01A}$), and a weighted mean cumulative germination rate (R) (Lehle and Putnam, 1982). To simplify quantification of bioassay responses, these three germination statistics were incorporated into a single numerical index (I_G) defined as:

$$I_G = \frac{AR}{t_{0.01A}}$$

Reductions of 50% in the index value (I_{G50}) were estimated from linear calibrations of test solution concentrations (weight of recovered residue basis) versus index values for each replicate separately.

A single unit dose of 50% inhibition (UD_{50}) was defined as the weight of recovered fraction residue required to inhibit the germination response of a single seed 50% as extrapolated from the replicate I_{G50} concentration. Total inhibitory potential for a particular fraction was based on the total weight of recovered fraction residue corrected for dry weight losses accumulated during succeeding purification steps starting with the crude aqueous extract (I). The specific inhibitory activity of any fraction was expressed as total UD_{50}/g of recovered residue.

RESULTS AND DISCUSSION

Residue Recovery. The overall percentage recovery of extracted materials on a dry weight basis for the major stages of purification averaged 86.9 ± 7.4%; individual procedures ranked as follows: silica gel chromatography of VII, 93.5%; sequential partitioning of acidic residue (V), 91.5%; dialysis, 85.5%; and cation exchange chromatography followed by ethyl acetate partitioning, 77.1%. Dry weight of residues recovered for individual fractions corrected on the basis of the above extraction efficiencies and the removal of sample for bioassay are graphically illustrated in Figure 2. About 20% of the dry weight of ground sorghum was removed by leaching with water. The bulk of materials recovered from the aqueous extraction of sorghum herbage were below 2000 MW (II). About 28% of the dry weight of this low-molecular weight fraction was retained by the cation exchange resin (IV, IVa, IVb and IVc). About 91% of the dry weight of residues recovered from the cation exchange resin was eluted with the NH_4OH gradient (IV). Of the material not retained by the resin (about 56% of the total dry weight recovered by aqueous extraction), about 26% partitioned into ethyl acetate

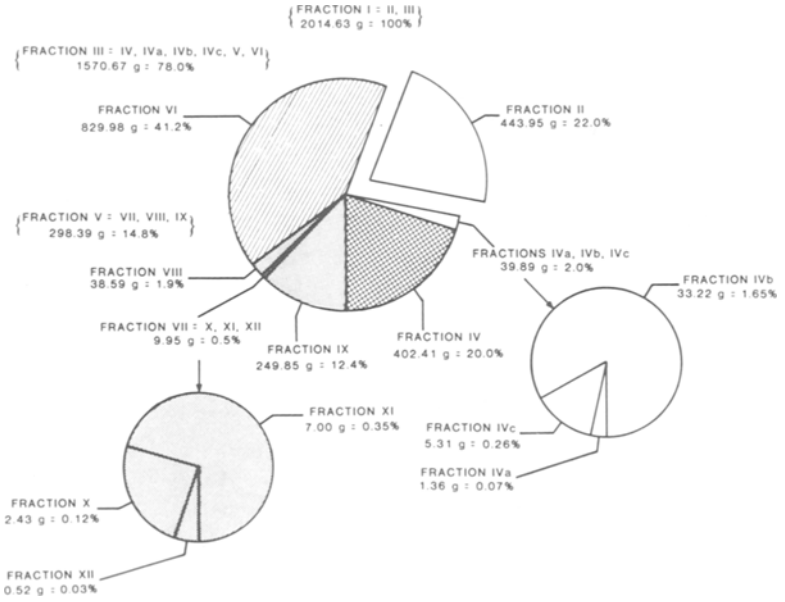

FIG. 2. Dry weights of residues recovered from fractions separated from Bird-a-boo sorghum herbage. Fraction residue weights were corrected to reflect dry weight losses accumulated during succeeding purification steps starting with the crude aqueous extract (I).

(V). Sequential extraction with hexane and toluene of an aqueous solution of the acidic residue obtained after ethyl acetate partitioning removed only 16% of the dry weight contained in this fraction. The hexane-soluble residue, further purified by silica gel chromatography, was separable into three minor fractions which together accounted for only 0.5% of the dry weight of all recovered residues.

Toxicity of Recovered Residues. The total inhibitory potentials and specific inhibitory activities of obtained fractions corrected for extraction efficiency and sampling for bioassay are presented in Table 1. Based on a comparison of cress germination bioassay results, the inhibitory potency of the crude aqueous extract (I) was about 80% greater than that reported previously for a similarly prepared extract of 4-week-old 'Bird-a-boo' herbage (Lehle and Putnam, 1982). Since the bulk (80%, 1980 harvest) of the herbage used in the present study was harvested at a later stage of maturity (10.4 weeks after planting), this result suggests that the reported decline in inhibitory potential of Bird-a-boo herbage beginning four weeks after planting is substantially reversed later in the growing season (Lehle and Putnam, 1982).

TABLE 1. INHIBITORY POTENTIAL AND POTENCY OF FRACTIONS SEPARATED FROM CRUDE AQUEOUS EXTRACT OF BIRD-A-BOO SORGHUM HERBAGE AS QUANTIFIED BY CUMULATIVE CRESS SEED GERMINATION BIOASSAY[a]

Fraction	$[I_{G50}]$ (ppmw ± SD)	Total inhibitory potential ($UD_{50} \times 10^{-6}$)	Specific inhibitory activity ($UD_{50} \times 10^{-3}$/g residue)
I	750 ± 64	108.0	53.5
II	429 ± 15	147.0	93.3
III	500 ± 52	35.7	80.5
IV	453 ± 42	36.6	88.7
IVa	1150 ± 79	<0.1	34.9
IVb	2380 ± 121	0.6	16.9
IVc	182 ± 4	1.2	220.7
V	1350 ± 9	9.1	29.8
VI	3220 ± 101	10.6	12.4
VII	86 ± 9	4.8	469.0
VIII	780 ± 80	2.1	51.7
IX	1580 ± 135	6.5	25.4
X	488 ± 164	0.3	90.0
XI	171 ± 3	2.2	235.0
XII	15 ± 1	2.0	2670.0

[a]Inhibitory potential and specific inhibitory activity of each fraction was extrapolated from I_{G50} concentration and corrected for extraction efficiency and removal of samples for bioassay. Each value represents the mean of two replications.

The cause for such an increase in inhibitory potential is not clear, but a contributing factor may have been the stress of several weeks of low temperatures prior to the 1980 harvest.

Similar to previous findings (Lehle and Putnam, 1982), most of the inhibition present in the crude aqueous extract was recovered in the low-molecular-weight fraction. Based on specific inhibitory activity, the low- (II) and high-molecular-weight (III) fractions were about equally inhibitory; the inhibitory potential of the low-molecular-weight fraction was greater due to its larger share of recovered residues. The combined inhibitory potentials and individual specific inhibitory activities of both the low- and high-molecular-weight fractions were greater than that expressed by the crude aqueous extract, suggesting that both fractions were antagonistic to each other in terms of expression of inhibitory activity. This conclusion was supported by a separate experiment in which fractions II and III were recombined in proportion to their dry weight recoveries. The I_{G50}, total inhibitory potential, and specific inhibitory activity values (units as in Table 1) of the resulting mixture were 1643 ± 361, 50.3, and 24.9, respectively.

Only about 40% of the inhibitory potential present in the low-molecular-weight fraction was recovered after cation exchange and ethyl acetate partitioning. The basis for the loss of inhibitory activity is not clear, but several possible causes were evaluated. The loss of inhibitory potential may have resulted from hydrophobic binding of inhibitory components to the resin matrix material. This possibility was suggested since only about 77% of the sample dry weight applied to the column was recovered. This was the lowest level of residue recovery measured for any purification procedure employed. Following the initial elution of the resin column with the NH_4OH gradient, an attempt was made to elute hydrophobically bound materials from the resin by washing the resin with several organic solvents. An additional 40 g of residue (IVa, IVb, and IVc) was recovered from the resin by this procedure, but the total inhibitory potentials of the obtained fractions were too small to account for major losses in inhibitory potential (Table 1). Despite the exhaustive elution procedures utilized, the possibility remains that a substantial proportion of the inhibitory components present in the low-molecular-weight fraction were still bound to the resin.

An alternative explanation for the large loss of inhibitory activity in fractions recovered from the low-molecular-weight fraction (II) is that the total inhibitory potential present in the low-molecular-weight fraction represented a synergistic expression of inhibition resulting from the combination of the six fractions (IV, IVa, IVb, IVc, V, and VI) derived from it. This possibility was evaluated in a separate experiment in which these six fractions were recombined in proportion to their dry weight recoveries. The I_{G50}, total inhibitory potential, and specific inhibitory activity values (units as in Table 1) of the resulting mixture were 1278 ± 43, 49.2, and 31.3, respectively. This

result suggests there was no synergism of inhibitory expression when the fractions were combined, since the total inhibitory potential expressed after recombination was similar to that which could be obtained by simple addition of the individual fraction inhibitory potentials (Table 1).

These results suggest the possibility that a substantial proportion of the inhibitory components present in the low-molecular-weight fraction decomposed or were otherwise chemically altered to less inhibitory substances as they passed through the resin. The relatively low specific inhibitory activities of the residues partitioned from the cation exchange resin effluent (V) as well as those recovered from the remaining aqueous phase (VI) are consistent with this possibility.

The low specific inhibitory activity of the residue partitioned from the cation exchange resin effluent (V) was surprising, since the phytotoxic properties of *Sorghum* species' residues have previously been attributed to several phenolic acids (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1966). Less of the loss of inhibitory potential present in the low-molecular-weight fraction can be associated with residues retained by the cation exchange resin since the specific inhibitory activity of the bulk of these residues was essentially equivalent to that of the low-molecular-weight fraction.

Preliminary chemical characterization of the acidic residue (V) indicated that aconitic acid accounted for about 14% of the dry weight of this fraction (results not shown). Sequential extraction with hexane and toluene of the acidic residue (V) partitioned from the cation exchange column effluent resulted in two fractions of enriched inhibitory potential. Residues recovered from the hexane-soluble fraction (VII) were the most inhibitory and had a specific inhibitory activity about 16 times that present in the acidic residue. The specific inhibitory activity of the residues recovered from the toluene fraction (VIII) were about twice that present in the acidic residue. The elution profile of residues recovered from the hexane-soluble fraction (VII) after further purification by silica gel chromatography is presented in Figure 3. Of the three fractions obtained, XI and XII were the most inhibitory and combined accounted for most of the inhibitory potential of VII. The specific inhibitory activity of XII was the highest obtained for any fraction and was about 50 times greater than that expressed by the crude aqueous extract (I). The spectrometric analysis and identification of the biologically active components of XI and XII is currently under investigation and will be reported elsewhere.

Results presented here indicate that the inhibitory basis of crude extracts prepared from sorghum residues is substantially more complex than previously reported and reflects the combined expression of numerous individual components of diverse chemical compositions and potencies. Some of these components have not been recognized previously. For example, previous

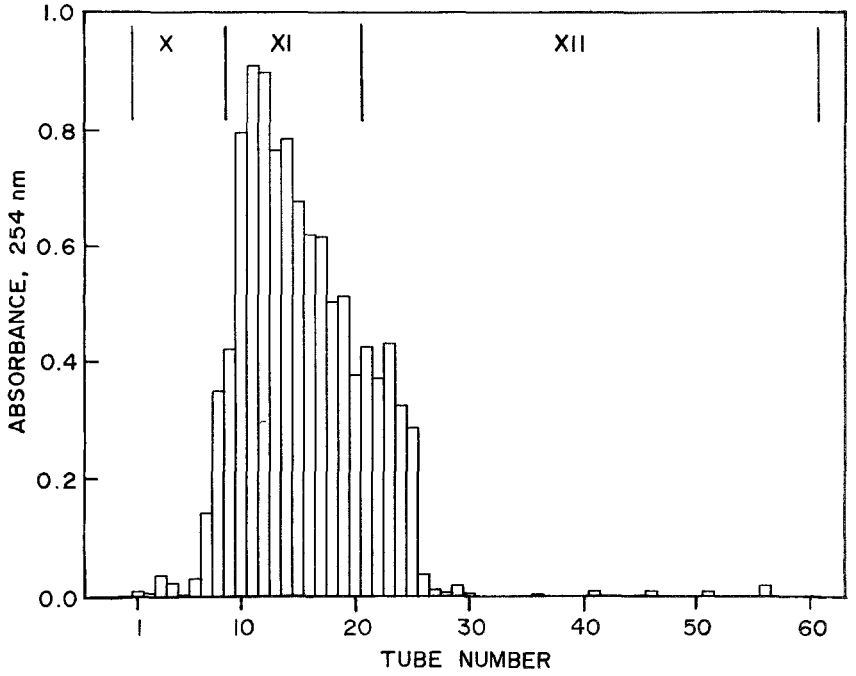

FIG. 3. Absorbance profile for silica gel chromatography of fraction VIII.

reports have attributed most of the phytotoxicity of sorghum residues to compounds of relatively low molecular weight, whereas, in the present study, about 20% of the total inhibitory potential recovered after dialysis was expressed by a high-molecular-weight fraction. Further work is needed to clarify if the inhibitory potential expressed by this high-molecular-weight component occurs as a direct result of the larger constituents or from products produced during their metabolism.

Results presented here indicate that a portion of the expressed inhibitory potential of extracted sorghum herbage results from both hydrophilic and basic components. Substantial inhibitory potential was present in several isolated aqueous fractions after these were partitioned with nonpolar solvents. Although the specific inhibitory activities of the residues recovered from these aqueous fractions were relatively low, this was offset in terms of contributed inhibitory potential by their greater abundance; the combined residue weight of these fractions accounted for about 55% of the dry weight of all recovered residues. With the exception of dhurrin, all previously identified inhibitors isolated from sorghum residues are lipophilic in terms of solubility (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1966). Basic components also contributed a substantial portion of the total inhibitory potential of

the crude aqueous extract as evidenced by the levels of inhibitory potential expressed by residues retained by the cation exchange resin. The majority of previously isolated inhibitors from sorghum residues have been identified as phenolic acids (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1966).

It is clear from the present study that several factors, not adequately addressed in the past, need to be considered in future studies in this area. Foremost among these is that the contribution any isolated fraction or purified inhibitor makes to the expressed inhibitory potential of the extract from which it was derived can only be assessed by knowledge of both its quantity and specific inhibitory activity. Purified components of low specific inhibitory activity may play a greater role in expressed inhibitory potential than those of high specific inhibitory activity by virtue of being present in greater quantity and vice versa. Only by knowing both the quantity and specific inhibitory activity of an isolated fraction can a reasonable estimation of its significance relative to the total expressed inhibitory potential of the crude extract be made.

Finally, the possibility is ever present that the separation techniques themselves may alter the expression of biological activity through such factors as chemical modification of labile constituents or their binding to a chromatographic support medium. The possibility of such deleterious effects during sample purification needs to be examined if changes in biological activity cannot be related to either antagonistic or synergistic interactions among the separated components. Only through careful quantification of biological activity at each juncture of the purification process can rational choices be made relative to the appropriateness of the isolation procedure employed.

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ALLELOPATHIC POTENTIAL OF WILD OAT (*Avena fatua*) ON SPRING WHEAT (*Triticum aestivum*) GROWTH[1]

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Abstract—Wild oat plants may produce toxic substances that suppress the growth and development of desirable species, thus accounting for severe yield loss in infested fields. The purpose of this study was to determine the allelopathic potential of wild oat (*Avena fatua*) on the growth of spring wheat (*Triticum aestivum* var. Fieldwin) in the absence of plant competition. Wild oat and spring wheat plants were grown separately in 250-ml beakers in a sand medium. Root exudates were extracted from wild oat medium at the 1-, 2-, 3-, and 4-leaf stages of wild oat development and added to beakers containing spring wheat in temporally corresponding stages of development. Spring wheat root and leaf dry weights were measured to determine if one or more allelochemical agents were released from wild oat roots. Spring wheat leaf and root dry weights were significantly reduced by exudates from wild oat plants at the 2- and 4-leaf stages of development, respectively. Allelochemicals were isolated from wild oat root exudates at various stages of plant development. Paper chromatography analysis indicated that at least two unknown compounds were present. R_f values in benzene–acetic acid–water of the two unknown compounds (0.825 and 0.930) were similar to scopoletin (7-hydroxy-6-methoxycoumarin) and vanillic acid (4-hydroxy-3-methoxybenzoic acid), respectively. Additional tests using diazotized *p*-nitraniline, ultraviolet absorption spectra, and gas chromatography analysis also indicated that the unknowns were coumarin-related compounds such as scopoletin and vanillic acid.

Key Words—Scopoletin, vanillic acid, root exudates, wild oat, *Avena fatua* L., Spring wheat, *Triticum aestivum* var. Fieldwin.

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INTRODUCTION

Wild oat (*Avena fatua* L.) continues to plague crop production in the western and north-central regions of the United States in spite of the increased use of modern integrated control tactics. In Idaho, more than 1.35 million hectares of cropland are infested with wild oat, resulting in crop losses estimated to exceed $34 million annually (Lee et al., 1981). These losses are primarily from yield reductions, but also include losses associated with dockage, storage, transportation, and cultural and chemical control practices.

Researchers (Bell and Nalewaja, 1968; Chancellor and Peters, 1976; Coleman-Harrell, 1978; Nalewaja, 1974) have shown that the interference of wild oat with other crops for light, moisture, and nutrients can decrease cereal grain yields. Crop yield losses from wild oat do not appear to be attributable solely to competition. Wild oat plants may produce toxic substances that suppress the growth and development of desirable species (Fay and Duke, 1977; Guenzi and McCalla, 1966; Martin, 1957; Tinnin and Muller, 1971, 1972) and contribute to yield losses. Most of the allelopathic research on the genus *Avena* has been conducted with the cultivated oat species and the allelopathic nature of wild oat has largely been inferred from this research (Fay and Duke, 1977; Martin, 1957).

The objectives of this research were to (1) evaluate the effect of exudates from intact wild oat roots on the growth of spring wheat in the absence of plant competition, (2) determine the plant growth stage at which wild oat becomes allelopathic to spring wheat, and (3) attempt isolation and identification of allelochemicals present in wild oat root exudates.

METHODS AND MATERIALS

Plant Material and Growing Conditions. Spring wheat *Triticum aestivum* L.) and wild oat seedlings were grown from caryopses in 250–ml glass beakers covered with aluminum foil to prevent the photodegradation of allelochemicals. Growing media was 300 g of sand (0.7 mm or smaller particle size) which had been autoclaved for 15 min at 121° C. The breakers were covered with cellophane until plant emergence and then incubated in a controlled environment chamber at 20° C constant temperature and a 14-hr photoperiod using incandescent and fluorescent lights (375 $\mu E/cm^2/sec$).

Experimental controls consisted of beakers containing six spring wheat plants that were surface watered every second day following plant emergence with 25 ml of a 0.1 concentration of Hoaglund's solution (Hoaglund and Aron, 1950). Dilute nutrient solutions were used throughout the experimental period to avoid interfering with the allelopathic effect. The nutrient concentration was, however, high enough to allow for normal plant growth and

development. Each beaker containing six wild oat plants was watered every second day with 50 ml of 0.1 concentration of Hoaglund's solution. Immediately after watering, 25 ml of the solution surrounding the wild oat roots were extracted from each beaker using a 35-ml syringe that was placed over a glass tube inserted to the bottom of the beaker. This solution was then added to a beaker containing six wheat plants. Root exudates were extracted from wild oat medium from emergence through the 1-, 2-, 3-, and 4-leaf stage of development and added to beakers containing spring wheat in temporally corresponding stages of development. Untreated and treated beakers containing spring wheat plants were paired for comparing differences in growth.

When wild oat plants reached the 1-, 2-, 3-, and 4-leaf stages of development, the wild oats and the corresponding treated and untreated wheat plants in temporally corresponding stages of growth were harvested. The wheat roots were washed free of the sand media with water, then separated from shoots. Root and shoot dry weights were determined after oven-drying them at 65° C for 48 hr.

The experiment was conducted as a randomized block design with eight replications and was repeated twice. Data were subjected to an analysis of variance and treatment means were separated by Duncan's multiple-range test.

Collection of Water Extracts. Wild oats were grown from caryopses in aluminum foil-covered, 250-ml glass beakers in the same manner as previously discussed. The beakers were watered with 50 ml of a 0.1 concentration of Hoaglund's solution every second day. Immediately after watering, 25 ml of the solution surrounding the wild oat roots was extracted from each beaker using a 35-ml syringe placed over a glass tube inserted to the bottom of the beaker, filtered through Whatman No. 1 filter paper, and then frozen at 0° C. The individual water samples from each replicate were bulked together to make a single sample for each stage of wild oat development (0–1, 1–2, 2–3, and 3–4 leaf stages). A water sample was also collected from the time of wild oat emergence through the fourth leaf stage of development. The frozen samples were then dried in a Virtis lyophilizer (model 10-140-DC). After lyophilizing, the sample was reextracted with 100 ml of diethyl ether and then allowed to evaporate slowly at room temperature. The samples were then redissolved in 5 ml of 95% ethanol, evaporated to a final volume of 1 ml, and chromatographed.

Chromatography Apparatus and Procedure. One-way descending paper chromatography analysis at 21 ± 0.5° C was conducted on each sample in glass tanks as outlined by Swain (1953). The chromatography tank and trough were constructed of glass. Chromatograms constructed of Whatman No. 1 filter paper were used throughout the experiments. A 10-μl sample of scopoletin (Sigma Chemical Company, St. Louis, Missouri 63178) dissolved

in 95% ethanol served as a reference and was spotted on each chromatogram. A 50-μl sample from each growth stage was spotted 5 cm from the bottom of the paper on separate chromatograms.

Several solvents were used for the one-way descending chromatography technique. Benzene–acetic acid–water (6:7:3 v/v/v) produced the clearest spots and most reproducible R_f values. The development time was approximately 15–16 min. The chromatograms were removed from the tank and allowed to air dry and then were viewed under ultraviolet light for the presence of phenolic compounds. Most fluorescent spots were observed without treating the paper. When the fluorescence was relatively weak, or when detecting for the presence of coumarin, the paper was lightly sprayed with 2 N NaOH.

Identification and Quantification of Allelochemicals. After locating fluorescent spots on the chromatograms under ultraviolet light, the unknown compounds were eluted in 1 ml of 95% ethanol. Ultraviolet spectra of the eluates were measured from 190 to 300 nm with a Hitachi 10A instrument (Hitachi Scientific Products, Mountain View, California 94043) using 1-cm cuvettes. A sample of 95% ethanol served as the reference.

The eluates were also subjected to gas chromatography analysis using a method described by Kirk and Adler (1970). The eluates were either methylated using 5 ml diazoethane in ether (Kirk and Adler, 1970) to form methyl esters of the compounds, or mixed with 5 ml of dimethylformamide (DMF) to form DMF derivatives of the compounds. The column consisted of 200 × 0.3 cm OD stainless-steel tubing. Solid support was 80–100 mesh Chromosorb G (Sigma) that was acid-washed and treated with dimethyldichlorosilane. Stationary phases were (1) silicone elastomer OV-1 (Applied Sciences Lab, Ann Arbor, Michigan 48107) for the methyl ester compounds or (2) Apiezon L, 5% by weight of solid support, for the DMF compounds. Temperature for column A was 187° C and for Column B 230° C. Injection and detector temperatures were 280 and 240° C, respectively. The gas carrier was N_2 at a flow rate of 25 ml/min. A flame ionization detector and dual matched columns were used throughout the analysis.

Qualitative determinations of the unknown compounds were made comparing relative retention times with that of vanillic acid, an internal standard. The retention times from the eluates were then compared to known compounds subjected to both the OV-1 and Apiezon L columns for positive identification.

RESULTS AND DISCUSSION

Root Exudate Experiment. Wild oat root exudates had no effect on spring wheat root weight until the wild oats reached the 4-leaf stage of development (Figure 1). At this stage of development, a 34% decrease in root

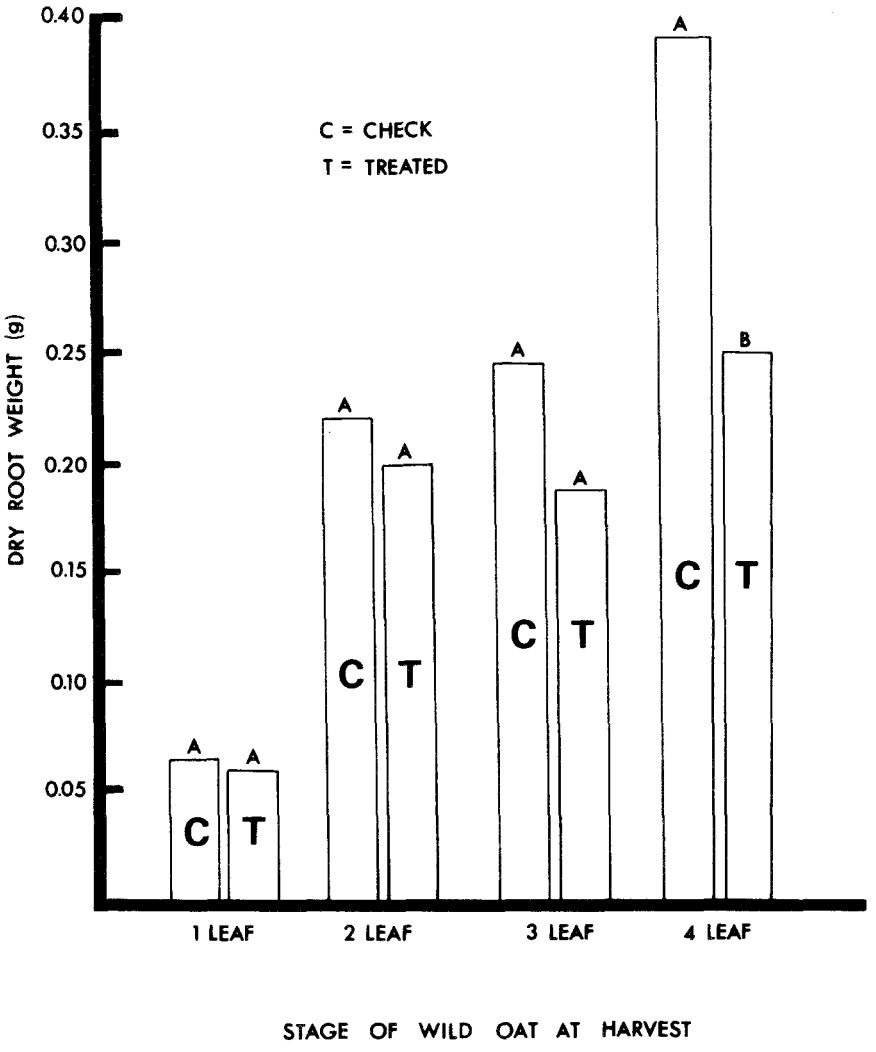

FIG. 1. The influence of wild oat root exudates on the dry root weight of six spring wheat plants.

dry weight was observed in spring wheat plants treated with wild oat root exudates compared to untreated spring wheat plants. Although not significantly different, root dry weights of the treated plants measured at the other three growth stages were always less than the untreated plants.

Wild oat root exudates did not affect spring wheat leaf weights at the 1-leaf stage of wild oat development, but at the other three growth stages, the treated spring wheat plants had a significantly lower leaf dry weight than the untreated plants (Figure 2). The results showed a 7, 19, and 26% difference

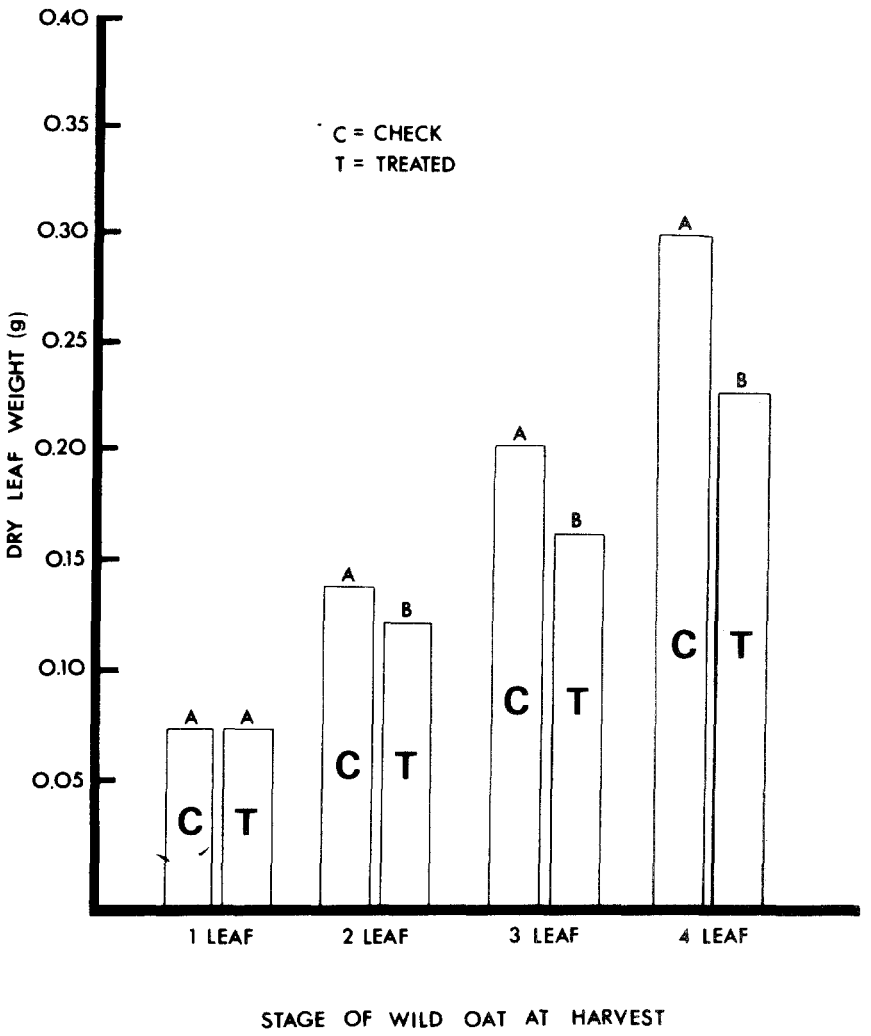

FIG. 2. The influence of wild oat root exudates on the dry leaf weight of six spring wheat plants.

between the treated and untreated spring wheat plants corresponding to the 2-, 3-, and 4-leaf stages of wild oat development, respectively.

Because these experiments were conducted in the absence of plant competition, the results strongly indicate that wild oat does produce a water-soluble, phytotoxic substance in its roots that can influence spring wheat growth. This phytotoxin appears more detrimental to leaf growth than root growth. Separation of the influence of wild oat competition from allelopathy

on spring wheat growth in the field would be difficult. Both are probably important in causing reduced crop yields.

Isolation and Determination of Allelochemicals. Two spots were detected on the paper chromatograms using ultraviolet light when the benzene–acetic acid–water (6:7:3 v/v/v) solvent system was used. The spot located nearest the origin had an R_f value of approximately 0.825, while the spot located furthest from the origin had an R_f value of approximately 0.93. These two spots were found to be present at all four wild oat growth stages and had approximately the same R_f values (0.825 ± 0.003; 0.93 ± 0.007). The reference sample of scopoletin had an R_f value of 0.83 on all chromatograms. Comparison of R_f values of the unknowns to known R_f values of phenolic compounds (Swain, 1953) showed that the 0.825 and 0.93 R_f values corresponded closely to that of scopoletin and *p*-hydroxybenzoic or vanillic acid, respectively. A colorimetric test using diazotized *p*-nitraniline (Block, 1952) gave a brown color for the R_f 0.825 compound and a pink to purple color for the R_f 0.93 compound. Again, the color reactions of the unknown compounds correspond closely to those for scopoletin and *p*-hydroxybenzoic or vanillic acid.

Ultraviolet (UV) absorption spectra of the R_f 0.825 compound showed a major peak located at approximately 202 nm, a minor peak at approximately 280 nm, and a broad shoulder from 215 to 240 nm (Figure 3). Again, this corresponds closely to that of scopoletin. The UV spectra of the R_f 0.93 sample showed a major peak located at approximately 207 nm (Figure 3), which corresponds closely to that of vanillic acid but not *p*-hydroxybenzoic acid. Vanillic acid has a second peak at approximately 265 nm that was not detected in the unknown sample.

Gas chromatographic analysis of the two unknowns resulted in approximate retention times for the OV-1 column of 2.42, 4.10, 5.64, and 11.10 and for the Apezion L column at 1.03, 3.88, 8.55, and 24.52 (Table I). Scopoletin had a retention time of 5.41 for the OV-1 column and 12.63 and 24.29 for the Apezion L column. Umbelliferone was analyzed as a standard through the gas chromatograph. Umbelliferone had retention times of 3.08 and 11.08 for the OV-1 and 3.88, 6.98, and 24.39 for the Apezion L column. When the unknown samples were compared to scopoletin and umbelliferone on the OV-1 column, none contained the same retention times for scopoletin but both did contain the retention time of 11.08 found in umbelliferone. However, this was a major peak for the unknown samples but not for umbelliferone. Also, the unknowns had a predominant retention time of 4.10 which was not found in the standards.

Comparing the unknowns to scopoletin and umbelliferone on the Apiezon L column showed the unknowns had retention times of 1.03 and 3.89, both found in scopoletin and umbelliferone, and 24.45 which corresponds closely to the retention time of 24.39 found in umbelliferone. Again the

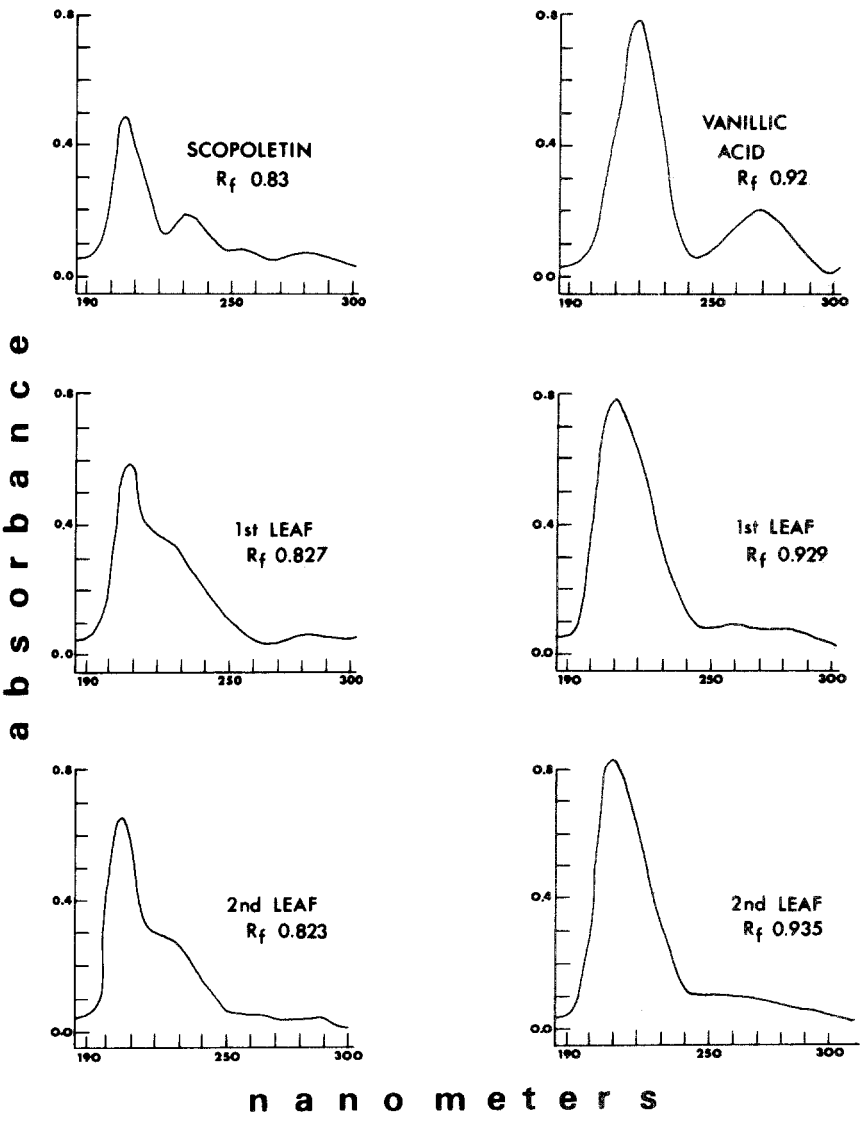

FIG. 3. Ultraviolet absorption spectrum of compounds obtained from wild oat root exudates at different stages of wild oat development and of reference samples of scopoletin and vanillic acid.

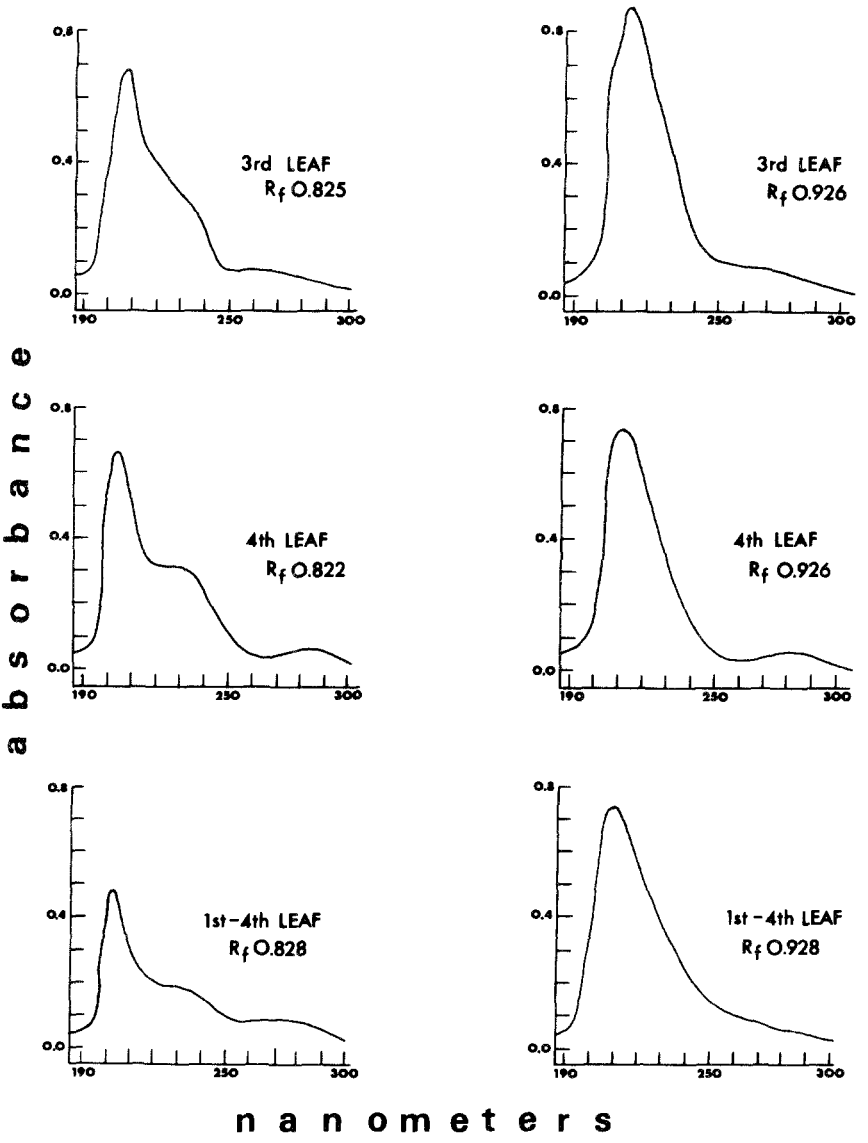

FIG. 3. Continued.

TABLE 1. GAS CHROMATOGRAPHIC ANALYSIS OF STANDARD PHENOLICS AND WILD OAT ROOT EXTRACTS

Compound	Relative retention time	
	OV-1 column	Apezion L column
Scopoletin	4.07[a]	3.89[b]
	5.41	7.07[a]
		12.63
		24.29
Umbelliferone	3.08	1.02
	4.08[a]	3.88[b]
	6.36[a]	6.98
	11.08	24.39
R_f 0.825	2.41[b]	1.02
	4.10	3.88[b]
	5.64	8.54
	11.10	24.48
R_f 0.930	2.42[b]	1.03
	4.10	3.88[b]
	5.64	8.56
	11.11	24.55
	15.53	

[a] Unknown peaks.
[b] Vanillic acid.

unknowns did not contain the retention time of 5.41 or 12.63 found in scopoletin and umbelliferone, respectively.

The retention times of the unknown compounds were compared to those of other known phenolics. On the OV-1 column the retention times of 2.42 and 11.10 correspond to vanillic acid and the coumarin compounds, respectively. On the Apezion L column, the retention times of 3.88 and 24.50 again correspond to vanillic acid and the coumarin compounds. Identical retention times in both unknown samples could be due to impurities or conjugation of the unknowns with such compounds as glycosides. Scopoletin has been reported to exist in wild oat in the glycoside form (Martin and Rademacker, 1960) which could account for the multiple peaks found in the unknown samples.

Scopoletin and vanillic acid have been reported to be present in wild oat (Banting, 1974; Molisch, 1937). The UV absorption spectra, color tests, and chromatographs of the unknown compounds support the presence of scopoletin and vanillic acid. The GC analysis of the R_f 0.825 compound did not show the major peak for scopoletin, but the vanillic acid peak was found on both columns.

Coumarin-related compounds such as scopoletin and others related to vanillic acid were tentatively identified in this study. Scopoletin has been found in root exudates from several cultivated oat accessions, but the concentration varied between cultivars (Fay and Duke, 1977). The same situation may exist between different biotypes of wild oat. Further analysis using mass spectrophotometry and gas chromatography are needed to identify precisely and to quantify the unknown compounds found to be present in wild oat root exudates.

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QUANTITATION OF A BIOACTIVE METABOLITE IN UNDISTURBED RHIZOSPHERE—BENZYL ISOTHIOCYANATE FROM *Carica papaya* L.[1]

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Abstract—A "continuous root exudate trapping system" was recently developed in this laboratory by which the allelopathic metabolites from the undisturbed rhizosphere of *Hemarthria altissima* (Poir.) Stapf. et Hubb. were collected. Twelve phenolic compounds were identified by capillary GC-MS (Tang and Young, 1982). In this report, we further describe its use for the quantitation of benzyl isothiocyanate (BITC), a highly bioactive volatile compound, in the rhizosphere of growing papaya plants. Samples were collected from 64 individual papaya (*Carica papaya* L.) trees of Waimanalo and Higgins cultivars. Hydrophobic compounds collected on XAD-4 columns were eluted with acetone and the levels of BITC were determined by a gas chromatograph equipped with a sulfur-specific flame photometric detector. For 2-month-old trees, the rate of BITC released from the root system of Waimanalo was $2.03 \pm 0.85\,\mu g$/tree/day. From Higgins, it was $2.36 \pm 1.06\,\mu g$/tree/day. It has been reported that Waimanalo is resistant whereas Higgins is susceptible to *Phytophthora palmivora* Butl., the major root rot fungal pathogen of papaya trees in Hawaii. Our results lead us to conclude that the rate of BITC released alone cannot account for differences in the resistance of these two cultivars to the pathogen.

Key Words—Bioactive metabolite, *Carica papaya*, L. rhizosphere, benzyl isothiocyanate, root exudate, soil organics, root–soil interface.

INTRODUCTION

With a large surface area, the plant root system continuously discharges organic matter into its medium. The chemical environment thus created affects the rhizospheric organisms including soil flora, fauna, and neighboring

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plants. In this respect, secondary plant metabolites are of particular interest due to their possible biological activities (Whitaker and Feeny, 1971). To assess the effects of these compounds at the root–soil interface, one must address them both qualitatively and quantitatively. However, due to their usually low concentrations, the presence of high background organic contaminants, and the lack of reliable analytical methods, the organic chemistry of these metabolites in the rhizosphere remains largely unexplored.

A "continuous root exudate trapping system" was recently developed in this laboratory (Tang and Young, 1982). Using hydrophobic Amberlite XAD-4 resin (Rohm and Haas) columns and capillary GC-MS, 12 phenolic compounds were identified from the undisturbed rhizosphere of *Hemarthria altissima* (Poir.) Stapf. et Hubb., a tropical forage grass with allelopathic properties (Young and Bartholomew, 1981). In the present report we describe the use of this continuous trapping system for the quantitation of benzyl isothiocyanate (BITC) in the rhizosphere of *Carica papaya* L.

Isothiocyanates are found in more than 11 families of plants (Ettlinger and Kjaer, 1968), including some important crops such as cabbage (*Brassica oleracea*) and rape (*B. napus*). These sulfur-containing, volatile compounds are usually found in macerated tissues due to mixing of the precursory glucosinolates and the hydrolytic enzyme thioglucosidase. However, we previously observed trace amounts of BITC in the cuticle wax of undamaged papaya fruits, suggesting that this compound also exists as a normal metabolite in intact plant tissue (Tang, 1971).

BITC has been reported as being a highly potent germicidal plant metabolite (Virtanen, 1962, 1965), being insecticidal (Seo et al., 1982), herbicidal (Syed, 1975), and enzyme inhibitory (Tang and Tang, 1976). It has also been shown to inhibit ethylene production in ripening papaya fruits (Patil and Tang, 1974). The continuous release of BITC from normal, growing papaya roots would suggest an active role of this metabolite in the rhizospheric ecosystem.

METHODS AND MATERIALS

Establishment of Papaya Plants. Mature seeds of *Carica papaya* L. (cv. Waimanalo and cv. Higgins) obtained from the Department of Horticulture, University of Hawaii, were soaked for 2 hr followed by surface sterilization in 0.3% sodium hypochlorite for 15 min. They were planted in 59-ml styrofoam cups containing vermiculite and kept in a greenhouse (21–32°C) under natural light. The plants were irrigated with Hoagland's solution twice a week and supplemented with water as needed. After 30 days, the seedlings wre transplanted into the continuous root exudate trapping system similar to that described previously (Tang and Young, 1982), except that a perforated Teflon

sheet barrier and a thick layer of glass wool were used to retard protrusion of roots. The pots were irrigated with circulating Hoagland's solution at a rate of ca. 1 liter/hr. To obtain the best growth, it was necessary to replace the nutrient solution every 10 days. Pots without plants treated in a similar manner were used as controls.

Collection of Root Exudates. Collection was started 30 days after transplanting; the plants were ca. 200 mm tall with healthy and proliferate root systems. The pots were first washed by slowly leaching with 2 liters distilled water to rinse off accumulated residues. A column containing 15 ml of cleaned XAD-4 resin (Junk et al., 1974) was attached to each pot, and fresh nutrient solution was circulated at 1 liter/hr for 48 hr. The columns were then removed for elution. Four consecutive collections were made from each plant. Height of the plants was recorded at the beginning of each collection period.

Quantitation of BITC. The charged column was disconnected from the pot and washed with 150 ml distilled H_2O followed by elution with 80 ml acetone. The eluate was concentrated to ca. 10 ml at 50°C using a rotary evaporator, and the resultant solution was extracted twice with 10 ml ethylacetate. The combined organic extract was dried over anhydrous Na_2SO_4 and concentrated to ca. 1 ml for GC analysis.

A Bendix 2500 gas chromatograph equipped with a sulfur-specific flame photometric detector (FPD; Tracor Co., Inc., Austin, Texas) and a 2-m × 2.5-mm ID glass column packed with 2% OV-17 on 80–100 mesh Chromosorb W AW HMDS was used for quantitations. The gas flow rates were: N_2, 30 ml/min; H_2, 155 ml/min; and air, 175 ml/min. Temperatures were: injector, 235°C; column, 200°C; and detector, 210°C. The levels of BITC in samples were determined by comparison of the peak heights with that of a standard peak.

To obtain the volatile profile of the root exudates, the sample was concentrated to ca. 0.1 ml. A Varian 1800 gas chromatograph equipped with a 30-m OV-17 SCOT glass capillary column (Scientific Glass Engineering, Inc., Austin, Texas) and flame ionization detectors (FID) was used. The gas flow rates were: He, 12 cm/sec; H_2, 30 ml/min and air, 300 ml/min. The temperatures were: injector, 200°C; detector, 220°C; and column, linearly programed from 85°C to 200°C at a rate of 4°C/min.

RESULTS AND DISCUSSION

From December 1981 to June 1982, six separate experiments were conducted. A total of 230 collections were made from 64 papaya trees. The rates of BITC released from the root systems in four experiments are shown in Table 1. In summary, our data indicate that during the collection period, viz., from 60 to 68 days of age, the Waimanalo cultivar had an average tree height

TABLE 1. RATE OF BITC RELEASE FROM ROOTS OF 2-MONTH-OLD HIGGINS AND WAIMANALO PAPAYA TREES

Data collected	BITC [μg/tree/day, mean $\pm$ SD (N)][a]	
	Higgins	Waimanalo
January 1982	2.30 $\pm$ 0.88 (20)	2.13 $\pm$ 0.83 (15)
February*	2.29 $\pm$ 0.90 (20)	2.01 $\pm$ 0.61 (18)
April**	3.09 $\pm$ 0.94 (8)	2.28 $\pm$ 1.15 (17)
June	2.37 $\pm$ 1.02 (16)	2.07 $\pm$ 1.51 (30)

[a]The mean of Higgins is significantly greater than that of Waimanalo at 0.1 (*) and 0.05 (**) levels according to the standard t test.

of 212 $\pm$ 29 mm and its root system released BITC at a rate of 0.57 $\pm$ 0.24 nmol/hr, equivalent to 2.03 $\pm$ 0.85 μg/day. Higgins, with an average tree height of 212 $\pm$ 31 mm, released 0.66 $\pm$ 0.30 nmol/hr, or 2.36 $\pm$ 1.06 μg/day.

The functions for the least-square linear regression plots (Figure 1) of BITC releasing rate vs. tree height are $y = 1.853 + 0.179x$; $r = 0.296$ for Waimanalo, and $y = 1.808 + 0.205x$; $r = 0.409$ for Higgins. Although small, the positive slopes corresponded to the expectation that larger trees produced more BITC. The correlations for the plots were poor for both cultivars as shown by the scatter diagrams and the low correlation coefficient r. This is not surprising because tree height may not be an accurate index of the surface area

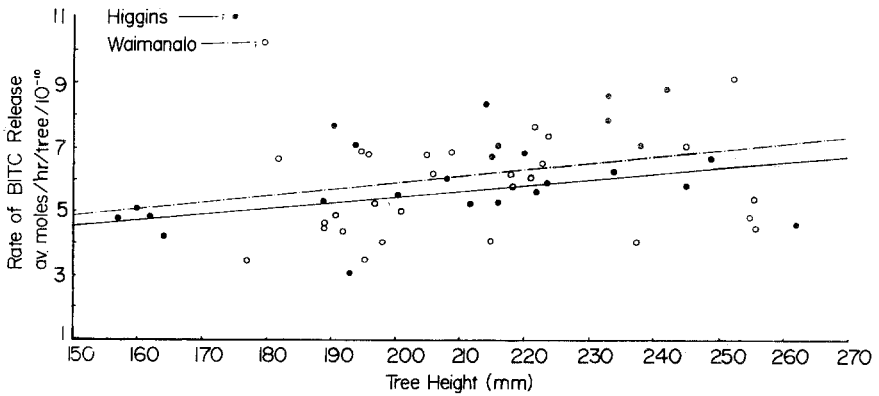

FIG. 1. Scatter diagrams and linear regression plots of papaya tree height vs. rate of BITC release from the roots: Each data point is the mean of 3–4 measurements taken between 60 and 68 days after planting. The regression functions and correlation coefficient r for Waimanalo and Higgins are $y = 1.853 + 0.179x$; $r = 0.296$, and $y = 1.808 + 0.205x$; $r = 0.409$, respectively.

of a root system. Furthermore, individual differences may exist within the same inbred line (Nakasone and Aragaki, 1973), and the enviromental factors may also vary from batch to batch and among the pots under the present experimental conditions. Substitution of the tree height with the dry weight of roots also showed poor correlation, probably due to the inconsistency of the weight to surface area ratio from plant to plant. The ideal parameter would be the surface area of the root system which, unfortunately, cannot be easily obtained.

The use of BITC production from papaya roots as a demonstration of the continuous root exudate trapping method has two major advantages: First, because BITC is a specific plant metabolite, the possibility that it originated from sources other than the root system of papaya can be readily ruled out. Secondly, quantitation of the trapped BITC can be carried out by a GC equipped with a sulfur-specific FPD without any precleaning procedures. BITC was the only major peak on the chromatogram. In constrast, a chromatogram (Figure 2) obtained from GC equipped with nonspecific flame ionization detectors (FID) showed more than 50 peaks. The FPD was approximately 10 times more sensitive than FID for BITC detection.

Due to its low water solubility, BITC released from the root surface would diffuse slowly into the soil solution, resulting in a concentration gradient at the root–soil interface. Organisms which interact successfully with the root system are those resistant to the inhibitor at concentrations maintained by an equilibrium between the release and the removal of BITC. Methods for the determination of this concentration gradient are presently

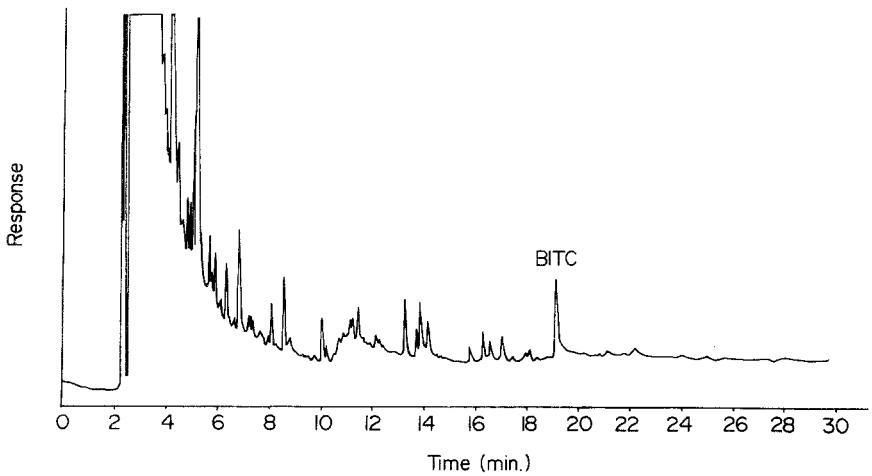

FIG. 2. A typical gas chromatogram of papaya root exudates on a 30-m OV-17 SCOT glass capillary column. For conditions see text.

not available. However, an in vitro experiment showed that BITC was lethal at 0.01 M to the mycelia of *Phytophthora palmivora*, the most serious papaya root rot pathogen (Tang and Aragaki, unpublished data). One may assume, accordingly, that with an exudation rate of ca. 2 μg/day, the concentration of BITC at the rhizosphere of a 2-month-old papaya tree did not reach 0.01 M. One may also speculate that, due to the presence of this gradient, the chemical environment of the papaya rhizosphere discriminates against organisms susceptible to BITC, and factors that affect this chemical equilibrium modify the existing ecological balance at the root-soil interface.

It has been reported that the Waimanalo is resistant whereas the Higgins is susceptible to *P. palmivora* (Mosqueda-Vazques et al., 1981). Results in Table 1 showed that the former did not release BITC at a higher rate than the latter, suggesting that the rhizospheric BITC levels alone cannot account for differences in the resistance of these two cultivars.

To our knowledge, the present work is the first attempt to measure the rate at which a bioactive secondary metabolite is released from the undisturbed plant root system. Experiments using the continuous root exudate trapping system under more strictly controlled conditions, together with a GC-MS-DS system for identification and quantitation, would further improve the reliability of this method.

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INFLUENCE OF IONIC THIOCYANATE ON GROWTH OF CABBAGE, BEAN, AND TOBACCO

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Abstract—Plant weight and contents of chlorophyll, ionic thiocyanate (SCN^-), and hydrogen cyanide (HCN) were determined in cabbage (*Brassica oleracea* var. *capitata* L. cv. Early Greenball), bean (*Phaseolus vulgaris* L. cv. Contender), and tobacco (*Nicotiana tabacum* L. cv. Delhi 76) grown hydroponically in modified Hoagland's nutrient solution with six concentrations of SCN^- (supplied as KSCN) (0, 5, 25, 50, 100, and 200 mg/liter). Whereas tobacco plants did not grow with any level of SCN^- in the culture solution, beans grew with 5 mg/liter and cabbages grew with between 5 and 50 mg/liter. Increasing levels of SCN^- in the culture solution resulted in decreased growth and chlorophyll content, accompanied by consistently increasing amounts of SCN^- in cabbage. Small amounts of HCN found only in tissues of cabbage were not influenced by levels of SCN^-. The greater insensitivity of cabbages to the presence of SCN^- compared with beans is apparently related to the presence of endogenous glucosinolates which are capable of being degraded into SCN^-. Accumulation of SCN^- and occurrence of leaf chlorosis in cabbage and beans and death of tobacco plants supplied with SCN^- in hydroponic culture confirm the capacity of SCN^- as an allelopathic agent, but its effect mechanism in ecology needs to be demonstrated.

Key Words—*Brassica oleracea* var. *capitata*, *Phaseolus vulgaris*, *Nicotiana tabacum*, thiocyanate, allelopathy.

INTRODUCTION

Investigators have reported that thiocyanate ion (SCN^-) appears to be toxic to plants (Evanari, 1949; Harvey, 1931; Landen, 1934; Reger, 1944; Wu and

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Basler, 1969). Thiocyanates applied as sprays to foliage and fruit of apple influenced development of chlorosis in leaves and red color in fruits (Dustman and Duncan, 1939, 1940). A high concentration (2%) of NH_4SCN inhibited germination and respiration in potato tubers (Ranjau and Kaur, 1954).

Thiocyanate ion has been implicated directly or indirectly as an allelopathic agent. Low yields of grass and other crops have been reported in fields where *Brassica* had previously been cultivated (Campbell, 1959; Horricks, 1969; Kuťacek, 1964; Stewart, 1939). The invasion of grassland by mustard (*Brassica nigra* L.) on the slopes of southern California was postulated to be due to rainwater leachates from residues of the *Brassica* crop from the previous year (Bell and Muller, 1973). Heavy residue of rape (*B. campestris* L.) reduced yields of wheat, barley, and oats (Horricks, 1969).

Kutácek (1964) postulated that one of the inhibiting factors acting in the *Brassica* effect may be indole glucosinolates (or their degradation products) released from parts of plants left in the soil. Woad (*Isatis tinctoria* L.), which has exceptionally high levels of indole glucosinolates, often was not grown because of its effect on later crops (Elliot and Stowe, 1971). Brassicaceous plants characteristically contain glucosinolates which can be degraded into isothiocyanates (mustard oils), thiocyanates, and other related derivatives (Ju, 1980; Ju et al., 1982).

With a view to learning more about the allelopathic role of thiocyanates, we examined in this study the growth of cabbage, bean, and tobacco plants in hydroponic culture supplied with SCN^- (as KSCN) and also the measured contents of chlorophyll, SCN^-, and hydrogen cyanide (HCN) in tissues of these plants.

METHODS AND MATERIALS

On December 17, seeds of Delhi tobacco were sown in fiber flats (25 × 15 cm) containing silica sand (size No. 24). On January 7 and January 21, seeds of Early Greenball cabbage and Contender beans, respectively, were sown in similar flats containing a 1:1 (v/v) mixture of peat moss and vermiculite. After emergence, seedlings were watered and fertilized every other day with a dilute solution of 20–20–20 fertilizer (0.5 g/liter H_2O). Plants at all stages of growth were kept under greenhouse conditions at a mean daily temperature of 22 ± 2°C.

On February 21, seedlings of each species were removed from the flats, washed with distilled water (22°C), wiped free of excess water, and weighed. Seedlings of uniform weight (tobacco, ca. 1.6 g; cabbage, ca. 1.8 g; beans, ca. 4.0 g) were transplanted to white styrofoam platforms (2.5 cm thick × 30 cm wide × 30 cm long) placed over 27-cm-diameter pots, each lined with polyethylene film and containing 9 liters of nutrient solution. There were five

TABLE 1. MODIFIED HOAGLAND'S NUTRIENT SOLUTION

Nutrients	SCN^- Treatment (ppm)[a]					
	0	5	25	50	100	200
KNO_3	0.004[b]	0.004	0.004	0.004	0.00325	0.00175
$Ca(NO_3) \cdot 4H_2O$	0.005	0.005	0.005	0.005	0.005	0.0055
NH_4NO_3	0.001	0.001	0.0005	0.00025	0.0005	0.0005
$MgSO_4 \cdot 7H_2O$	0.002	0.002	0.002	0.002	0.002	0.002
KH_2PO_4	0.001	0.001	0.005	0.00025	0.000	0.000
$NH_4H_2PO_4$	0.000	0.000	0.0005	0.00075	0.001	0.001

[a]5 ppm Fe added as FeEDTA and Hoagland's micronutrients were added to each treatment.
[b]Each datum is expressed in molar concentration.

seedlings per platform at a spacing of 13.5 cm between seedlings. The nutrient solution consisted of Hoagland's solution modified to obtain six SCN^- treatments (0, 5, 25, 50, 100, and 200 ppm) using KSCN (Table 1). The SCN^- treatments were arranged according to a randomized complete block design with three replications of each treatment for each species. During the experiment, nutrient solutions were aerated continuously and were renewed at weekly intervals, at which times the weight of plants was obtained (Ju et al., 1982).

Cabbage and bean plants only were harvested after 5 weeks of growth in hydroponic culture; tobacco plants did not grow in any SCN^- treatments. At harvest, plants were divided into top and roots. Top fresh weight, root fresh weight, and number of leaves of cabbage and beans, and also number of pods and pod weight of beans were recorded. The combined tops or roots of two plants of each species within each treatment and replication were divided longitudinally into two portions, one for chemical analysis and the other for dry weight determination. Chlorophyll content was determined by the spectrophotometric procedure of Arnon (1949) in lamina tissue samples (ca. 0.25 g) taken by a cork borer from both young and older leaves. HCN content was determined from similar tissue samples by the spectrophotometric procedure of Gilchrist et al. (1967). SCN^- was extracted with water from the remainder of the top and from root samples and was determined spectrophotometrically in duplicate as previously described by Chong and Bible (1974). Readings of SCN^- content were corrected for the percentage moisture in each sample and were expressed as micrograms of KSCN per gram dry weight of tissue.

Two other plants of each species within each treatment and replication were divided as follows: young leaves, older leaves, and stem for cabbage; pod, leaves, petioles, and stem for beans. These tissues were analyzed similarly for chlorophyll, SCN^-, and HCN.

TABLE 2. EFFECT OF SCN^- SUPPLIED AS KSCN ON GROWTH OF EARLY GREENBALL CABBAGE AND CONTENDER BEANS IN HYDROPONIC CULTURE

SCN^- in hydroponic culture (mg/liter)	Cabbage			Beans				
	Fresh weight (g/plant)		Number of leaves	Fresh weight (g/plant)			Number of leaves	Number of pods
	Top	Root		Top	Root	Pod		
0	109.0 ± 14.0[a]	13.5 ± 3.5	14 ± 0.6	56.9 ± 9.8	20.8 ± 4.6	20.0 ± 7.5	12 ± 1.2	8 ± 2.3
5	89.1 ± 17.9	14.8 ± 2.9	13 ± 1.2	37.1 ± 5.2	15.3 ± 2.3	9.3 ± 4.0	10 ± 1.2	6 ± 2.3
25	48.9 ± 8.7	7.6 ± 1.7	13 ± 0.6	1.2 ± 0.6	1.8 ± 0.6	0.0 ± 0.0	3 ± 1.2	0 ± 0.0
50	29.0 ± 5.2	6.5 ± 1.7	12 ± 1.2	—[b]	—	—	—	—
100	2.7 ± 1.2	1.2 ± 0.6	6 ± 1.2	—	—	—	—	—
LSD ($P = 0.05$)	15.2	1.8	2.3	14.7	7.1	7.1	2.9	3.5

[a] Each datum represents the mean ±SE of three replications each with five plants recorded after five weeks of growth in hydroponic culture.
[b] Plants died in SCN^- treatments > 25 mg/liter.

RESULTS AND DISCUSSION

While tobacco plants died in all SCN^- treatments, cabbages and beans were less sensitive to the presence of SCN^-. However, beans were more sensitive than cabbages (Table 2). Beans grew in SCN^- treatment with 5 mg/liter; growth was drastically impaired with 25 mg/liter, and death of plants occurred in treatments with higher levels of SCN^-. Cabbages grew in SCN^- treatments between 5 and 50 mg/liter, and growth was drastically impaired with 100 mg/liter SCN^-.

While number of leaves in cabbage was little affected by increasing levels of SCN^- up to 50 mg/liter, growth (both top and roots) (Table 2) and degree of chlorosis as measured by chlorophyll content in leaf tissue (Table 3) increased with increasing SCN^- levels in the culture solution. In contrast, SCN^- content analyzed in cabbage tops and in young leaves increased with increasing levels of SCN^- in the culture solution (Table 4).

Small amounts of HCN (expressed in μg/g fresh weight) found in tissues of cabbage (top, 6.7–9.2; young leaves, 8.1–10.8; older leaves, 6.1–8.3; stem, 5.7–10.7) were not significantly different due to SCN^- treatments except in stem tissue ($P = 0.05$). HCN was not detected in bean tissues.

Harvey (1931) suggested that NH_4SCN acted as a protoplasmic poison by coagulating proteins or combining with cell constituents. Landen (1934) showed that the activity of catalase was reduced by 65% with 0.1 M NH_4SCN in plant tissue preparations and suggested that the affinity of NH_4SCN for iron may, in part, account for its toxicity. According to Reger (1944), thiocyanate-induced chlorosis may be corrected by spraying with soluble iron.

TABLE 3. INFLUENCE OF SCN^- SUPPLIED AS KSCN IN HYDROPONIC CULTURE ON ANALYZED CONTENTS OF CHLOROPHYLL IN EARLY GREENBALL CABBAGE AND CONTENDER BEANS

SCN^- in hydroponic culture (mg/liter)	Chlorophyll in leaf tissue (mg/g fresh weight)	
	Cabbage	Beans
0	1.08 ± 0.03[a]	1.47 ± 0.08
5	1.06 ± 0.08	1.29 ± 0.05
25	1.03 ± 0.06	0.69 ± 0.02
50	0.84 ± 0.09	—[b]
100	0.80 ± 0.05	—
LSD ($P = 0.05$)	0.13	0.10

[a]Each datum represents the mean ± SE of three replications each with two plants harvested after five weeks of growth in hydroponic culture.
[b]Plants died in SCN^- treatments >25 mg/liter.

TABLE 4. INFLUENCE OF SCN^- SUPPLIED AS KSCN IN HYDROPONIC CULTURE ON ANALYZED CONTENTS OF SCN^- IN DIFFERENT PLANT TISSUES OF EARLY GREENBALL CABBAGE AND CONTENDER BEANS

SCN^- in hydroponic culture (mg/liter)	SCN^- (μg/g dry weight)								
	Cabbage				Bean				
	Top	Young leaves	Older leaves	Stem	Top	Leaves	Petioles	Stem	Pods
0	171 ± 23[a]	366 ± 28	96 ± 13	140 ± 30	6 ± 26	9.3 ± 23	0.7 ± 0.2	0.6 ± 0.2	0 ± 0
5	166 ± 36	656 ± 80	143 ± 17	120 ± 12	201 ± 69	225 ± 45	3.6 ± 0.9	4.3 ± 0.1	6.5 ± 1.2
25	236 ± 40	641 ± 63	99 ± 26	106 ± 13	343 ± 140	—	—	—	—
50	236 ± 31	1526 ± 230	199 ± 24	188 ± 19	—[c]	—	—	—	—
100	430 ± 64	—[b]	—	—	—	—	—	—	—
LSD ($P = 0.05$)	125	438	NS	NS	NS	202	NS	NS	5.5

[a]Each datum is the mean ± SE of three replications with two plants harvested after five weeks of growth in hydroponic culture.
[b]Samples were insufficient for chemical analysis.
[c]Plants died in SCN^- treatments > 25 mg/liter.

Wu and Basler (1969) reported that high concentrations of NH_4SCN inhibited the photosynthetic O_2 release in intact tissue and caused a rapid inactivation of isolated chloroplasts in their ability to carry out the Hill reaction. This evidence indicates that the physiological influence of SCN^- is related to its ability to combine with iron, making it less available for chlorophyll synthesis.

In comparison with the control treatment, there was marked relative accumulation of SCN^- in leaves of beans which had a low tolerance for SCN^- (5 mg/liter). In contrast, there was correspondingly low relative accumulation of SCN^- in leaves of cabbage which had a high tolerance for SCN^- ($\leqslant$ 50 mg/liter). This result in cabbage is noteworthy in view of the presence of thiocyanate-yielding indole glucosinolates, which are the major glucosinolates in this species (VanEtten et al., 1979). In fact, comparative analysis of different plant parts indicated that young leaves of cabbage and also leaves of beans were the major organs in which SCN^- accumulated (Table 4). This evidence confirms previous findings that young vegetative tissue, such as leaves, is one of the major sites of synthesis or accumulation of parent glucosinolates or their enzyme(s) of hydrolysis in brassicaceous plants (Chong and Bible, 1974).

Ju (1980) suggested that the markedly higher synthesis or accumulation of SCN^- derived from indolyl glucosinolates during the early seedling stage might function allelopathically in suppressing competition of weeds during ontogeny in turnip (*B. rapa* L.) and rutabaga (*B. napobrassica* L.). Elliot and Stowe (1971) postulated that indolyl glucosinolates released or degraded from dead cells of woad may act as allelopathic agents.

Any phytotoxin could be an allelopathic agent if there exists a mechanism that releases it in sufficient concentration and if it persists long enough to inhibit growth of another plant. The present study demonstrates contrasting degrees of growth inhibition of cabbage, bean, and tobacco as influenced by SCN^- in culture solution. Clearly, the accumulation of SCN^- and occurrence of leaf chlorosis in cabbage and beans and death of tobacco plants supplied with SCN^- in hydroponic culture confirm the capacity of SCN^- as an allelopathic agent. Brassicaceous species contain various phytotoxins resulting from breakdown of glucosinolates. While there may exist an effective mechanism that releases such phytotoxins as a result of the rotting of brassicaceous plant residue (Bell and Muller, 1973), it remains to be shown that SCN^- is an allelopathic agent under such circumstances.

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GROWTH ANALYSIS OF CORN AND SOYBEAN RESPONSE TO ALLELOPATHIC EFFECTS OF WEED RESIDUES AT VARIOUS TEMPERATURES AND PHOTOSYNTHETIC PHOTON FLUX DENSITIES

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Abstract—The effects of redroot pigweed (*Amaranthus retroflexus* L.) and yellow foxtail [*Setaria glauca* (L.) Beauv.] residues on corn (*Zea mays* L.) and soybeans [*Glycine max* (L.) Merr.] were evaluated at various temperatures and photosynthetic photon flux densities (PPFD) in a Biotron. Mathematical growth analysis techniques were used for the evaluation. Redroot pigweed markedly reduced leaf area duration (LAD), leaf weight ratio (LWR), and total dry matter production in both crops. Yellow foxtail residue inhibited total dry matter production in corn and soybeans 20 and 30 days after planting (DAP). It also reduced growth rate (GR) and LAD in corn and biomass increment (Δ W) in soybeans. Biomass increment was more closely correlated to LAD than net assimilation rate (NAR) in soybeans, whereas in corn NAR contributed more to Δ W than LAD. The 30/20° C temperature with a PPFD of 380 μE/m^2/sec produced a larger Δ W with a greater NAR and larger LAD in corn, resulting in maximum dry matter accumulation than 20/10° C and other levels of PPFD. Under similar conditions, soybeans showed little or no response to the changes. The results demonstrate the allelopathic effects of weed residues on growth and dry matter production, affecting LAD, LWR, and GR. The results also show that environmental temperature and PPFD may alter the allelopathic effects of weeds on crops. The possible interference with photosynthesis and the partitioning of biomass into leaf component relative to the total biomass produced by the plant may be the inhibitory effects of allelochemicals present in redroot pigweed and yellow foxtail residues.

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Key Words—*Amaranthus retroflexus*, *Setaria glauca*, allelochemicals, temperature, light condition, *Zea mays*, *corn*, *glycine max*, soybeans.

INTRODUCTION

The evidence that allelopathy may be a part of weed and crop interactions has been documented (Rice, 1974). Allelopathic relationships may change the composition of species in a cropping system. Putnam and Duke (1978) suggested that allelopathy may be implicated in shifts in species distribution and increases in dominance by certain annual and perennial weeds in a variety of crop ecosystems.

The allelopathic effects of giant foxtail (*Setaria faberi* Herrm.) reduced corn growth in a greenhouse study (Bell and Koeppe, 1972). The field studies of Chambers and Holm (1965) demonstrated that green foxtail [*Setaria viridis* (L.) Beauv.] and redroot pigweed reduced the growth and P uptake in snap beans (*Phaseolus vulgaris* L.). Lower crop yields sometimes observed in stubble-mulch systems have been attributed in part to the release of growth-inhibiting substances from crop residues (Guenzi and McCalla, 1966; Cochran et al., 1977).

Greenhouse experiments have shown that residues of common lambsquarters (*Chenopodium album* L.) redroot pigweed, velvetleaf (*Abutilon theophrasti* Medic.), and yellow foxtail inhibited corn and soybean growth (Bhowmik and Doll, 1979, 1982). Growth reductions were not due to allelopathic interference on mineral uptake (Bhowmik, 1981). Thus, the growth reductions may be attributed to effects on other physiological processes involved in dry matter production.

Various phenolic acids have been reported to inhibit net photosynthetic rate (Einhellig et al., 1970; Rice, 1974, Patterson, 1981). Einhellig et al. (1970) reported that scopoletin, a coumarin derivative, inhibited dry matter production, leaf area expansion, and photosynthesis in tobacco (*Nicotiana tabacum* L.)., sunflower (*Helianthus annuus* L.), and redroot pigweed. Recently, Patterson (1981) investigated the effects of various phenolic compounds on the physiological responses of soybeans. He found several phenolic acids that significantly reduced dry matter production, leaf area expansion, height, leaf production, net assimilation rate (NAR), and leaf area duration (LAD). These same compounds also caused a marked reduction in leaf chlorophyll concentration.

Environmental factors affect the amount of phytotoxins produced in plants. Moore et al. (1967) reported that reducing the photosynthetic photon flux density (PPFD) received by a plant can increase its alkaloid content. Deficiency of N produced large increases in chlorogenic acid in the stem and leaves of sunflower (del Moral, 1972; Lehman and Rice, 1972). However, no information is available as to how environmental factors could alter the

response of test species to allelopathic compounds. This research project was initiated to determine if temperature and PPFD could alter the response of corn and soybeans to the effects of weed residues and to further investigate the inhibitory action of allelochemicals present in weed residues. This study reports the effects of two weed residues on corn and soybeans grown under two temperatures and four PPFDs.

METHODS AND MATERIALS

Collection of Weed Residue. Mature redroot pigweed and yellow foxtail plants (whole plants) were collected in September 1979 from the field at the University of Wisconsin Arlington Experimental Farm. The samples were oven-dried at 70° C for 24 hr and ground in a Wiley mill to pass a 1.27-mm^2 screen. Ground samples were stored at room temperature in plastic bottles.

Growing Conditions. Corn and soybeans were grown in 12.5-cm plastic pots (500 ml) containing 500 g silica sand (grade No. 70, Portage Silica Sand Co., Rockton, Illinois) at the University of Wisconsin Biotron. In one 2.6 × 3.6-m room, day-night temperatures were 20 and 10° C and in the other 30 and 20° C. Both rooms received a 14-hr photoperiod and had relative humidities of 70 ± 5%. Incandescent and fluorescent lamps were the light source. In each temperature regime four levels of PPFD were established. The highest level was 760 $\mu E/m^2/sec$ 20 cm above the pots as determined with a quantum meter (Lambda LI-185 Quantum/Radiometer/Photometer, Lambda). In the other groups, light reductions to 25, 50, or 75% of maximum PPFD were obtained using layers of cheese cloth. Thirty-six pots were included for each level of PPFD.

Weed residues were incorporated into silica sand at 1.0 and 0.75% (w/w) for the corn and soybean experiments, respectively. Corn (W5452) and soybean (Steele) seeds were planted 2.5 cm deep. After emergence, seedlings were thinned to two plants per pot. The pots were watered daily to field capacity by weight with half-strength Hoagland's solution (Hoagland and Arnon, 1950). Control with commercial peat moss and with silica sand were used. Since there were no significant differences between the two types of controls, the results were combined and the averages were used as a control for comparisons.

Harvest Procedures. At 10, 20, and 30 days after planting (DAP), eight plants from each residue and PPFD were harvested. The heights to the longest extended leaf for corn and to the growing point for soybeans, leaf areas, dry weights (70° C oven-dried) of leaves, shoots, and roots were determined. The leaf area (LA) was determined by an automatic area meter (Type AAM5, Tokyo Hayashi Denco, Ltd., Tokyo, Japan).

Mathematical Analysis of Growth. The procedures of Radford (1967), Kvet et al. (1971), and Patterson et al. (1979) were used to carry out a

mathematical growth analysis of corn and soybean growth. The following formulas were used to calculate the necessary growth parameters:

1. Relative growth rate (RGR) or the amount of dry matter produced per unit of dry matter present per unit of time (g/g/day).

$$RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1)$$

where t_1 and t_2 = time in days; and W_1 and W_2 = total dry weight at time t_1 and t_2.

2. Relative leaf area growth rate (RLAGR) or the amount of leaf area produced per unit of leaf area present per unit of time ($dm/dm^2/day$).

$$RLAGR = (\ln A_2 - \ln A_1)/(t_2 - t_1)$$

where A_1 and A_2 = total leaf area at time t_1 and t_2.

3. Net assimilation rate (NAR) or the amount of dry matter produced per unit of leaf area present per unit of time ($g/dm^2/day$).

$$NAR = [1/(t_2 - t_1)][(W_2/A_2) - (W_1/A_1)][\alpha/(\alpha - 1)]$$

where $\alpha = RGR/RLAGR$.

4. Biomass increment (Δ W) or the amount of dry matter produced over a period of time (g days).

$$\Delta W = (W_2 - W_1)$$

5. Growth rate (GR) or the amount of dry matter produced per unit of time (g/day)

$$GR = \Delta W/(t_2 - t_1)$$

6. Leaf area duration (LAD) or the total leaf area present during a particular time interval (dm^2 days).

$$LAD = [(A_2 - A_1)/(\ln A_2 - \ln A_1)](t_2 - t_1)$$

7. Leaf area ratio (LAR) or the amount of leaf area per unit of total plant dry weight (dm^2/g).

$$LAR = LA/W$$

where LA = total leaf area; and W = total plant dry weight.

8. Leaf weight ratio (LWR) or the amount of leaf weight per unit of total plant dry weight (g/g).

$$LWR = LDW/W$$

where LDW = leaf dry weight.

9. Specific leaf weight (SLW) or the amount of leaf dry weight per unit of leaf area (g/dm^2).

$$SLW = LDW/LA$$

Statistical Analysis. The experimental design was a randomized complete block with split-split plots (temperature as the main plot and PPFD and weed residue as sub-subplots) with four replications. A three-way analysis of variance was used to determine significant differences among treatments. The temperature effects averaged over PPFD and residue, the effects of PPFD averaged over temperature and residue, and the residue effects averaged over temperature and PPFD were determined. The pooled mean values for the treatments were separated by Duncan's multiple-range test at the 0.05 level for the single harvest data and the interval growth analysis data. The coefficient of determination was used as an indication of the amount of variation in the dependent variable that could be accounted for by variation in the independent variable (Steele and Torrie, 1960).

RESULTS

Plant Growth. At 10 DAP, corn height and leaf area were not affected by temperature (Table 1). Corn plants grown at 20/10° C accumulated more dry matter with a smaller shoot-root weight ratio than the plants grown at 30/20° C. At 20 DAP, temperature did not affect corn height, dry weight, and leaf area, although shoot-root ratio was greater at the high temperature than at the low temperature. At 30 DAP, corn grown at 30/20° C was taller, with greater dry weight and larger leaf area and shoot-root ratio than those at 20/10° C.

Increased PPFD did not affect corn growth at 10 DAP. However, at 20 and 30 DAP, corn plants receiving 380 μE/m^2/sec or higher PPFD produced more dry matter than the plants receiving the lowest PPFD (190 μE/m^2/sec). The effect of PPFD on corn shoot-root ratio was inconsistent at various growth stages.

Redroot pigweed residue reduced corn height, leaf area, and dry weights at all growth stages. Yellow foxtail residue reduced total dry weights 10, 20, and 30 DAP, and inhibited corn height and leaf area only at 30 DAP. Larger shoot-root weight ratios were observed with redroot pigweed residue, indicating a greater inhibitory effect on root growth.

At 10 and 20 DAP, soybeans grown at 20/10° C accumulated more dry matter than the plants grown at 30/20° C (Table 2). Leaf area expansion in soybeans was not affected by temperature. A higher shoot-root ratio was observed 10 DAP for the 20/10° C temperature than at 30/20° C. The shoot-root ratio was not affected by temperature 20 and 30 DAP.

Increased PPFD did not influence soybean dry weight or leaf area at any growth stage. As PPFD increased, the shoot-root ratio in soybeans generally decreased.

Redroot pigweed residue inhibited height, dry weight, and leaf area in soybeans at all growth stages. It also resulted in the highest shoot-root dry

TABLE 1. EFFECTS OF TEMPERATURE, PPFD, AND WEED RESIDUE ON GROWTH OF CORN 10, 20, AND 30 DAYS AFTER PLANTING

Source of variation	Parameter/plant											
	10 DAP[a]				20 DAP				30 DAP			
	Height (cm)	Dry weight (g)	Leaf area (dm^2)	S/R[b] ratio	Height (cm)	Dry weight (g)	Leaf area (dm^2)	S/R ratio	Height (cm)	Dry weight (g)	Leaf area (dm^2)	S/R ratio
Temperature (°C)												
20/10	21.4a[d]	0.44a	5.8a	0.8b	35.3a	1.45a	16.2a	1.0b	44.8b	3.11b	27.8b	1.1b
30/20	24.2a	0.28b	5.3a	1.4a	42.8a	1.31a	17.5a	1.5a	67.1a	4.18a	42.0a	1.6a
PPFD[c] ($\mu E/m^2/sec$)												
190	22.2a	0.29a	5.3a	1.1ab	36.2a	0.78b	13.0a	1.2bc	59.9a	2.32b	33.5bc	1.5a
380	22.8a	0.36a	5.4a	1.2a	43.7a	1.50a	19.8a	1.4a	58.5a	3.85a	36.9ab	1.3a
570	24.3a	0.40a	6.2a	1.1ab	43.0a	1.75a	19.9a	1.3ab	60.4a	4.46a	38.7a	1.3a
760	21.8a	0.39a	5.3a	1.0b	33.3a	1.47a	14.8a	1.1c	48.0b	3.94a	30.5c	1.2a
Weed residue												
Redroot pigweed	20.6b	0.30c	5.0b	1.6a	36.1b	1.15b	15.0b	1.5a	52.1b	3.27b	32.3b	1.4a
Yellow foxtail	23.3a	0.36b	5.7ab	1.0b	39.5a	1.36b	16.7ab	1.1b	54.8b	3.21b	32.6b	1.4a
Control	24.5a	0.41a	6.0a	0.8c	41.5a	1.61a	18.9a	1.1b	61.0a	4.43a	39.8a	1.2b

[a]DAP = days after planting.
[b]S/R ratio = shoot/root dry weight ratio.
[c]PPFD = photosynthetic photon flux density.
[d]For each source of variation, means within a column followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.

TABLE 2. EFFECTS OF TEMPERATURE, PPFD, AND WEED RESIDUE ON GROWTH OF SOYBEANS 10, 20, AND 30 DAYS AFTER PLANTING[a]

Source of variation	Parameter/plant											
	10 DAP				20 DAP				30 DAP			
	Height (cm)	Dry weight (g)	Leaf area (dm^2)	S/R ratio	Height (cm)	Dry weight (g)	Leaf area (dm^2)	S/R ratio	Height (cm)	Dry weight (g)	Leaf area (dm^2)	S/R ratio
Temperature (°C)												
20/10	5.7a	0.29a	2.7a	4.2a	10.0a	1.09a	12.2a	2.7a	15.0b	2.11a	22.0a	2.4a
30/20	5.8a	0.18b	2.0a	3.4b	11.5a	0.61b	9.2a	2.7a	26.4a	1.71a	23.5a	2.7a
PPFD ($\mu E/m^2/sec$)												
190	7.1a	0.23a	2.7a	4.3a	14.1a	0.80a	12.1a	3.3a	28.4a	1.86a	27.7a	3.5a
380	5.6bc	0.23a	2.3a	4.1a	10.5ab	0.85a	11.2a	2.6a	20.8a	2.05a	23.7a	2.5ab
570	5.9ab	0.27a	2.6a	3.7ab	10.9ab	0.99a	12.0a	2.4a	19.5a	1.96a	22.2a	2.4ab
760	4.3c	0.23a	1.9a	3.1b	7.6b	0.75a	7.3a	2.5a	14.1a	1.75a	17.5a	1.9b
Weed residue												
Redroot pigweed	4.9b	0.18b	1.8b	5.8a	10.0b	0.63c	8.1c	3.5a	17.9b	1.51c	18.1c	3.2a
Yellow foxtail	6.3a	0.26a	2.7a	3.2b	10.7b	0.87b	11.2b	2.4b	20.8a	1.91b	22.1b	2.2b
Control	6.0a	0.28a	2.7a	2.3b	11.5a	1.03a	12.7a	2.3b	23.5a	2.30a	28.0a	2.3b

[a] Abbreviations as in Table 1.

weight ratio in soybeans as compared to the control plants. Yellow foxtail residue did not affect soybean growth 10 DAP. However, soybean dry weights and leaf areas were reduced by yellow foxtail residue 20 and 30 DAP.

Mathematical Growth Analysis. During the first interval, temperature did not affect GR, NAR, LAD, or RGR in corn (Table 3). At this time interval, GR per plant was reduced by the lowest PPFD, and redroot pigweed residue significantly reduced GR and LAD in corn.

During the second interval, corn GR was greater at 30/20°C due to the larger LAD with a greater NAR and RGR. Increasing PPFD above 190 $\mu E/m^2/sec$ increased the GR and NAR in corn while LAD increased up to 570 $\mu E/m^2/sec$ of PPFD. Redroot pigweed and yellow foxtail residues reduced the GR and LAD in corn during the second interval.

Leaf area ratio and LWR in corn were unaffected by temperature during the first interval (Table 4). The 30/20°C temperature significantly reduced SLW. Leaf area ratio and LWR were unaffected by PPFD. However, the lowest PPFD resulted in the smallest SLW as compared to the other levels. Only redroot pigweed residue significantly increased LAR and LWR in corn with no effect on SLW.

The effects of temperature and PPFD on LAR and LWR in corn during the second interval were identical to those observed during the first interval. At this interval, SLW was unaffected by temperature. Redroot pigweed residue increased LAR and decreased SLW of corn. Yellow foxtail residue did not influence any growth parameter except LAR, which was larger than the control.

During the first interval, soybeans grown in the low-temperature regime had a greater GR that correlated to increased LAD (Table 5). Soybean growth parameters were not affected by PPFD. Redroot pigweed residue reduced GR and LAD, whereas yellow foxtail residue reduced only GR in soybeans.

Neither temperature nor PPFD affected the GR, NAR, LAD, or RGR in soybeans during the second interval except that RGR was greater at the high temperature than the low temperature. At this time interval, redroot pigweed residue inhibited the GR of soybeans. Leaf area duration in soybeans decreased 38 and 17% with redroot pigweed and yellow foxtail residues, respectively.

In soybeans, high temperature increased LAR and decreased LWR and SLW during the first interval (Table 6). The highest PPFD resulted in the lowest LAR and in the highest SLW in soybeans as compared to the lowest PPFD. The LWR and SLW increased with redroot pigweed residue, whereas LAR was unaffected.

Similar effects of temperature on LAR, LWR, and SLW during the first interval were observed at the second interval in that high temperature increased LAR and decreased LWR and SLW. The effect of PPFD on LAR was also similar to that obtained during the first interval. The LWR in

TABLE 3. EFFECTS OF TEMPERATURE, PPFD, AND WEED RESIDUE ON GR, NAR, LAD, AND RGR IN CORN AS DETERMINED BY GROWTH ANALYSIS

Source of variation	Parameter[a]							
	10–20 days				20–30 days			
	GR (g/day)	NAR (g/dm^2/day)	LAD (dm^{2}days)	RGR (g/g/day)	GR (g/day)	NAR (g/dm^2/day)	LAD (dm^{2}days)	RGR (g/g/day)
Temperature (°C)								
20/10	0.101a	0.010a	100.7a	0.118a	0.165b	0.008b	213.7b	0.073b
30/20	0.103a	0.010a	100.0a	0.145a	0.287a	0.011a	276.3a	0.130a
PPFD (μE/m^2/sec)								
190	0.048b	0.006a	85.1a	0.099a	0.154b	0.007b	213.5b	0.118a
380	0.115a	0.011a	109.1a	0.146a	0.234a	0.009ab	271.2a	0.097a
570	0.136a	0.011a	115.3a	0.155a	0.271a	0.009ab	280.6a	0.094a
760	0.109a	0.011a	91.8a	0.126a	0.246a	0.011a	214.8b	0.098a
Weed residue								
Redroot pigweed	0.086b	0.009a	89.1b	0.130a	0.212b	0.009a	221.8b	0.114a
Yellow foxtail	0.100a	0.010a	100.6a	0.129a	0.185b	0.008a	234.7b	0.090b
Control	0.120a	0.011a	111.3a	0.136a	0.282a	0.010a	278.5a	0.102ab

[a]For each source of variation, means within a column followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.

TABLE 4. EFFECTS OF TEMPERATURE, PPFD, AND WEED RESIDUE ON LAR, LWR, AND SLW IN CORN AS DETERMINED BY GROWTH ANALYSIS

Source of variation	Parameter[a]					
	10–20 days			20–30 days		
	LAR (dm^2/g)	LWR (g/g)	SLW (g/dm^2)	LAR (dm^2/g)	LWR (g/g)	SLW (g/dm^2)
Temperature (°C)						
20/10	12.5a	0.30a	0.0025a	10.6a	0.31a	0.0031a
30/20	20.0a	0.34a	0.0021b	14.8a	0.33a	0.0029a
PPFD ($\mu E/m^2/sec$)						
190	22.5a	0.32a	0.0018b	18.6a	0.35a	0.0023b
380	16.2a	0.33a	0.0024a	12.6a	0.32ab	0.0030a
570	14.3a	0.32a	0.0024a	10.3a	0.32ab	0.0032a
760	12.1a	0.31a	0.0026a	9.3a	0.30ab	0.0033a
Weed residue						
Redroot pigweed	18.3a	0.34a	0.0022a	13.6a	0.33a	0.0028b
Yellow foxtail	16.6ab	0.32b	0.0024a	13.6a	0.32a	0.0029ab
Control	13.9b	0.30b	0.0024a	10.9b	0.32a	0.0031a

[a]For each source of variation, means within a column followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.

TABLE 5. EFFECTS OF TEMPERATURE, PPFD, AND WEED RESIDUE ON GR, NAR, LAD, AND RGR IN SOYBEANS AS DETERMINED BY GROWTH ANALYSIS

Source of variation	Parameter[a]							
	10–20 days				20–30 days			
	GR (g/day)	NAR ($g/dm^2/day$)	LAD (dm^2days)	RGR (g/g/day)	GR (g/day)	NAR ($g/dm^2/day$)	LAD (dm^2days)	RGR (g/g/day)
Temperature (°C)								
20/10	0.079a	0.014a	62.0a	0.129a	0.102a	0.007a	164.5a	0.069b
30/20	0.042b	0.010a	46.5b	0.120a	0.110a	0.007a	150.4a	0.105a
PPFD ($\mu E/m^2/sec$)								
190	0.057a	0.010a	61.5a	0.130a	0.106a	0.006a	185.6a	0.088a
380	0.062a	0.012a	54.6a	0.120a	0.120a	0.007a	164.5a	0.097a
570	0.072a	0.012a	60.9a	0.129a	0.097a	0.006a	164.1a	0.072a
760	0.052a	0.013a	39.8a	0.119a	0.101a	0.009a	115.7a	0.091a
Weed residue								
Redroot pigweed	0.046c	0.012a	39.8b	0.127a	0.088b	0.008a	121.2c	0.096a
Yellow foxtail	0.061b	0.011a	58.6a	0.119a	0.104ab	0.006b	158.8b	0.081a
Control	0.075a	0.012a	64.2a	0.128a	0.127a	0.007ab	192.3a	0.084a

[a] For each source of variation, means within a column followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.

TABLE 6. EFFECTS OF TEMPERATURE, PPFD, AND WEED RESIDUE ON LAR, LWR, AND SLW IN SOYBEANS AS DETERMINED BY GROWTH ANALYSIS

Source of variation	Parameter[a]					
	10–20 days			20–30 days		
	LAR (dm^2/g)	LWR (g/g)	SLW (g/dm^2)	LAR (dm^2/g)	LWR (g/g)	SLW (g/dm^2)
Temperature (°C)						
20/10	10.5b	0.54a	0.0061a	11.2b	0.51a	0.0048a
30/20	13.6a	0.38b	0.0029b	14.5a	0.46b	0.0033b
PPFD ($\mu E/m^2/sec$)						
190	14.1a	0.47a	0.0036b	15.7a	0.50a	0.0035c
380	12.4ab	0.46a	0.0048ab	13.2ab	0.49a	0.0039bc
570	11.8ab	0.46a	0.0045ab	12.1b	0.48a	0.0043ab
760	9.9b	0.45a	0.0050a	10.4b	0.46a	0.0046a
Weed residue						
Redroot pigweed	12.2a	0.48a	0.0053a	12.5a	0.51a	0.0043a
Yellow foxtail	12.2a	0.45b	0.0041b	12.9a	0.47b	0.0040b
Control	11.7a	0.44b	0.0041b	13.0a	0.47b	0.0039b

[a]For each source of variation, means within a column followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.

soybeans was unaffected by PPFD. Specific leaf weight increased significantly as the PPFD level increased. Redroot pigweed residue increased LWR and SLW in soybeans as compared to the control plants. These growth parameters were not affected by yellow foxtail residue.

Since biomass increment (ΔW) = $NAR \times LAD$, this relationship can be used to determine the relative importance of changes in dry matter production per unit leaf area (NAR) and the amount of leaf area present during a time interval (LAD) in accounting for changes in biomass increment (Patterson et al., 1979). In general, dry matter accumulation in corn was more closely related to NAR than to LAD, especially in the second interval, whereas for soybeans the reverse was true (Table 7).

To evaluate the effects of temperature and photosynthetic photon flux density on the allelopathic effects of weed residues in corn and soybeans, plant dry weights were compared at 30 days after planting. At 20/10° C temperature, corn was least inhibited by redroot pigweed residue with a PPFD of 570 $\mu E/m^2/sec$ (Table 8). Dry matter production in corn at the high temperature (30/20° C) increased as the PPFD increased up to 380 $\mu E/m^2/sec$ with redroot pigweed residue. In the presence of yellow foxtail residue, PPFD had no effect on dry weights at the low temperature, although dry weights increased as the PPFD increased at the high temperature. Control plants responded significantly to PPFD of 380 $\mu E/m^2/sec$ or greater at both temperatures.

Soybean plants showed no response to PPFD with redroot pigweed residue at either temperature (Table 9). In the presence of yellow foxtail residue, soybean dry weights increased as the PPFD increased up to 570 $\mu E/m^2/sec$ at the 20/10° C temperature. With 30/20° C temperature, soybeans responded negatively to increased PPFD. Soybean plants from the silica control showed no response to either temperature or PPFD.

TABLE 7. CORRELATION ANALYSIS AMONG BIOMASS INCREMENT (ΔW), NET ASSIMILATION RATE (NAR), AND LEAF AREA DURATION (LAD) IN CORN AND SOYBEANS AT TWO HARVEST INTERVALS

Harvest interval	Dependent variable	Independent variable	Coefficient of determination (%)	
			Corn	Soybean
First				
Day 10 to day 20	ΔW	NAR	61**[a]	40**
		LAD	49**	50**
Second				
Day 20 to day 30	ΔW	NAR	56**	28**
		LAD	10*	47**

[a]Level of significance * = 0.05 and ** = 0.01 level.

TABLE 8. INFLUENCE OF TEMPERATURE AND PPFD ON ALLELOPATHIC EFFECTS OF WEED RESIDUES ON CORN DRY WEIGHT 30 DAYS AFTER PLANTING

Temperature (°C)	PPFD ($\mu E/m^2/sec$)	Total dry weight (g/plant)		
		Weed residue		
		Redroot pigweed	Yellow foxtail	Control (no residue)
20/10	190	1.91b[a]	1.89a	2.58b
	380	3.19ab	2.91a	4.10a
	570	4.18a	2.89a	4.61a
	760	2.50b	2.05a	4.47a
Average over PPFD		2.95B[b]	2.44B	3.94A
30/20	190	1.89b	2.24c	3.40b
	380	3.79a	3.71b	5.38a
	570	4.31a	5.22a	5.57a
	760	4.45a	4.79ab	5.37a
Average over PPFD		3.61B	3.99B	4.93A
Average over temperature and PPFD		3.27B	3.21B	4.43A

[a] Means within a column followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.
[b] Means within a row followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.

DISCUSSION

Mathematical analysis of growth (Radford, 1967; Kvet et al., 1971) provides a means of determining the relative importance of leaf area and net assimilation rate (NAR) in accounting for differences in growth rate per plant (GR) or biomass increment (ΔW) among species or among plants of the same species. Therefore, any differences in total dry matter production as influenced by temperature, PPFD, or weed residue would be detected and consequently would be attributed to RGR, NAR, or LAD. In general, dry matter production in corn was more closely related to NAR than LAD, especially at the later growth stage, whereas LAD contributed more to soybean dry matter production.

The response of plants to allelochemicals may be altered by environmental factors of temperature and PPFD. The results show that the 30/20°C temperature and PPFD of 380–570 $\mu E/m^2/sec$ resulted in higher biomass increment, greater NAR, and larger LAD in corn. This led to higher dry matter production as compared to the other PPFDs and reflects the ability of corn plants to respond to environmental factors.

TABLE 9. INFLUENCE OF TEMPERATURE AND PPFD ON ALLELOPATHIC EFFECTS OF WEED RESIDUES ON SOYBEAN DRY WEIGHT 30 DAYS AFTER PLANTING

		Total dry weight (g/plant)		
		Weed residue		
Temperature (°C)	PPFD ($\mu E/m^2/sec$)	Redroot pigweed	Yellow foxtail	Control (no residue)
20/10	190	1.58a[a]	1.78b	2.24a
	380	1.95a	2.60a	2.93a
	570	1.56a	2.27ab	2.29a
	760	1.68a	1.81b	2.58a
Average over PPFD		1.69C[b]	2.12B	2.51A
30/20	190	1.64a	1.98a	1.93a
	380	1.21a	1.74ab	1.88a
	570	1.30a	1.88ab	2.47a
	760	1.19a	1.20b	2.06a
Average over PPFD		1.34C	1.70B	2.09A
Average over temperature and PPFD		1.51C	1.91B	2.30A

[a] Means within a column followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.
[b] Means within a row followed by the same letter are not significantly different at the 0.05 level as determined by Duncan's multiple-range test.

Under similar conditions, soybeans showed little or no response to changes in temperature or PPFD. The lack of response by soybeans to increased light and temperature was unexpected, as normally plant growth is correlated with temperature and PPFD increases.

Photosynthetic photon flux density influences the size and longevity of a leaf. In general, leaf area expansion is closely linked with PPFD (Kvet et al., 1971). Higher PPFD results in less expansion of a leaf. Partial shade or reduced levels of PPFD, therefore, could enhance its leaf area expansion. The observed changes in LAR in response to PPFD are important because LAR is one of the two components of RGR, which is the product of NAR and LAR. The results demonstrate that corn plants with PPFD of 380 $\mu E/m^2/sec$ or greater produced more biomass with greater NAR and larger LAD as compared to those with the lowest PPFD.

The rate of expansion of photosynthetic area is an important factor in determining productivity or survival of a species. The growth rate of a plant depends on the amount of leaf area present during a time interval (LAD) and the rate of dry matter increased per unit leaf area (NAR). The results of mathematical growth analysis show that redroot pigweed residue significantly

reduced LAD in both crops at both intervals, while yellow foxtail residue reduced LAD only during the second interval. Neither residue affected the NAR of either crop. This illustrates that biomass increment (ΔW) was primarily related to LAD, since a reduction in LAD led to reduced GR and ΔW. Similar results on itchgrass (*Rottboellia exaltata* L. f.) were reported by Patterson et al. (1979).

A close relationship between photosynthesis and chlorophyll concentration of soybeans has been reported (Buttery and Buzzell, 1977; Colton and Einhellig, 1980). Sprague and Curtis (1933) found that the amount of chlorophyll present in leaves or whole plants of corn was correlated with both total leaf area and growth rate. Since weed residues reduced GR and LAD in corn and soybeans in the present studies, it is reasonable to believe that there was a concomitant reduction in chlorophyll. Allelochemicals may interfere with chlorophyll metabolism (Einhellig and Rasmussen, 1979), and this could account for the observed reduction in dry matter production. However, no attempt was made to quantify chlorophyll concentration in the present studies.

Various phenolic acids have been reported to inhibit net photosynthetic rate (Einhellig et al., 1970; Rice, 1974; Patterson, 1981). Colton and Einhellig (1980) reported that aqueous extracts of freshly collected velvetleaf leaves inhibited growth of soybean seedlings. The chlorophyll content of treated soybean plants was reduced and inhibited plants gave evidence of water stress. It is possible that the inhibitory effects of redroot pigweed and yellow foxtail residues on crop growth are related to reduced chlorophyll synthesis and photosynthetic rate. In both crops, ΔW, GR, and LAD were reduced by these residues. Similar results were reported by Patterson (1981), who found that several phenolic acids reduced dry matter production, LA, NAR, and LAD in soybeans.

Redroot pigweed residue significantly increased LWR during the first interval in corn and during both intervals in soybeans. This illustrates that LWR in soybeans was affected for a longer period of time. The increase in LWR reflects changes in the partitioning of plant biomass into the leaf component relative to the total biomass produced by the plant so that less biomass was distributed into the roots as evidenced by the larger shoot–root dry weight ratio.

The differences in SLW reflect changes in the structure of the leaves or the distribution of leaf biomass as leaf surface area. High temperature (30/20°C) and the lowest PPFD (190 $\mu E/m^2/sec$) significantly decreased SLW in soybeans during both intervals, whereas in corn only the lowest PPFD decreased SLW. The reduction in SLW is evidence for less dense or thinner leaves under these conditions. This demonstrates that more biomass was utilized for the leaf area rather than leaf weight, since LWR was

unaffected by PPFD. The changes in biomass partitioning or distribution into leaves could account for the observed differences in reduced crop growth.

The results suggest that environmental factors of temperature and PPFD may alter the allelopathic effects of weeds on some crops. The inhibitory effects of weed residues on corn were reduced when the plants grew with a moderate PPFD (380–570 $\mu E/m^2/sec$) at 30/20°C temperature. Soybeans, on the other hand, showed no recovery from the inhibitory effects of weed residues at higher levels of PPFD or temperature. The possible interference with photosynthesis and the partitioning of biomass into leaf component relative to the total biomass produced by the plant may be the inhibitory effects of allelochemicals present in redroot pigweed and yellow foxtail residues.

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EFFECT OF VARIETY AND STAGE OF GROWTH ON POTENTIAL ALLELOCHEMIC COMPOUNDS IN SOYBEAN ROOTS

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Abstract—HPLC was used to obtain fingerprint patterns of organic compounds extracted from roots of five soybean varieties at seven stages of growth. Fifteen major peaks were observed in all varieties at most growth stages. With increasing stage of growth, the less polar compounds accounted for a greater relative percentage of total peak area. Concentration of compounds extracted from roots increased dramatically as plants approached nodulation and flowering and then decreased to initial levels as plants matured.

Key Words—Allelopathy, roots, soybean fingerprint, growth stage, HPLC, soybeans.

INTRODUCTION

Several mechanisms exist whereby allelochemicals are introduced into soils. These include leaching of above-ground tissues, decomposition of plant residues (Koeppe et al., 1976; Shindo et al., 1979; Shindo and Kuwatsuka, 1975; Kimber, 1973; Lodhi, 1978; Patrick et al., 1963, 1964; Cochran et al., 1979; Guenzi et al., 1967; Toussoun et al., 1968; Patrick, 1971) and exudation of compounds through plant roots (Barber and Martin, 1976; Martin, 1977; Odunfa, 1979; Rovira, 1969; Vancura and Hanzlikova, 1972; Prikryl and Vancura, 1980; Bokhari et al., 1979).

Chemicals reported to be released from plants include several classes of compounds such as sugars, amino acids, proteins, and organic acids. Phenolic acids are often cited as active allelochemicals from living or decomposing plant tissues (Kuc, 1972; Rovira, 1969; Vancura and Hanzlikova, 1972; Prikryl and Vancura, 1980; Bokari et al., 1979). Several researchers report benzoic and cinnamic acid derivatives in natural systems or the allelochemic

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action of these acids as they affect germination or plant growth (Einhellig and Rasmussen, 1978, 1979; Lee, 1980; Slominski, 1980; Toussoun et al., 1968; Koeppe et al., 1976; Glass, 1975; Wang et al., 1967; Sparling et al., 1981; Pareek and Gaur, 1973; Carballeira, 1980; Kovacs, 1971; Shindo et al., 1979; Shindo and Kuwatsuka, 1975; Eussen and Niemann, 1981).

Exudates from soybean roots represent a source of potential allelochemicals. These include compounds such as phenolics and chemically related compounds that have demonstrated biological activity (Preston et al., 1954; Mitchell et al., 1959, 1961; Linder et al., 1957, 1963).

Several investigators (Whitehead et al., 1981; Walter and Schadel, 1981; Carballeira, 1980) have attempted to examine roots at a selected growth stage for specific phenolic compounds. The study reported herein was undertaken to test methods to extract and separate (fingerprint) a large number of potential allelochemicals from soybean roots. These fingerprints were used to assess varietal differences as well as the effect of stage of growth on fingerprint patterns and relative amounts of compounds present.

METHODS AND MATERIALS

Will, Pella, Pixie, Gnome, and Corsoy soybean varieties were grown in 15-cm greenhouse pots (15 plants/pot) using Drummer soil (typic haplaquoll). These varieties represent a range in maturity groups and growth habits (Table 1). Three replicates of each variety were harvested for analysis at each of seven different growth stages ranging from emergence to senescence (Table 2). Roots of soybean plants were rinsed free of soil with water and extracted for 30 min with dimethylsulfoxide (2:1 DMSO to roots on a weight basis) in stainless-steel centrifuge tubes. Samples were then centrifuged at 17,000 rpm for 10 min and filtered through a 5-μm filter prior to analysis. The extract (20 μl sample) was analyzed by a modification of the method of Murphy and Stutte (1978) using a Beckman HPLC gradient elution system with mixtures of solvent B (68% water, 25% methanol, 5% butanol, and 2% glacial acetic acid in 0.018 M ammonium acetate) and solvent A (98% water and 2% glacial

TABLE 1. MATURITY GROUP AND GROWTH HABIT OF SELECTED SOYBEAN VARIETIES

Variety	Maturity group	Growth habit
Corosoy	II	Indeterminant
Gnome	II	Determinant
Will	III	Semideterminant
Pella	III	Indeterminant
Pixie	IV	Determinant

TABLE 2. GROWTH STAGES SELECTED FOR ROOT ANALYSIS

Growth stage[a]		Description
I	(VE)	Emergence
II	(V1)	Pretrifoliate
III	(V2)	Posttrifoliate
IV	(V3)	Nodulation
V	(R2)	Flowering
VI	(R5)	Pudding
VII	(R8)	Senescence

[a]Number in parenthesis designates growth stage as outlined by Fehr and Caviness (1977).

acetic acid 0.018 molar in ammonium acetate). The following gradient was used: (1) 0–1 min isocratic at 20% B; (2) 1–21 min linear gradient from 20 to 35% B; (3) 21–36 min linear gradient from 35 to 45% B; (4) 36–50 min linear 45 to 90% B; (5) 50 min flow rate increased from 1.0 to 1.2 ml/min; (6) 50–60 min isocratic at 90% B. Solvent B was then increased to 100% and returned to 20% B to equilibrate the column for the next run. A 25-cm Ultrasphere C_{18} column was used for all separations. Compounds were detected by UV absorption at 280 nm.

RESULTS AND DISCUSSION

The HPLC method employed to survey organics in soybean roots is a modification of that developed by Murphy and Stutte (1978) to separate benzoic and cinnamic acid derivatives. Figure 1 shows the separation of seven benzoic and cinnamic acid derivatives achieved with this gradient. Examples of other classes of natural products that are separated by this gradient are shown in Figure 2. In an attempt to identify individual compounds, retention times and relative retention times were established using this gradient for nearly 50 standard compounds including the following classes: benzoic acids, benzaldehydes, benzoates, cinnamic acids, coumarins, phenols, benzene carboxylic acids, acetic acid derivatives, as well as flavones, isoflavones, flavanones, and their associated glycosides.

An HPLC fingerprint pattern observed for the DMSO extracts of soybean roots is shown in Figure 3. The pattern shown represents a large number of compounds with retention times similar to the range observed for the potential allelochemicals listed above. The fingerprint pattern for soybean roots (Figure 3) is very different than that shown for corn roots subjected to the same extraction (Figure 4). The extracts for corn show a larger concentration of peaks in the earlier portion of the chromatogram (more polar compounds) and a distinct lack of later eluting peaks (less polar

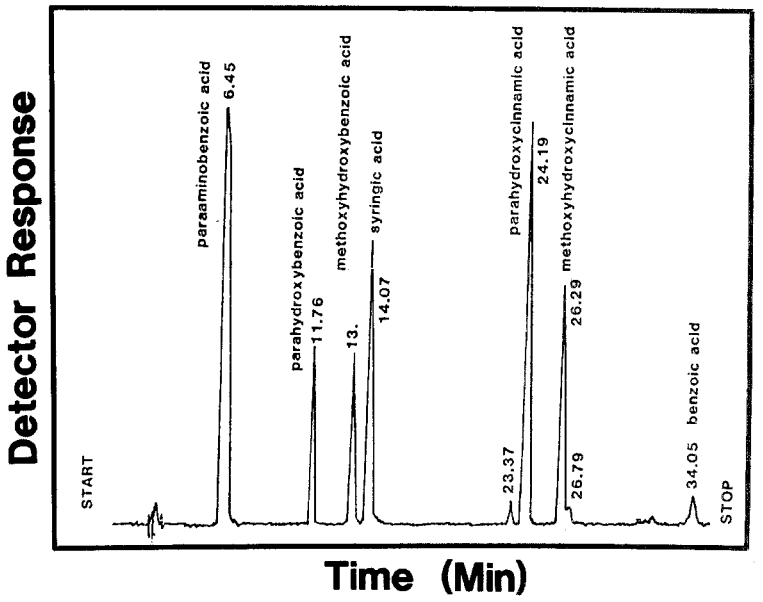

FIG. 1. HPLC separation of a standard solution containing seven benzoic and cinnamic acid derivatives.

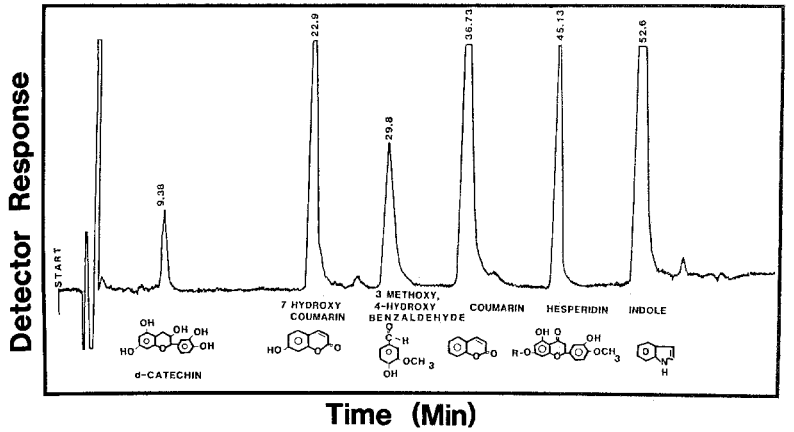

FIG. 2. HPLC separation of a standard solution of six natural products representative of compounds detected.

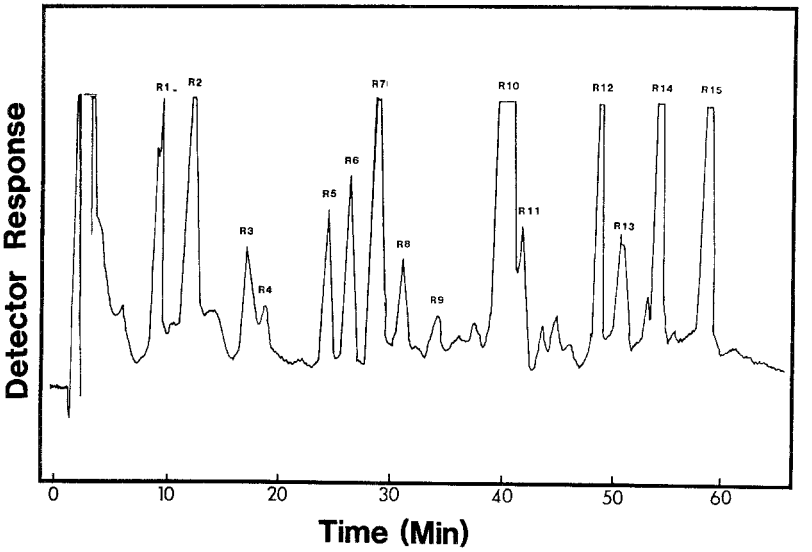

FIG. 3. HPLC fingerprint of a DMSO root extract of soybeans (stage III).

compounds). Data from our laboratory for several other plant species confirm the uniqueness of the soybean root fingerprint to that species. Some of the peaks shown in Figure 3 may represent more than one compound, but these fingerprints are very reproducible and are unique to soybean roots. Each of 15 major peaks observed was assigned a reference number (R1 to R15) to examine the effect of variety and growth stage on the fingerprint pattern.

Results from Table 3 emphasize the similarity of fingerprint patterns for

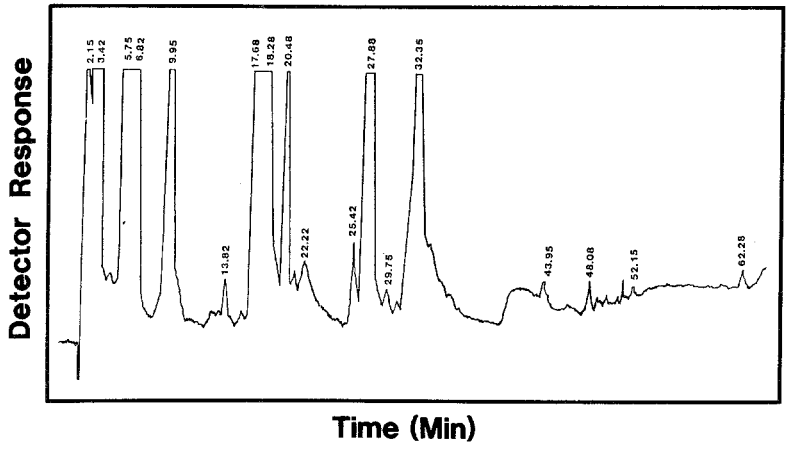

FIG. 4. HPLC fingerprint of a DMSO root extract of an 8-week-old corn plant.

TABLE 3. FINGERPRINT PEAKS DETECTED FOR FIVE SOYBEAN VARIETIES AT SEVEN STAGES OF GROWTH

Variety	Growth stages	Major Fingerprint Peaks[a]														
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
Corsoy	I	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
	II	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	−	+	+	+	−	+	+	+	+	+	+
	VI	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
	VII	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+
Gnome	I	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
	II	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
	VI	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
	VII	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+

Pella	I	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
	II	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	VI	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
	VII	+	−	+	−	+	+	+	+	−	+	+	+	+	+	+
Pixie	I	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
	II	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	IV	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	VI	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
	VII	+	−	−	−	+	+	+	+	−	+	+	+	+	+	+
Will	I	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+
	II	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+
	III	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
	IV	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
	V	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+
	VI	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
	VII	+	−	−	−	+	+	+	+	−	+	+	+	+	+	+

[a]Detected (+), not detected (−).

five soybean varieties including determinant, indeterminant, and semi-determinant varieties as well as maturity groups II, III, and IV. All of the 15 major peaks examined were found in each variety. R11 and R13 were not detected in any variety at growth stage 1 but were detected in all varieties by growth stage III. Some of the more polar components R2, R3, and R4, as well as R9, were not detected in all varieties at the later stages of growth. Hence, there appears to be a slight shift toward less polar components with stage of growth.

These results are emphasized by Figure 5 which compares fingerprints for three stages of growth for the Pella soybean. Fingerprint patterns are similar for all three growth stages. Most peaks and especially the late-eluting compounds appear to increase with stage of growth. Peak R11, for example, is hardly detected at growth stage I but is a significant peak by growth stage V. Most of the less polar compounds (R12, R13, R14, R15) appear as much broader peaks by growth stage V.

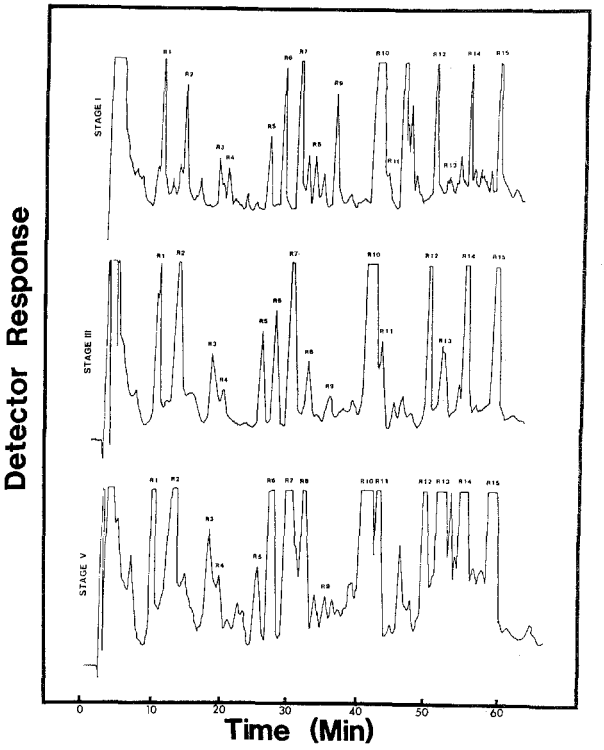

FIG. 5. Comparison of the HPLC fingerprints for DMSO root extracts of Pella soybeans at three different growth stages.

TABLE 4. RELATIVE PEAK AREA FOR INDIVIDUAL PEAKS AS AFFECTED BY STAGE OF GROWTH (AVERAGE OF ALL VARIETIES)

	Stage of growth						
Peak	I	II	III	IV	V	VI	VII
R1	7.6	8.4	2.6	0.8	1.2	2.1	1.8
R2	1.2	2.7	2.5	0.7	2.3	1.4	2.0
R3	1.0	2.2	1.8	1.5	1.8	1.7	0.7
R4	0.6	1.1	0.9	0.9	0.4	0.9	0.8
R5	0.7	2.0	2.2	0.8	0.9	0.9	5.3
R6	2.9	3.0	3.3	4.6	4.6	4.8	3.4
R7	4.5	6.2	5.8	6.3	5.9	5.6	4.5
R8	1.4	2.8	1.8	2.0	2.4	3.1	2.8
R9	2.1	1.8	0.8	0.8	0.3	0.3	0.0
R10	50.6	44.3	46.2	46.1	34.2	29.8	39.5
R11	0.0	0.9	1.5	1.7	3.4	4.6	1.8
R12	5.2	4.5	4.0	4.6	3.8	3.7	4.5
R13	0.0	2.5	3.1	4.7	7.6	7.6	3.5
R14	2.4	6.1	8.6	10.3	11.7	13.0	5.6
R15	4.1	4.4	8.6	8.6	10.0	6.5	17.8

The changes in area for the various fingerprint components with stages of growth are emphasized in Table 4 where relative concentrations of each of the 15 peaks labeled were calculated as a percentage of the total peak area recorded for each chromatographic run. Peak R10 resulted in by far the largest detector response, representing nearly 50% of the total peak area (average for all varieties) at growth stage 1. The relative area of this peak decreased, however, with stage of growth such that it represented above 30% of the total peak area at growth stage VI. This peak appears to have properties closely related to a glycoside of daidzein. The relative decrease in peak R10 with stage of growth is accounted for primarily by increases in the relative concentrations of the less polar peaks (R11, R13, R14, R15) as shown in Table 4. Although trends are not entirely consistent, some of the peaks, such as R9, R3, and R1, showed a decrease in relative peak area with stage of growth (Table 4).

Of the nearly 50 standard compounds tested, only two could be tentatively identified as components of the soybean root extract by comparing relative retention times on three separate gradients and by comparing wavelength ratios. Peak R15 (Figures 3 and 5) has been tentatively identified as daidzein and subsequent studies have shown a peak eluting later than R15 to be coumestrol. It can be speculated that at least some of the peaks observed are glycosides since our procedure does not involve acid hydrolysis. It should

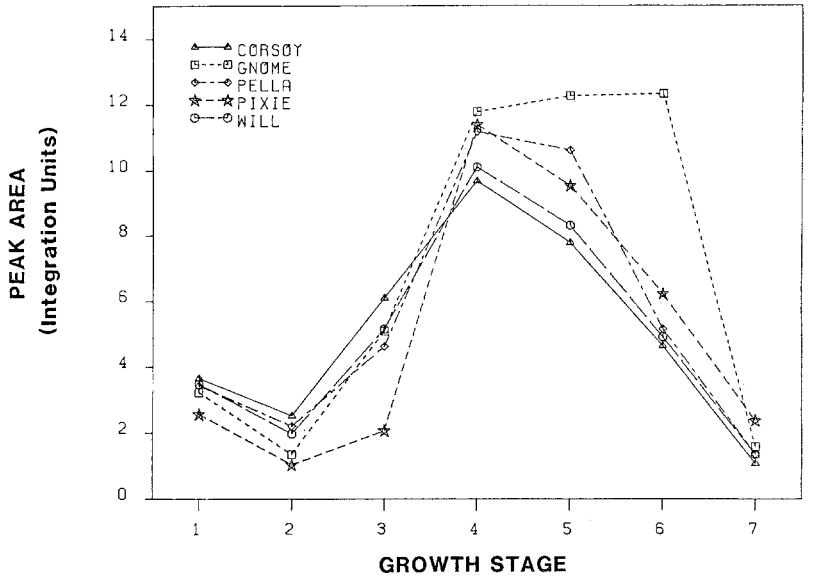

FIG. 6. Total peak area (relative integration units) for five soybean varieties as affected by growth stage.

be noted that no detectable levels of free cinnamic or benzoic acid derivatives tested were found in the DMSO extract of soybean roots. Failure to positively identify any fingerprint peaks as these derivatives reduces the probability of soybean roots being a direct source of these potential allelochemicals.

The effect of stage of growth on the total peak areas recorded for each of the varieties is shown in Figure 6. These data indicate a dramatic increase in concentration of DMSO extractable organics in the roots of soybeans as root nodulation and flowering occurs. This dramatic increase in total peak areas observed, however, is not the result of "new" peaks associated with advancing growth stage (Table 3). As plants approach senescence, the total peak areas recorded dropped to levels near those found in the roots at growth stages I and II (2:1 w/w extracts were used in all cases). No significant differences were observed among varieties although Gnome appeared to maintain a higher concentration of total extractable organics through growth stage VI. Additional work is needed to positively identify the major fingerprint compounds observed in this study.

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IDENTIFICATION OF THE DEFENSIVE SECRETION FROM SOLDIERS OF THE NORTH AMERICAN TERMITE, *Amitermes wheeleri* (DESNEUX) (ISOPTERA: TERMITIDAE)

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Abstract—Head capsule extracts of *Amitermes wheeleri* soldiers yielded an isomeric mixture (67 μg/soldier) of three sesquiterpene hydrocarbons identified by EIMS, [^{13}C]-, and [^{1}H]NMR as (+)-(*S,Z*)-α-bisabolene (53%), (+)-(*R*)-β-bisabolene (16%), and (−)-(*Z*)-α_2-bisabolene (31%). When alarmed, the termite soldiers secreted the fluid onto the head surface surrounding the efferent pore of the frontal gland reservoir. A defensive function for the soldier secretion was indicated by the avoidance behavior displayed toward alarmed soldiers by the antagonistic ants, *Pogonomyrmex rugosus* and *Iridomyrmex humilis*. Laboratory studies demonstrated that the sesquiterpene mixture is repellent to foraging *I. humilis* workers.

Key Words—*Amitermes wheeleri*, Isoptera, Termitidae, termite soldiers, defensive secretion, ant repellents, α-bisabolene, β-bisabolene, α_2-bisabolene, *Iridomyrmex humilis*, *Pogonomyrmex rugosus*, sesquiterpenes.

INTRODUCTION

Chemical analyses of termite soldier cephalic secretions have resulted in the identification of a substantial number of novel as well as previously known natural products (see review by Prestwich, 1979). The ecological role of these secretions is usually associated with defensive strategies employed by the soldier caste to protect the termite colony from ants or other predators. Only two investigations of the soldier secretions of Nearctic termites have been published compared to the many extensive chemical studies of the more numerous and conspicuous tropical species. Nutting et al. (1974) identified

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three monoterpene constituents in the volatile fraction of pentane extracts from the nasute soldiers of *Tenuirostritermes tenuirostris* (Desneux). Zalkow et al. (1981) characterized two major components from cephalic extracts of *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) as the sesquiterpene hydrocarbon γ_1-cadinene and its 14-aldehyde. However, a defensive role for these latter compounds could not be demonstrated.

Prestwich (1979) reported the unpublished findings of Prestwich and Nutting that soldiers of *Amitermes wheeleri* (Desneux) collected in Arizona produced a single, nearly pure ($\geqslant$99%) sesquiterpene hydrocarbon. Our preliminary bioassays using California populations of this species convincingly showed a defensive role for the fluid emitted by soldiers. We herein report our findings on the chemical composition and the repellent activity against ants of the defensive secretion from the soldiers of *Amitermes wheeleri*.

METHODS AND MATERIALS

Termite Collection and Extraction. Termites were collected between March 30 and April 3, 1982, within 1 km of the campus of the University of California, Riverside. Foraging parties of *A. wheeleri* were taken from dead root systems of fire-burned *Encelia farinosa* Gray, a common local desert shrub. Only workers, brachypterous nymphs, and soldiers were present in the minings of unearthed roots and root crowns; reproductives and early instar forms were never encountered. *Amitermes minimus* Light and *Reticulitermes* sp. also inhabited these root systems but only one species was ever present in any single root network. Roots infested with *A. wheeleri* were taken to the laboratory where the termites were immediately excised from the wood. Soldiers (ca. 1–2% of the total foraging populations) were separated from the other castes, decapitated, and the head capsules immersed in 4.6 ml distilled CS_2. A total of 393 head capsules were crushed in a reactor vial using a form-fitted glass rod. The extract and 4 ml of CS_2 rinse were eluted through a Sep Pak® silica cartridge to remove particulates and water. The solution was then concentrated to 0.5 ml at 65° C in a Kuderna-Danish type evaporative concentrator.

GLC Analysis and Isolation. All gas–liquid chromatography (GLC) was carried out isothermally with a Hewlett-Packard model 402 gas chromatograph equipped with flame ionization detectors and 2 m $\times$ 6 mm ID glass columns. All liquid phases were coated onto 100–120 mesh, acid-washed Chromosorb W. β-Caryophyllene was used as an external standard for quantitative analyses, and peak areas were calculated by height times width at one-half height. After recording the retention time(s) of the desired fraction(s), the detector was removed from the instrument and the exit end of

the column capped with a perforated septum. Samples were collected by inserting into the exhaust septum glass capillary tubes (26 cm × 1 mm ID) which were lightly packed with glass wool and cooled with dry ice. Immediately after a fraction was collected, the capillary was internally rinsed three times with 25 μl of spectral grade CCl_4. After numerous collections, the pooled CCl_4 rinses were concentrated under a stream of nitrogen to a volume compatible with NMR requirements. The recovery rate by this method, estimated with β-caryophyllene, was ca. 60%, as some of the effluent was visibly lost as an aerosol vapor.

The three major components present in the termite head extract could not be separated by any single column packing we tested, so the following isolation scheme was developed. A column containing 16.7 g of 3% cholesteryl cinnamate (Heath et al., 1979) run at 150° C, N_2 flow 32 ml/min, gave adequate separation[3] of (1) from (2) and (3) (Figure 2A). The latter two eluted simultaneously with this packing. This system was used to collect preparative quantities (ca. 300 μg total) of the two fractions, (1) and (2) + (3). The cholesteryl cinnamate phase bled profusely so the isolate of (1) was rechromatographed and collected on a column of 2% Dexsil 300 GC on silanized Chromosorb W at 165° C, N_2 flow 36 ml/min. This procedure allowed for the retention of the higher mass cholesteryl cinnamate contaminant while eluting pure (1) suitable for NMR analysis. A column containing 17.7 g of 10% Silar 10C run at 150° C, N_2 flow 28 ml/min, was used to separate the (2) + (3) isolate (the Silar column did not resolve (1) and (2), see Figure 2B). Baseline separation of (2) and (3) at preparative loads was achieved and each compound was separately trapped in nearly pure (>98%) form. The Silar packing did not bleed significantly to interfere with spectral analyses and, as with Dexsil packing, prevented the elution of the cholesteryl cinnamate contaminant.

NMR and MS Analysis. Proton nuclear magnetic resonance spectra were obtained with a Bruker WM-500 500 MHz high-resolution spectrometer. Broad-band decoupled ^{13}C spectra for (1) and (3) were recorded on a Jeol FX-200 instrument. ^{13}C data for (2) were obtained with the Bruker instrument from a (1) + (2) mixture in CCl_4 + 10% $CDCl_3$. All other samples were run in CCl_4 plus 10% d_6-acetone for a deuterium signal and internal reference. Electron-impact mass spectra were recorded at 20 eV from neat samples with a Nicolet FT/MS-1000 mass spectrometer. Optical rotations were determined from NMR ready samples with a Perkin-Elmer 241 polarimeter set at 589 nm.

Biological Activity. Initial field observations were carried out with live soldiers placed near foraging ants. The behavioral reactions by workers of a native harvester ant, *Pogonomyrmex rugosus* Emery, and an introduced pest,

[3] Resolution with the cholesteryl cinnamate packing decreased after use each day but could be regenerated by several minutes of heating to 180° C with carrier gas flow off.

the Argentine ant, *Iridomyrmex humilis* (Mayr), were observed. Laboratory studies used extracted chemicals only to negate the influence of mechanical defense by soldiers on ant aggression. A colony of Argentine ants, reared by the method of Markin (1970), was connected to its food source, a 25% sucrose solution, by 50 cm of 2-mm-ID Teflon® tubing. A junction near the center of the tubing contained a removable glass ring (6 mm × 2.2 mm ID) through which the ants had to pass. After the ants had established a trail in the tubing, the number of ants passing the junction from either direction was counted for 5 min. The ring was then removed and internally coated with diluted extract solution quantitatively determined by GLC. The solvent was evaporated and the ring replaced in the junction to again complete the foraging route. The time required for the first ant to cross the treated junction from either side and subsequent foraging traffic were recorded.

RESULTS AND DISCUSSION

The mandibular morphology of *Amitermes* soldiers is categorized as a reaping type by Deligne et al. (1981) because of the curved, pointed mandibles which crisscross each other. This mechanical defense in *Amitermes* soldiers is supplemented by subtle chemical weaponry. In the case of *A. wheeleri* soldiers, the defensive secretion is contained in a shallow frontal gland reservoir suspended from the dorsum of the head capsule and visible from above as a pale yellow region amidst the orange-colored head (Figure 1). The secretion is delivered through a minute fontanellar pore (20 μm diam) situated on a shallow frontal mound. When the secretion is exuded, the fluid collects onto a bed of setae which surround the fontanelle and extend beyond the tip of the labrum. This allows for an evaporative surface on which the soldier pools the fluid during attack. The labrum of *A. wheeleri* serves as an anterior extension of the fluid-coated surface which can be directed toward an attacker analogous to the more developed labra of some rhinotermitid soldiers (Quennedey and Deligne, 1975).

Exhaustive GLC of the soldier head capsule extract indicated that it consisted of the three major components (+)-(*S*,*Z*)-α-bisabolene (1), (+)-(*R*)-β-bisabolene (2), and (−)-(*Z*)-α_2-bisabolene (3) in the relative proportion 53:16:31, respectively (Figures 2 and 3). A 500-MHz [^{1}H]NMR (CCl_4) recording of the crude head extract was qualitatively identical to the superimposed traces of the GLC-isolated bisabolenes (Figure 3). This verified that the isolation procedure had not altered the NMR properties of the extract (i.e., no degradation, isomerization, etc.) and also ensured that nonvolatile constituents, which would not be detected by GLC, were absent from the extract. The β-bisabolene (2) could not be resolved with either the Silar 10C or cholesteryl cinnamate columns (Figure 2). Additionally, columns of 5%

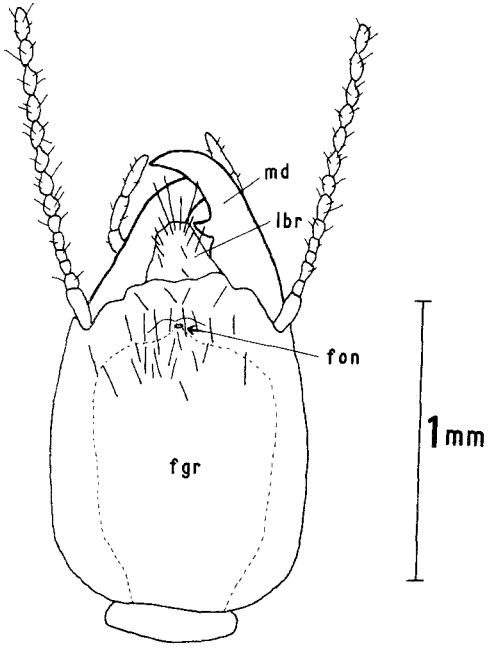

FIG. 1. Dorsal view of the head capsule of an *Amitermes wheeleri* soldier: md, mandible; lbr, labrum; fon, fontanelle; fgr, frontal gland reservoir (drawn from scanning electron and light micrographs).

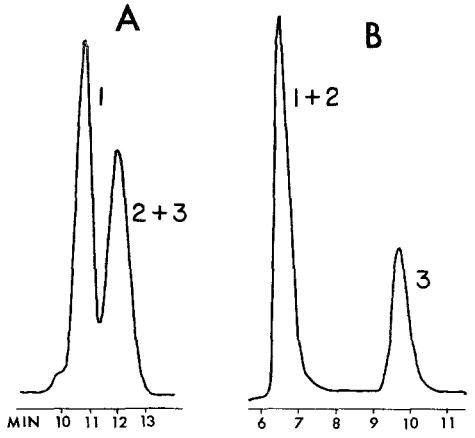

FIG. 2. Chromatograms of *A. wheeleri* soldier defensive secretion; (+)-(*S*,*Z*)-α-bisabolene (1), (+)-(*R*)-β-bisabolene (2), and (−)-(*Z*)-α_2-bisabolene (3). (A) 3% cholesteryl cinnamate. (B) 10% Silar 10C. Both run at 150° C, N_2 flow 32 ml/min.

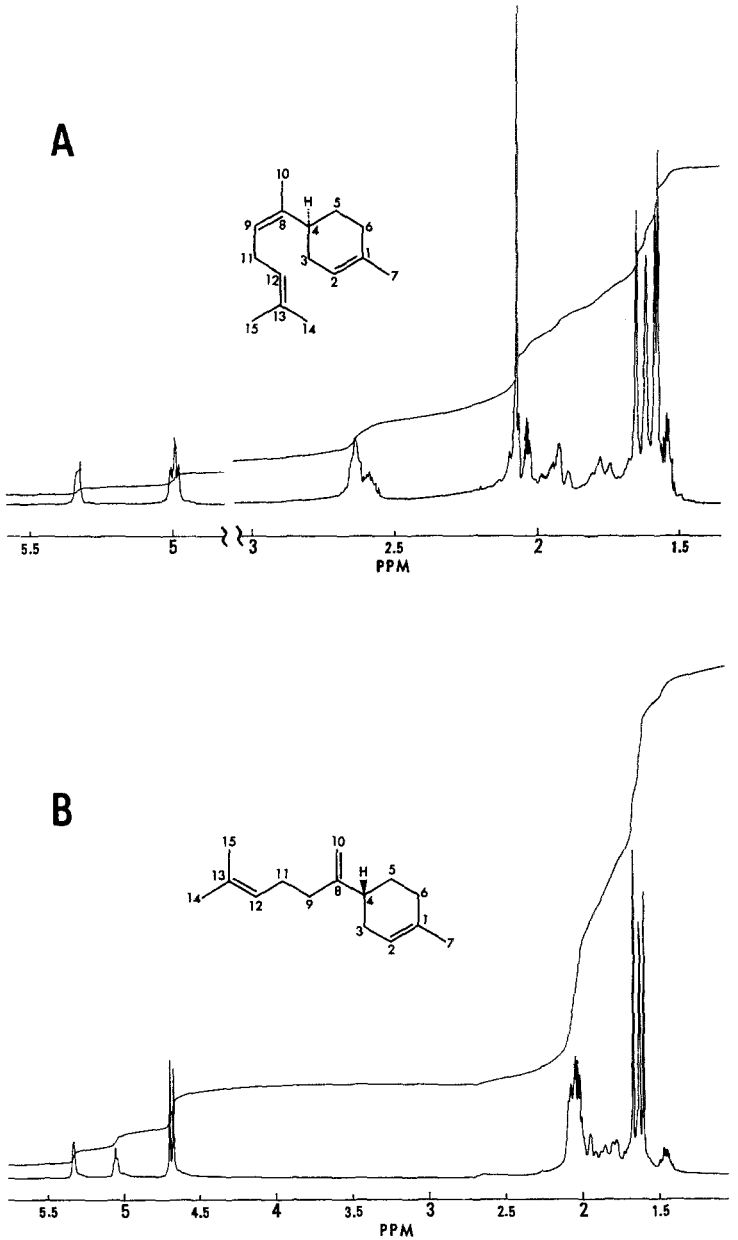

FIG. 3. 500-MHz [^{1}H]NMR spectra and structures of sesquiterpene hydrocarbons isolated from *A. wheeleri* soldier defensive secretion. (A) (+)-(*S*,*Z*)-αbisabolene (1). (B) (+)-(*R*)-β-bisabolene (2). (C) (−)-(*Z*)-α_2-bisabolene (3). The large singlet at δ2.07 in A is a d_6-acetone impurity. Chemical shifts are not depicted to same scale.

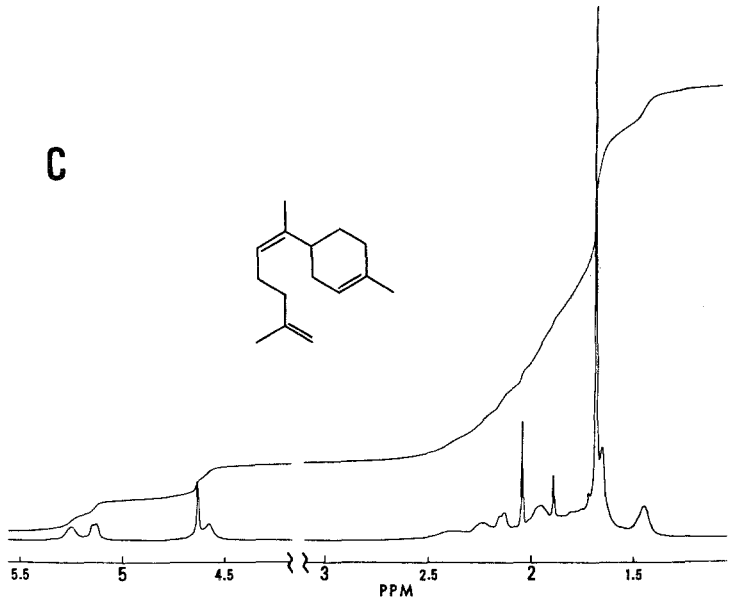

FIG. 3. Continued.

Carbowax 20 M, 2% Dexsil 300 GC, and 2% SF-96 did not resolve (1) from (2); however, the Carbowax column separated a minor component responsible for the leading shoulder on the (1) peak (Figure 2A). Due to the small amount present (<2%), this fourth component was not identified. Specific rotations, $[\alpha]_D^{21}$ CCl_4), for (1), (2), and (3) were calculated as $+10° \pm 5°$ (0.0010 g/ml), $+41° \pm 3°$ (0.0022 g/ml), and $-30° \pm 3°$ (0.011 g/ml), respectively.

Mass spectrometric analysis of the major components produced identical molecular ions of m/e 204 and similar fragmentation patterns (Table 1) indicative of tetraunsaturated sesquiterpene hydrocarbons ($C_{15}H_{24}$). [^{1}H]NMR spectra of each compound lacked signals upfield of 1.40 ppm which rules out nonallylic methyl or axial cyclic proton resonances of bicyclic systems. The acyclic farnesene structures were eliminated since no more than four protons with olefinic shifts were integrated for each compound. Ultimately, all spectral data conformed with the monocyclic bisabolene structure as the rudimentary carbon skeleton for each of the compounds.

Both α- and β-bisabolene are known constituents of the perfume essence, oil of opoponax (Wenninger and Yates, 1969, and references therein). On the basis of NMR data, Delay and Ohloff (1979) unequivocally differentiated the two geometrical isomers of α-bisabolene obtained by stereospecific synthesis. From their findings, we have assigned the (+)-(*S*,*Z*) configuration to (1), the same *Z* enantiomer which Delay and Ohloff (1979) identified in opoponax oil. [^{13}C]NMR chemical shifts for 14 carbons of (1) (Figure 4) coincided with

TABLE 1. MASS SPECTRAL VALUES OF MAJOR FRAGMENTS ≥30% RELATIVE INTENSITY ABOVE 50 MASS UNITS AND MOLECULAR IONS OF *A. wheeleri* DEFENSIVE SECRETION COMPONENTS

Compound	*m/e* (% relative intensity)			
(1) (+)-(*S,Z*)-α-bisabolene	69(30)	93(100)	94(61)	95(33)
	105(71)	106(38)	107(50)	109(42)
	119(75)	121(31)	133(67)	147(58)
	148(43)	161(81)	189(56)	
	204(M^+,63)			
(2) (+)-(*R*)-β-bisabolene	67(41)	69(69)	79(30)	93(100)
	94(65)	105(46)	107(42)	109(56)
	119(67)	161(75)	189(32)	
	204.1863 (M^+,29) calc. for $C_{15}H_{24}$ 204.1877			
(3) (−)-(*Z*)-α_2-bisabolene	67(36)	79(35)	81(44)	93(100)
	94(31)	105(78)	107(64)	108(52)
	119(74)	121(37)	125(70)	133(34)
	147(83)	148(30)	161(44)	189(49)
	204.1863 (M^+,11) calc. for $C_{15}H_{24}$ 204.1877			

those reported for the Z isomer. We did not observe a signal for carbon No. 13 (131.0 ppm, Delay and Ohloff, 1979) on our trace and cite a combination of weak quaternary carbon resonance and a low signal-to-noise ratio for its undetectability. Our 500-MHz [^{1}H]NMR spectrum of (1) (Figure 3A) indicated three protons on trisubstituted double bonds, one cyclic at δ5.33 (br s, 1H, C-2) and two alicyclic with equivalent shifts (δ4.99, br t, 2H, C-9, C-12, $J = 7$ Hz). A poorly resolved triplet at δ2.64 (2H, C-11, $J = 7.6$ Hz) is characteristic of a doubly allylic methylene. The proton on the tertiary allylic C-4 shifts downfield at δ2.59 (b m, 1H). A series of multiplets between δ2.12 and 1.70 accounted for five methylene protons. Four allylic methyls at C-7, 10,

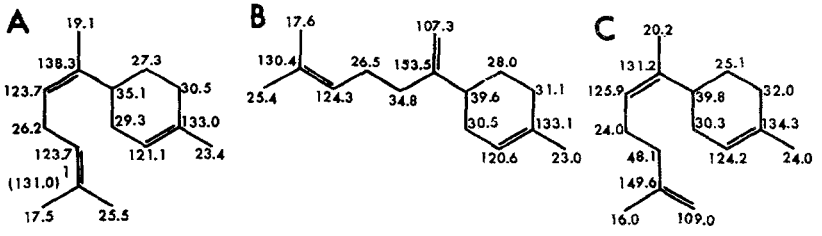

FIG. 4. [^{13}C]NMR chemical shift assignments for sesquiterpene hydrocarbons isolated from *A. wheeleri* soldier defensive secretion. (A) (+)-(*S,Z*)-α-bisabolene (1). (B) (+)-(*R*)-β-bisabolene (2). (C) (−)-(*Z*)-α_2-bisabolene (3). [1]Delay and Ohloff (1979).

14, and 15 were observed (δ1.60, s, 3H; 1.61, s, 3H; 1.64, s, 3H; and 1.67, s, 3H), and the axial C-5 proton appeared as a multiplet at δ1.56. Mass spectral data of (1) (Table 1) is in agreement with that of (*Z*)-α-bisabolene (Delay and Ohloff, 1979).

Low-field [^{1}H]NMR spectra for β-bisabolene have been published independently by Nigam and Neville (1968) and Giraudi et al. (1974). Both are superimposable and in agreement with our 500-MHz trace of (2). [^{1}H]NMR data (Figure 3B) revealed a broad singlet (δ5.33, 1H, C-2) and triplet (δ5.06, 1H, C-12, J = 7 Hz) which represent the olefinic hydrogens of trisubstituted double bonds and are analogous with their (*Z*)-α-bisabolene counterparts. The terminal methylene protons at C-10 appear as two singlets (δ4.69, 1H; 4.67, 1H). Seven allylic protons were integrated in the unresolved multiplet at δ2.04 in addition to the sharp absorption bands of d_5-acetone (d_5-acetone impurity) superimposed at its center. Three more protons fall as multiplets between δ1.97 and 1.72. The three allylic methyl protons at C-7, 14, and 15 occur as prominent singlets (δ1.67, 3H; 1.63, 3H; 1.60, 3H). The axial nonallylic C-5 proton is represented by the upfield multiplet at δ1.46. Additionally, all 15 [^{13}C]NMR chemical shifts for (2) (Figure 4) were congruous ($\leqslant$0.5 ppm) with those published for β-bisabolene (Wenkert et al., 1976). The dextrorotary property of (2) was correlated with the *R* enantiomer by Mills (1952).

The structure for α_2-bisabolene (Figure 3C) was first proposed by Wenninger and Yates (1969) for a compound isolated from the dehydrochlorination reaction products of bisabolene trihydrochloride. Unlike our third component, the GLC retention time of α_2-bisabolene was reported to be shorter than the other bisabolenes (Wenninger and Yates, 1969); however, evidence supporting their proposed structure was omitted. Spectral data of (3) are consistent with the geometrically unspecified α_2-bisabolene structure, and we have further assigned it the *Z* configuration on the basis of the C-4 and C-10 [^{13}C]NMR shift similarities with (2) (Figure 4), disparity with analogous values in the *E* isomer shown by Delay and Ohloff (1979) to have a diagnostically greater difference in shift (16.3 ppm for *Z*, 28.7 for *E*), and similar arguments for identifying the geometry of other terpenoids by [^{13}C]NMR (Wiemer et al., 1979). The [^{13}C]NMR spectrum of (3) (Figure 4) indicated two trisubstituted double bonds (δ134.3, 131.2, 125.9, and 124.2) and a terminal methylene (δ149.6 and 109.0). These were verified by four single proton NMR resonances (Figure 3C) (δ5.26, b s; 5.14 b s; 4.64 s; 4.58, b s). Upfield absorptions produced by nonolefinic protons were unresolved and not of much use in verifying the proposed structure; however, overall shift positions and corresponding integrations were consistent with expectations. Additionally, the mass spectral data of (3) (Table 1) indicated considerable similarity in fragmentation with respect to other bisabolenes.

The granivorous ant, *Pogonomyrmex rugosus*, was chosen as a

representative sympatric desert species for field evaluation of the *A. wheeleri* soldier chemical defense. Foraging workers of *P. rugosus* were baited with termite workers which the ants swiftly killed and carried back to their nest. When soldiers were placed among the ants, initial confrontation produced a mandibular snapping frenzy and liberation of an undetermined amount of defensive fluid by the soldiers. Frontal approach or contact by the much larger (9 mm length) ants caused them to retreat rapidly followed by vigorous antennal grooming behavior. Harvester ants that approached a soldier posteriorly sometimes bit into its abdomen, inflicting a mortal wound. Some carried the soldier a few centimeters only to reject it and scurry off to begin the grooming activity. Similar results occurred under field conditions with the smaller (2.5 mm length) Argentine ant, *Iridomyrmex humilis*. Foraging ants approached within a few millimeters of the alarmed soldier and then quickly retreated while frantically grooming their antennae by drawing them through their mouthparts and forelegs. When presented with the soldier's decapitated body, the ants grasped the corpse and carried it away, as they did with the termite workers. The severed head was completely avoided by the ants. Both ant species have been reported to actively seek termite prey under natural conditions: *P. rugosus* in New Mexico (Davidson, 1980) and *I. humilis* in South Africa (Bouillon, 1970).

Gas-liquid chromatography revealed that an average of 67 μg of secretion was extracted per soldier, about 2% of their biomass. Results of laboratory bioassays with Argentine ants using the soldier secretion are given in Table 2. Immediately after inserting the ring treated with 16 or 32 μg of extract, ants approached within 0.1–2.0 cm of the deposit and, upon detecting the extract, quickly crawled in reverse for several centimeters and initiated their characteristic antennal grooming behavior. Ants congregated on both sides of the junction, but gradually attempted fewer crossing approaches. After 60 min without any crossing, the ring was removed, rinsed with acetone, and replaced. Since foraging recruitment had ceased for 1 hr, the ensuing crossing traffic rates were low at first but increased as sugar-fed ants reinitiated nestmate recruitment. At lower concentrations, some ants traversed the chemical barrier; however, overall traffic was reduced due to the number of ants that continually avoided the deposit. The solvent control treatments did not noticeably disrupt foraging traffic.

The results of both field and laboratory bioassays indicate that avoidance behavior is the dominant response elicited among potential ant predators by the *A. wheeleri* sesquiterpene mixture; i.e., the soldier secretion acts as a defensive repellent. Since *A. wheeleri* forages in an exclusively underground habitat and has a small soldier force, a repellent type of chemical defense would be ecologically advantageous. Hypothetically, termite soldiers could use repellents to effectively "close off" subterranean routes of travel which

TABLE 2. DISRUPTION OF ARGENTINE ANT FORAGING TRAFFIC BY HEAD CAPSULE EXTRACT OF *A. wheeleri* SOLDIERS

Deposit quantity (μg)	Ant traffic across junction ring							
	Pretreatment traffic[a]	Time until first crossing (min:sec)	Successive traffic after first crossing at minutes[a]					
			5	10	15	20	25	30
33.0	11.4	—[b]	1.0	1.4	2.0	4.0	3.0	6.4
16.5	8.6	—[b]	0.6	2.4	4.0	6.2	7.0	
8.0	16.0	4:15	1.2	0.8	4.4	5.6	8.6	10.0
6.0	15.6	0:31	6.2	7.4	7.8	10.4		
4.0	8.4	0:12	0.6	1.4	0.2	2.0	1.8	2.0
3.0	12.8	1:54	3.0	6.0	11.0	8.4		
2.0	16.0	0:50	5.0	6.6	10.2	12.6	9.6	11.0
0.0[c]	9.5	0:07	9.4					

[a] Mean number of ant crossings/min during 5-min intervals.
[b] No crossings after 60 min; successive traffic recorded after acetone rinse of junction ring.
[c] Mean values of four replicates bioassayed on different days.

ants might use to gain access into termite galleries. Relatively few soldiers could provide colony protection if they were stationed at strategic peripheral positions such as feeding locations or brood centers. We have often collected *A. wheeleri* soldiers near foraging gallery entrances. Avoidance of direct combat would prevent the altruistic self-sacrifice of soldiers, a common occurrence during battle among many termites, but costly to a small soldier force. The postulated defense strategy of *A. wheeleri* is comparable to the offensive deployment of "venom mace" by the European thief ant, *Solenopsis fugax* Latr. (Blum et al., 1980). During raids on neighboring ant nests, *S. fugax* workers deposit a repellent alkaloid which inhibits the invaded species from traversing contaminated passageways and eliminates interference by the resident defenders while *S. fugax* plunders their brood.

Foraging mode, soldier proportion, defense strategy, and predator threat appear to be interdependent in the Isoptera. Open-air foraging termitids, including the partially sympatric genus *Tenuirostritermes* and many tropical species, usually maintain 10-fold or greater soldier-to-worker ratios in foraging parties than does *A. wheeleri* (Haverty, 1977) and utilize incapacitating chemical weapons such as gluelike substances and/or toxicants to combat their freely moving and agile arthropod enemies. The use of solely repellent soldier secretions might be inadequate to protect workers of these termites during foraging expeditions. *A. wheeleri* is an example of an exclusively subterranean forager in which predator management appears to

be facilitated by the prudent use of a small soldier force equipped with repellent chemical armament.

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AGGREGATION OF LARVAE OF THE HOUSE CRICKET, *Acheta domesticus* (L.), BY PROPIONIC ACID PRESENT IN THE EXCRETA

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Abstract—Acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids have been identified in extracts of the excreta of the house cricket, *Acheta domesticus* (L.), by gas–liquid chromatography. Solutions of propionic acid applied to filter paper aggregated 1- to 2-week-old larvae, while solutions of acetic, butyric, valeric, and isovaleric acids were without effect.

Key Words—Aggregation, volatile fatty acids, house cricket, propionic acid, carboxylic acid, Orthoptera, Gryllidae, *Acheta domesticus*.

INTRODUCTION

An aggregating factor, as well as a dispersing factor, for adults of the house cricket, *Acheta domesticus* (L.), have been demonstrated by Sexton and Hess (1968) to be present on surfaces contaminated or "conditioned" by adults of the same species. Watler (1979) has shown that conditioned paper will aggregate immatures of *Acheta*. A similar factor aggregating larvae has also been found in *Blattella germanica* (Ishii, 1970). In this article, it will be shown that aggregation of larvae produced by excreta of the house cricket is at least in part due to the presence of propionic acid in the excreta.

METHODS AND MATERIALS

The stock culture of crickets was reared on a diet of pulverized Ralston Purina rabbit chow and maintained at 30 ± 2° C, 55 ± 5% relative humidity, and a photoperiodic regime of 14 hr light and 10 hr dark.

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The artificial diet contained 40 g casein, 30 g Alphacel nonnutritive cellulose, 20 g D-(+)-dextrose, 1 g cholesterol, 4 g of salt mixture, and the required water and fat-soluble growth factors (McFarlane and Distler 1981). The filter paper used was Whatman No. 1.

Testing for Presence of Aggregating Factor. Larvae 4–13 days old (first to third instars) were used in the experiments. Preliminary trials showed that younger larvae gave inconsistent results, whereas older larvae were more active and did not readily aggregate under the experimental conditions. Sixty larvae were placed in a culture dish (diam. 19.2 cm). Two 14 × 3.8-cm strips of filter paper folded twice (to form a W) were placed on edge and spaced evenly in the dish. One filter paper contained the substance to be tested while the other was an untreated control. The test dish was placed on a laboratory bench with overhead illumination and testing was done at room temperature. The numbers of larvae on each filter paper were recorded after 1.5 hr. There were always some larvae which were not on either filter paper; those not on filter paper were not included in the analysis of results. In four experiments less than half the larvae came to rest on the filter paper; these experiments were also not included. Mean numbers of larvae on each paper, together with standard deviations, are presented where applicable.

Where pellets of excreta were tested, a mound of pellets (0.3 g) was placed in the rearing jar and no papers were added. The numbers of insects on the pellets was recorded at hourly intervals for 3 hr.

Gas–Liquid Chromatographic Analysis. The composition of volatile fatty acids in the aqueous extracts was determined by gas–liquid chromatography using a Varian gas chromatograph (model 3700) equipped with a flame ionization detector. The conditions of chromatography were as follows: glass column (1.8 m long, 2 mm internal diameter); column packing material, Fluorad FC-431-1% H_3PO_4 on chromosorb W-HP 80/100 (Chromatographic Specialities Limited, Brockville, Ontario, Canada); nitrogen carrier gas; flow rate = 40 cc/min; temperature programing, initial temperature = 110° C, initial hold = 1 min, program rate = 8°/min, final temperature = 193° C, final hold = 4 min; injector temperature = 230° C, detector temperature = 250° C. Quantitive analysis was carried out by measurement of peak areas.

Testing for Aggregating Activity of Individual Volatile Fatty Acids. Individual fatty acids were tested by making serial dilutions from 1.0×10^{-1}% to 1.0×10^{-7}% and treating a strip of filter paper with 0.68 ml of the solution. This amount of solution completely wetted the paper and permitted uniform distribution of each acid. In one series of experiments, the papers were then dried in a steam of air and tested against untreated filter paper. In a second series of experiments, the papers were tested immediately after treatment, that is, while still wet, against strips of filter paper wetted with 0.68 ml of distilled water. Numbers on treated and control papers were recorded after 1.5 hr, by

which time the papers were dry. The larvae in these tests varied in age from 7 to 12 days.

RESULTS

Demonstration of Aggregating Factor Produced by Insects Reared on Chow and Artificial Diets. Strips of filter paper conditioned by the stock culture for 1–7 days aggregated the larvae, and this effect did not decline when the same papers were tested daily for two weeks (Table 1). However, contamination of the papers by the larvae used for the tests could have renewed the aggregating substance.

In aggregating, the larvae generally came to rest in clusters on the conditioned paper, often in just a single major cluster, but sometimes in several clusters.

Nutrition experiments run concurrently provided the conditioned strips used to test insects reared on the artificial diet. The strips had been conditioned for 6 weeks by larvae or for 4 weeks by adults. The conditioned paper strips aggregated the larvae as before.

TABLE 1. AGGREGATION ON FILTER PAPER ORIGINALLY CONDITIONED FOR 24 HR TESTED AT DAILY INTERVALS

	No. on conditioned paper			No. on untreated paper			
Day	#1	#2	#3	#1	#2	#3	Age of test larvae (days)
0	40	48	46	11	8	8	4
1	40	41	42	4	1	2	5
2	23[a]	30	32	0[a]	3	6	6
3	35	49	50	3	2	1	7
4	54	57	43	5	3	7	8
5	54	53	50	1	2	4	9
6	43	48	37	2	1	7	8
7	23	20	22	10	14	9	4
8	52	53	38	1	4	7	12
9	40	30	17[a]	0	2	4[a]	13
10	42	41	23	3	1	21	7
11	52	50	56	2	2	1	6
12	56	54	48	0	2	7	7
13	56	50	44	1	1	3	8
		44 ± 10	($N = 40$)		4 ± 4	($N = 40$)	

[a] Neglected in calculations as fewer than 30 insects were on papers.

Source of Aggregating Factor. A strip of filter paper placed in larval or adult cultures soon became spotted with anal excretions. The nature of the spotting was determined by observation. When defecating, the larva produced a pellet with a small amount of brown fluid which was quickly absorbed by the filter paper. Initially, the pellet of excreta stuck to the spot on the paper, but as pellet and spot dried, the pellet fell or was knocked by the larvae to the bottom of the rearing jar. Rarely was fluid produced without a pellet as well. The spotting of the paper was definitely not due to regurgitation. The spotting was much less colored when the artificial diet was fed.

Fresh pellets of excreta ($\leqq$ 48 hr old) taken from larval or young adult cultures did not lead to aggregation.

Extraction of Aggregating Factor. Single conditioned papers were extracted with water, and the extract concentrated, applied to fresh filter paper, and the filter paper dried. Aggregation resulted when these papers were tested. Chloroform–methanol extracts also gave positive results. Aqueous extracts of pellets of excreta consistently showed no aggregating or repellent effect.

Chemical Nature of Aggregating Factor. Aqueous extracts were prepared of untreated filter paper (189 strips), of paper conditioned by insects fed the artificial diet (135 strips), and of air-dried pellets of excreta from the larval stock culture maintained on the chow diet (0.3 g of pellets $\leqq$24 hr after deposition by the insects). Two milliliters of distilled water were used for the extraction of each filter paper, and 30 ml of distilled water for the extraction of the pellets of excreta. The combined extracts for each material were concentrated to a volume of about 5 ml in a stream of air, and then analyzed for volatile fatty acids by gas–liquid chromatography. Figure 1A shows a representative chromatogram obtained with the aqueous extract of the conditioned paper. The following fatty acids were identified: acetic, propionic, isobutyric, butyric, isovaleric, and valeric acid. With the extract of pellets of excreta, the proportions of propionic, butyric, and valeric acids were reduced, as were the unidentified components of the chromatogram (Figure 1B). Table 2 gives the fatty acid composition of the extracts. The amounts of acetic, propionic, butyric, valeric, and isovaleric acids estimated to be present on the average conditioned filter paper extracted are given in Table 3. The untreated filter paper contained only acetic acid, in a concentration about one tenth of that in which it was present in the excreta.

Acetic, propionic, butyric, valeric, and isovaleric acids were tested individually. When the treated paper was first dried, before testing, none of the fatty acids aggregated the larvae; in fact, all were repellent in concentrations in which they occurred on conditioned paper. The treated papers were then tested while wet. Aggregation was produced by propionic acid (Tables 4 and 5). The response was particularly strong at the $1 \times 10^{-4}\%$ level, the

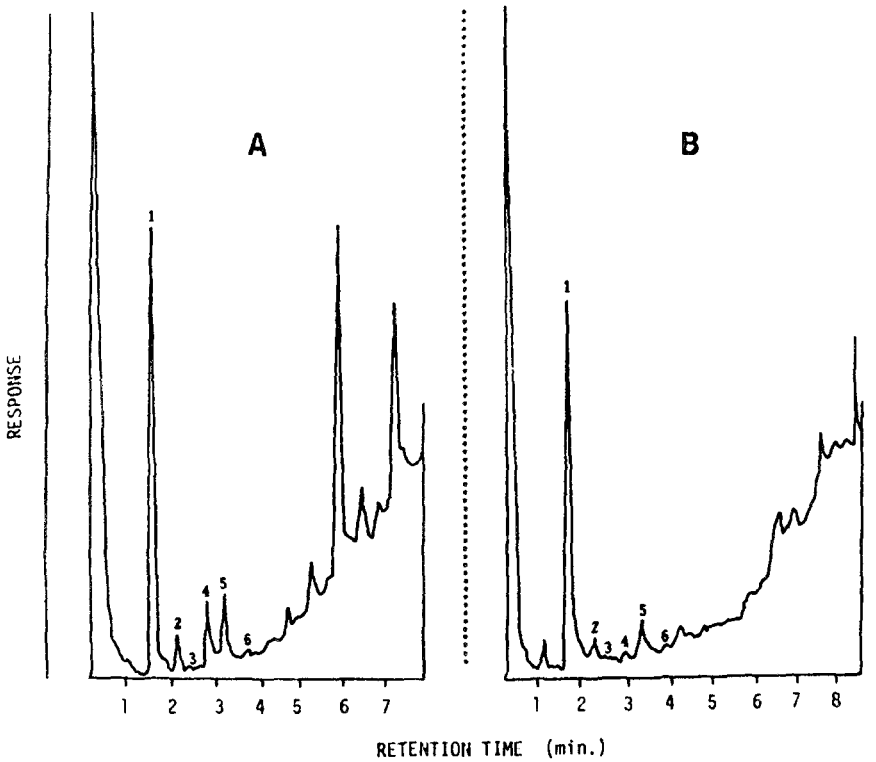

FIG. 1. Gas chromatograms of aqueous extracts of (A) filter papers conditioned by larvae and adults fed the artificial diet, and of (B) pellets of excreta from the larval stock culture: 1, acetic acid; 2, propionic acid; 3, isobutyric acid; 4, butyric acid; 5, isovaleric acid; 6, valeric acid.

TABLE 2. PERCENTAGE OF VOLATILE FATTY ACIDS IN EXTRACT OF CONDITIONED PAPER AND PELLETS OF EXCRETA

Acid	Total fatty acids in extract of (%)	
	Conditioned paper	Pellets of excreta
Acetic	58.8	72.5 (0.55 mg/g pellets)
Propionic	7.8	5.9 (0.045 mg/g pellets)
Isobutyric	trace	trace
Butyric	15.7	2.6 (0.02 mg/g pellets)
Isovaleric	15.7	17.8 (0.135 mg/g pellets)
Valeric	2.0	1.2 (0.009 mg/g pellets)

TABLE 3. AMOUNTS PER FILTER PAPER OF FATTY ACIDS EXTRACTED FROM CONDITIONED PAPER

Acid	Amount (μg) extracted/paper
Acetic	4.33
Propionic	0.57
Buytric	1.16
Isovaleric	1.16
Valeric	0.15

TABLE 4. RESPONSE OF *Acheta* LARVAE TO PROPIONIC ACID SOLUTIONS

Solution (%)	Total No. of trials	Mean No. on treated paper	Mean No. on control paper
1.0×10^{-1}	5	37 ± 12	14 ± 12
1.0×10^{-2}	3	23 ± 18	24 ± 13
1.0×10^{-3}	3	21 ± 7	23 ± 14
1.0×10^{-4}	6	35 ± 13	12 ± 6
1.0×10^{-5}	2	24 ± 8	28 ± 6
1.0×10^{-6}	7	30 ± 13	20 ± 12
1.0×10^{-7}	3	33 ± 5	19 ± 3
	29	30.5 ± 12.4[a]	18.2 ± 10.7

[a]Significantly greater than control mean at $P = 0.01$.

TABLE 5. RESPONSE OF *Acheta* LARVAE TO PROPONIC ACID SOLUTIONS

Solution (%)	Total No. of trials	Greater No. on treated paper			Greater No. on control paper		
		No. of trials	Mean No. on treated paper	Mean No. on control	No. of trials	Mean No. on treated paper	Mean No. on control
1.0×10^{-1}	5	4	41	9	1	21	31
1.0×10^{-2}	3	1	44	9	2	13	32
1.0×10^{-3}	3	1	29	8	2	17	31
1.0×10^{-4}	6	5	40	9	1	12	22
1.0×10^{-5}	2	1	29	24	1	18	32
1.0×10^{-6}	7	5	37	14	2	14	35
1.0×10^{-7}	3	3	33	19	0		
	29	20	37	13	9	15	31

TABLE 6. RESPONSE OF *Acheta* LARVAE TO SOLUTIONS OF FATTY ACIDS OTHER THAN PROPIONIC (1.0×10^{-1} % to 1.0×10^{-7} %)

Acid	Total No. of trials	Mean No. on treated paper	Mean No. on control
Acetic	14	24.0 ± 10.0	21.6 ± 7.1
Butyric	20	19.5 ± 11.7	25.4 ± 12.0
Valeric	33	22.0 ± 10.7	25.7 ± 11.8
Isovaleric	15	24.1 ± 12.8	26.7 ± 12.6

TABLE 7. RESPONSE OF *Acheta* LARVAE TO SOLUTIONS OF FATTY ACIDS OTHER THAN PROPIONIC (1.0×10^{-1} % to 1.0×10^{-7} %)

		Greater No. on treated paper			Greater No. on control papers			
Acid	Total No. of trials	No. of trials	Mean No. on treated paper	Mean No. on control	No. of trials	Mean No. on treated paper	Mean No. on control	Equal Nos.
Acetic	14	9	28	18	5	15	28	
Butyric	20	10	29	16	10	10	35	
Valeric	33	12	32	14	20	16	34	1
Isovaleric	15	6	37	16	8	15	35	1

concentration which corresponds to the amount present on conditioned paper. None of the other fatty acids were effective, although butyric and valeric acids possibly had slight repellent effects (Tables 6 and 7).

DISCUSSION

Propionic acid aggregates larvae of *Acheta* when presented in solution, but repels when presented in the dried, or pure state. Jacobson et al. (1968) have shown that valeric acid in solution attracts males of the sugar beet wireworm and is presumably the sex attractant for this species; in pure form it repels. In the wireworm, olfactory receptors only are involved, and the differing effects of valeric acid appear to be due to concentration differences. This may be true for aggregation and repellency in *Acheta* as well, or, since in aggregation there is contact with the stimulating surface, different receptors may be affected.

Conditioned papers are, however, dry, and dried papers to which extracts of conditioned papers have been applied also aggregate the larvae. There may

be attraction to these papers through the olfactory sense by a substance or combination of substances not yet tested. At the very least, there must be a factor which neutralizes the volatile repellent substance demonstrated by Sexton and Hess (1968). In point of fact, larvae will explore any paper presented to them, unless repelled, so the initial stimulus to aggregation may be that of an arrestant. As the larvae continue to excrete throughout the course of an experiment, newly recruited larvae could be responding to the fresh excreta of larvae already present on the paper, hence the observed clustering of larvae. Propionic acid may therefore be mimicking fresh excreta. It is also possible that the liquid part of fresh excreta dissolves a substance on conditioned paper which results in the initial arrest of exploring larvae.

Sexton and Hess (1968) interpret their results on aggregation and dispersion (repellency) in relation to population density control. Certainly, any increase in population density might produce a repellent concentration of substances attractive at a lower concentration. If nutrition were inadequate at high population densities, the production of less excreta, and hence less aggregating substance, might lead to dispersal.

The volatile fatty acids are the product of the fermentation of carbohydrate, and are similar to those produced by other cellulolytic insects (Breznak, 1982). The source of the fatty acids would be glucose or cellulose when the insects are fed the artificial diet. Cellulose is digested to the extent of 40–46% by the house cricket reared on an artificial diet (McFarlane and Distler, 1981). Microbial fermentation of carbohydrate in the gut of phytophagous insects may be more common than is generally supposed and may help to explain aggregation in other species, e.g., *B. germanica*. In *Blattella*, aggregation is produced by an ether extract, which would contain the volatile fatty acids (Ishii, 1970).

Fermentation may take place in the hindgut, but it remains to be determined in which divisions of the gut fermentation occurs and which is the principal site. Larvae of *A. domesticus* will not grow with cellulose as the sole carbohydrate in the diet; glucose or some other digestible sugar is also necessary (McFarlane and Ritchot, 1962). This may be due to a gustatory effect of the sugar.

The volatile fatty acids are present in the solid as well as the liquid part of the excreta, yet it is only the liquid part of the excreta that attracts or aggregates the larvae. There is slightly less propionic acid proportionately in the pellets than in the liquid part of the excreta, as well as less of some of the other fatty acids and unidentified substances. There may also be less attractive surface exposed to the insect, or possibly there is absorption of fatty acids by the solids of the excreta.

In *Locusta migratoria*, Nolte (1977) has found that locustol, 2-methoxy-5-ethyl phenol, produced by the action of microorganisms on lignin in the

crop, is excreted, volatilizes, and produces the morphological and physiological changes characteristic of gregarization. In view of the fact that filter papers conditioned by insects fed the artificial diet in our work were attractive to and led to aggregation of the larvae, locustol would seem to be ruled out as the aggregating substance in *Acheta*, as it is derived from lignin, which is not present in the diet.

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EXPERIMENTS WITH A TWO-COMPONENT SEX ATTRACTANT OF THE SILVER Y MOTH (*Autographa gamma* L.), AND SOME EVIDENCE FOR THE PRESENCE OF BOTH COMPONENTS IN NATURAL FEMALE SEX PHEROMONE

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Abstract—(*Z*)-7-Dodecen-1-yl acetate and (*Z*)-7-dodecen-1-ol were synthesized and tested on males of the silver Y moth (*Autographa gamma* L.) for sex attractant activity. The key step of the synthesis was the isomerization of acetylenic alcohol (III) with potassium 3-amino-propylamide. In EAG tests with a series of dodecen-1-yl acetates and alcohols, the highest activity was elicited by these two compounds. In field tests using three different kinds of dispensers, highest catches were achieved with a mixture of (*Z*)-7-dodecen-1-yl acetate and (*Z*)-7-dodecen-1-ol which contained 1–5% of the alcohol. Some evidence was also found for the presence of both compounds in extracts of the abdominal tip of females. The quantities of these components in the extract was 1.0 ng/female for the acetate, and 1.1 ng/female for the alcohol.

Key Words—Insect sex pheromone, sex attractant, *Autographa gamma* L., Lepidoptera, Noctuidae, silver Y moth, (*Z*)-7-dodecen-1-yl acetate, (*Z*)-7-dodecen-1-ol, ester, alcohol.

INTRODUCTION

The silver Y moth is a widespread polyphagous noctuid pest, occurring in Europe, North Africa, and the Middle East. The species is normally unable to

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overwinter north of the Mediterranean region, and populations in Central Europe develop each year from adults migrating from the south (Gozmány, 1970). Pheromone traps would provide a suitable means for detecting the occurrence of the pest in these areas. In the present paper we report on field and EAG tests of a two-component sex attractant of the species and present some evidence for their occurrence in the natural female sex pheromone.

METHODS AND MATERIALS

Chemical Synthesis. Instrumentation used in the chemical synthesis included a Spectromom 2000 infrared spectrophotometer, JEOL FX-100 FT NMR spectrometer, JEOL-20K and JMS-OLSG-2 combined GC-MS system, and a Pye 105 gas chromatograph.

The synthetic routes used for the preparation of (*Z*)-7-dodecen-1-yl acetate (I) and (*Z*)-7-dodecen-1-ol (II) are outlined in Figure 1. The readily

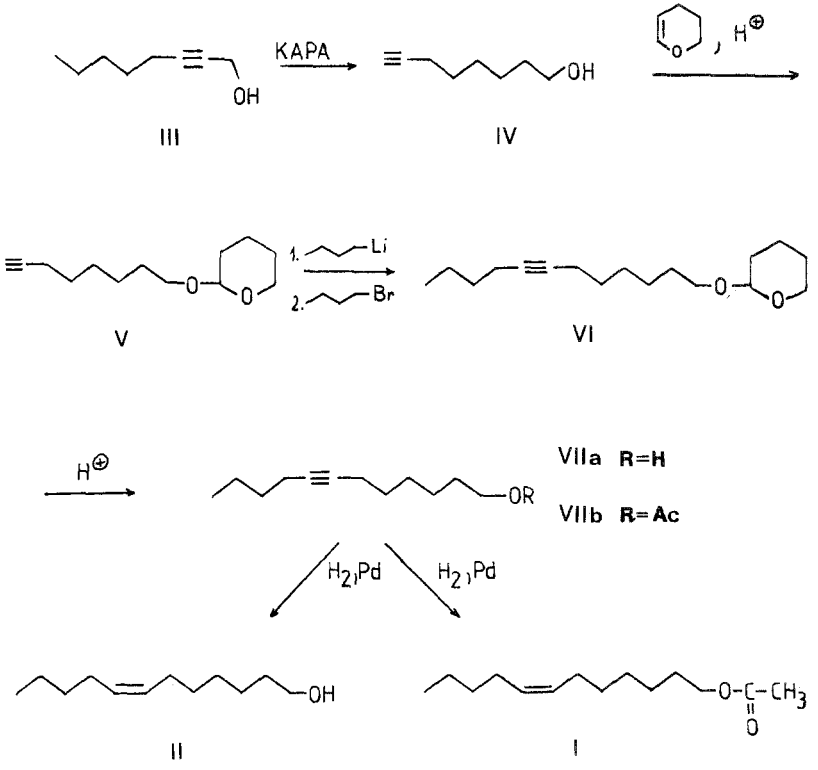

FIG. 1. Synthesis route for sex pheromone components of the silver Y moth.

available 2-octyn-1-ol (III) (Subramaniam and Fedor, 1975) was isomerized by potassium 3-aminopropylamide (KAPA) to give 7-octyn-1-ol (IV). Potassium hydride (18% dispersion in mineral oil, 28.0 g, 0.126 mol) was washed with dry hexane (600 ml) under argon. To this dry potassium hydride was added dropwise 3-aminopropylamine (44 g) at 0° C, and the resulting mixture was stirred at 0° C for 2 hr and then at room temperature for 8 hr. A solution of 2-octyn-1-ol (III) in dry tetrahydrofuran (10 ml) was added dropwise to the above cooled (0° C) solution of potassium 3-aminopropylamide and the mixture was allowed to stand at 0° C for 8 hr. The reaction mixture was poured into a well-stirred and cooled mixture of hexane (300 ml) and acetic acid (25 ml), the organic layer separated and washed successively with acetic acid and water, and dried. Removal of the solvent in vacuo afforded crude IV, which was purified by column chromatography (silica gel, hexane–ether). Yield: 2.0 g (78%).[^{1}H]NMR ($COCl_3$): δ 1.3 (8H, m, $4CH_2$), 1.95 (1H, t, J = 1.5 Hz, C≡CH), 2.5 (2H, m, CH2C≡C), 3.45 (2H, m, —O—CH_2), MS: 126 (<1) $[M]^{2+}$; m/z 111 (1) $[M-CH_3]^+$, 95 (10), 93 (28), 79 (44), 67 (41), 56 (34), 42 (100). IR (film): 3400 (OH), 1460, 1430, 1250, 1150 cm^{-1}). Crude IV was then transformed into the corresponding tetrahydropyranyl ether (V) by treatment with dihydropyran. Deprotonation of the latter with *n*-butyllithium gave the lithium salt of V, which was reacted with 1-bromobutane. Hydrolysis of the resultant ether (VI) yielded 7-dodecyn-1-ol (VII). This compound was hydrogenated in the presence of Pd-$BaSO_4$ catalyst to afford (*Z*)-7-dodecen-1-ol (II).

For the preparation of I, 7-dodecyn-1-ol (VII) was reacted with acetic anhydride in the presence of pyridine, and the resulting acetate (VII) was hydrogenated on Pd-$BaSO_4$ catalyst.

(*Z*)-7-Dodecen-1-yl acetate (I): GC: R_t = 7.6 min. IR (film): 1740 (CO), 1470, 1365, 1230, 1040 cm^{-1}. [^{1}H]NMR (CCl_4): δ 0.9 (3H, t, J = 6 Hz, CH_3), 1.35 (12H, m, 6 CH_2), 2.05 (3H, s, $COCH_3$), 2.0 (4H, m, CH_2—CH=CH—CH_2), 4.05 (2H, t, J = 7 Hz, O—CH_2), 5.35 (2H, t-d, J_t = 6 Hz, J_d = 1 Hz, CH=CH). MS: 226 (<1) $[M]^{+\cdot}$, m/z: 166 (35), $[M-AcOH]^{+\cdot}$, 123 (12), 110 (33), 109 (27), 96 (69), 95 (42), 82 (89), 81 (65), 68 (46), 66 (80), 55 (80), 43 (100), 41 (67).

(*Z*)-7-Dodecen-1-ol (II): GC: R_t = 5.4 min. IR (film): 4320 (OH), 1460, 1380, 1050 cm^{-1}. [^{1}H]NMR (CCl_4): δ 0.9 (3H, t, J = 7 Hz, CH_3), 1.4 (12H, m, 6 CH_2), 2.0 (4H, m, CH_2—CH=CH—CH_2), 2.3 (1H, s, exchangeable with D_2O, OH), 4.1 (2H, t, J = 7 Hz, O—CH_2), 5.34 (2H, t-d, J_t = 6Hz, J_d = 1 Hz, CH=CH). MS: 184 (2) $[M]^{+\cdot}$, m/z 166 (10) $[M-H2O]^{+\cdot}$, 140 (7), 111 (15), 110 (16), 97 (31), 96 (34), 95 (29), 83 (48), 82 (68), 81 (40), 69 (60), 55 (100), 43 (53), 41 (78). Purified (I) and (II) were shown to be 98% pure by GC analysis.

EAG Tests. EAG measurements were made using a method similar to that described by Roelofs (1977). The dose of compounds tested was 10/μg.

The amplitude of the antennal response for each test compound was normalized against a common standard, (*Z*)-5-dodecen-1-yl acetate which gave antennal responses of an amplitude of 1.0 ± 0.2 mV; blank: 0.1 mV), to produce EAG values in arbitrary units. Individual compounds were tested on one antenna of each of four male moths (four replicates).

Field Evaluation. The first unreplicated experiment was conducted at Pestimre (Budapest, Hungary) from July 1 to 30, 1979. Cylindrical traps (Tóth et al., 1979) were placed along the edge of a cabbage field, ca. 25 m apart and at a height of 0.5 m. The pheromone dispensers were 8-mm-ID glass vials containing 0.5 ml of sunflower-seed oil. Dispensers were replaced at weekly intervals during the test.

The second experiment was also conducted at Pestimre from June 12 to July 6, 1981. Pheromone traps (manufactured by EGYT and Reanal, Budapest, Hungary) were set up in three parallel lines in a cabbage field in a random layout. Trap spacing and placement were similar to those in the first experiment. The dispensers were sections of red rubber tubing (length 13 mm; 5 mm ID: 6 mm OD) a commercially available product purchased from Borászati Szaküzlet, Budapest, Hungary. There were three replicates of each treatment.

The third and fourth trials were conducted at Pestimre and at Vecsés, (Pest County, Hungary) from August 26 to October 27, 1981, in cabbage fields. Trap design and layout were the same as in the second experiment. The dispensers were 1-μl disposable micropipets (Microcaps, Drummond Scientific, Broomall, Pa.). About 0.5 μl of (*Z*)-7-dodecen-1-yl acetate and, in other treatments, a similar amount of mixtures of this compound with (*Z*)-7-dodecen-1-ol, was drawn into each micropipet. Mixtures of compounds were prepared beforehand in the required ratio. A single micropipet, with both ends open, was suspended in each trap. There were three replicates of each treatment.

Moths captured were collected weekly and their identity confirmed. Capture data were analyzed by means of Duncan's new multiple-range test using a $\sqrt{x + 0.5}$ transformation.

Analysis of Sex Pheromone Extracts. The moths came from a continously maintained laboratory culture reared on a semisynthetic diet (Szőcs and Tóth, 1982). For pheromone extraction, (2–5 days old) female moths were collected in the middle of the 6-hr dark period, when calling activity was strongest (Szőcs and Tóth, 1979). The last two distal abdominal segments of the females with the ovipositor were snipped off and extracted in the selected solvent for at least 24 hr. The supernatant extract was then removed and filtered through Na_2SO_4 and a layer of cotton wool.

An extract prepared in hexane from 250 females was analyzed on a Pye 105 type gas chromatograph (2 m × 2 mm glass column, 10% SE 54 on a

Chrom W 80–100 mesh, carrier gas nitrogen, at 30 ml/min, temperature program 180–250° C, at 4°/min). Another extract prepared in cyclohexane from 100 females was analyzed on a Varian Aerograph model 1400 gas chromatograph (4 m × 2 mm glass column, 5% Carbowax 20 M on Gas-Chrom Z 80–100 mesh, carrier gas helium, at 25 ml/min, 180° C), which was connected to a VG model 70/70 mass spectrometer set to scan repeatedly over m/z 166, which is a common ion for both (*Z*)-7-dodecen-1-yl acetate and (*Z*)-dodecen-1-ol. Before analysis the volume of the extracts was reduced to 0.1 ml in a nitrogen stream.

RESULTS

EAG Tests. Among the dodecen-1-yl acetates tested, (*Z*)-7-dodecen-1-yl acetate showed high EAG activity, producing a response amplitude 6.6 times that of the (*Z*)-5-dodecen-1-yl acetate standard, followed by (*E*)-7-dodecen-1-yl acetate (×2.1), (*Z*)-8-dodecen-1-yl acetate (×1.9), (*Z*)-9-dodecen-1-yl acetate (×1.5), and (*Z*)-6-dodecen-1-yl acetate (×1.4) (Figure 2). Other compounds produced only low to medium responses. In the alcohol series, (*Z*)-7-dodecen-1-ol was again the most active (×4.0), followed by (*E*)-7-dodecen-1-ol (×2.1), and (*Z*)-9-dodecen-1-ol (×1.3). The other compounds tested showed only limited activity (Figure 2).

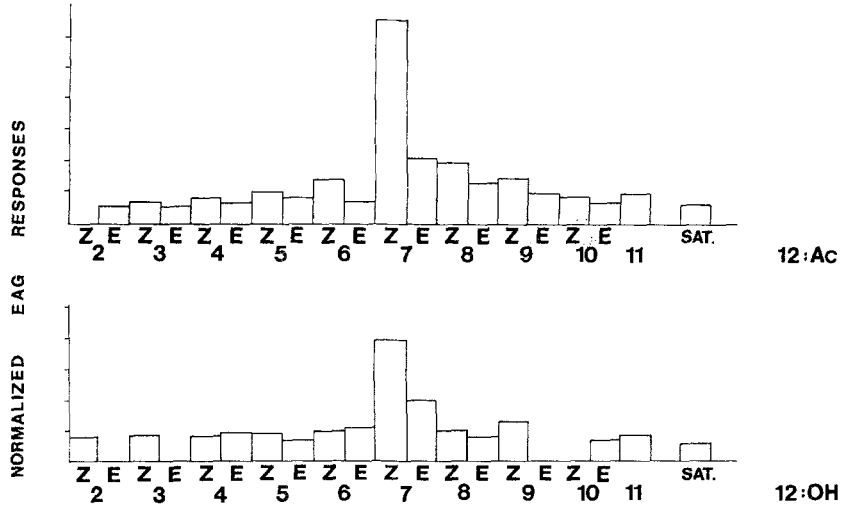

FIG. 2. Normalized EAG responses of male *Autographa gamma* (L.) antennae to a series of monounsaturated dodecen-1-yl acetates and alcohols. (*Z*)-5-Dodecen-1-yl acetate was used as standard.

TABLE 1. CAPTURES OF MALE SILVER Y MOTHS IN TRAPS BAITED WITH SYNTHETIC SEX PHEROMONE COMPONENTS AND MIXTURES AT PESTIMRE (BUDAPEST, HUNGARY), JULY 1–30, 1979 (1 REPLICATE)

Lure[a]	Number of males captured
(*Z*)-7-12:Ac (1000 μg)	0
(*Z*)-7-12:Ac (950 μg) + (*Z*)-7-12:OH (50 μg)	16
(*Z*)-7-12:Ac (800 μg) + (*Z*)-7-12:OH (200 μg)	0
(*Z*)-7-12:Ac (500 μg) + (*Z*)-7-12:OH(500μg)	0
Unbaited	0

[a] Dispenser: sunflower-seed oil in glass vials.

Field Evaluation. In the first unreplicated trial (Table 1), the only combination which caught males of the silver Y moth was a 95:5 mixture of (*Z*)-7-dodecen-1-yl acetate and (*Z*)-7-dodecen-1-ol. Mixtures in other ratios, the acetate alone, and the unbaited control traps caught no moths.

In the experiment where red rubber tubings was used as a dispenser (Table 2), highest catches were achieved with the 100:1 and 95:5 mixtures of (*Z*)-7-dodecen-1-yl acetate and (*Z*)-7-dodecen-1-ol, while the acetate alone captured significantly fewer males. Only two moths were captured at a 1:1 mixture, and no catches were recorded at the alcohol alone and the unbaited traps.

The two experiments with 1-μl micropipets (Table 3) gave very similar

TABLE 2. CAPTURES OF MALE SILVER Y MOTHS IN TRAPS BAITED WITH SYNTHETIC SEX PHEROMONE COMPONENTS AND MIXTURES AT PESTIMRE (BUDAPEST, HUNGARY) JUNE 12–JULY 6, 1981 (3 REPLICATES)

Lure[a]	Number of males captured
(*Z*)-7-12:Ac (1000 μg)	14 ac
(*Z*)-7-12:Ac (1000 μg) + (*Z*)-7-12:OH (10 μg)	38 b
(*Z*)-7-12:Ac (950 μg) + (*Z*)-7-12::OH (50 μg)	29 bc
(*Z*)-7-12:Ac (500 μg) + (*Z*)-7-12:OH (500 μg)	2 a
(*Z*)-7-12:OH (1000 μg)	0 a
Unbaited	0 a

[a] Dispenser: red rubber tubing. Captures followed by the same letter are not significantly different at the 5% level (Duncan's NMRT).

TABLE 3. CAPTURES OF MALE SILVER Y MOTHS IN TRAPS BAITED WITH SYNTHETIC SEX PHEROMONE COMPONENTS AND MIXTURES AT PESTIMRE (BUDAPEST, HUNGARY) AND VECSÉS (PEST COUNTY, HUNGARY), AUGUST 26–OCTOBER 27, 1981 (3 REPLICATES)

Lure[a]	Number of males captured	
	Vecsés	Pestimre
(Z)-7-12:Ac	6 ac	8 a
(Z)-7-12:Ac + (Z)-7-12:OH 95:5	44 b	25 b
(Z)-7-12:Ac + (Z)-7-12:OH 7:3	18 a	6 a
(Z)-7-12:Ac + (Z)-7-12:OH 1:1	8 ac	3 ac
Unbaited	0 c	0 c

[a] Dispenser: 1-μl micropipet (Drummond Scientific). Captures followed by the same letter within one column are not significantly different at the 5% level (Duncan's NMRT).

results. In both cases the highest catches were recorded at the 95:5 mixtures of (Z)-7-dodecen-1-yl acetate and (Z)-7-dodecen-1-ol. Relatively few moths were caught at mixtures in other ratios and the acetate alone. Unbaited controls caught no moths.

Analysis of Sex Pheromone Extracts. On the 10% SE 54 column, two major peaks of about the same height were detected, one in the region of dodecen-1-yl acetates and the other in that of dodecen-1-ols. Their retention times coincided well with those of synthetic (Z)-7-dodecen-1-yl acetate and (Z)-7-dodecen-1-ol (7.6 and 5.4 min for acetate and alcohol, respectively).

Selective ion monitoring with the GC-MS (5% Carbowax 20 M column) also revealed two peaks, the retention times of which again coincided with those of synthetic (Z)-7-dodecen-1-yl acetate and (Z)-7-dodecen-1-ol (synthetic: 3.56 min for acetate, 4.42 min for alcohol; extract: 3.57 min and 4.42 min). Injections of known quantities of these two compounds and the extract, and comparisons of peak heights, allowed the pheromone content of the extract to be calculated as 1.0 ng/female and 1.1 ng/female for (Z)-7-dodecen-1-yl acetate and (Z)-7-dodecen-1-ol, respectively.

DISCUSSION

On the basis of the above results, we conclude that (Z)-7-dodecen-1-yl acetate and (Z)-7-dodecen-1-ol may constitute part of the silver Y moth natural female sex pheromone. Ghizdavu et al. (1979) have earlier reported, as a result of a chance finding, that (Z)-7-dodecen-1-yl acetate attracts males of

the silver Y moth in the field. According to Priesner (1980), sensilla on the antennae of silver Y moth males contain specialist sensillum cells for (*Z*)-5-decen-1-yl acetate, (*Z*)-5-dodecen-1-yl acetate, (*Z*)-9-tetradecen-1-yl acetate, (*Z*)-7-dodecen-1-yl acetate, and (*Z*)-7-dodecen-1-ol. Priesner (1980) also mentioned that a 95:5 mixture of the latter two compounds was an effective attractant in the field, but presented neither data on the mixture's attractivity as related to that of the acetate alone or mixtures in other ratios, nor actual capture figures. These two reports are in accord with our findings. Many moths of the Plusiinae subfamily appear to have (*Z*)-7-dodecen-1-yl acetate as a pheromone component. Its presence in the female has been reported in *Pseudoplusia includens* (Walk.) (Tumlinson et. al., 1972), *Autographa biloba* (Steph.), *Rachiplusia ou* (Guenée) (Berger and Canerday, 1968), and *Trichoplusia ni* (Hbn.) Berger, 1966; Bjostad et al., 1980).

(*Z*)-7-Dodecen-1-ol is a component of the sex attractant of *Anagrapha falcifera* (Kirby) (Underhill et al., 1977) and *Autographa californica* (Speyer) (Steck et al., 1979), but until now its presence has not been verified in any female-secreted sex pheromone of Plusiinae.

The present study revealed a discrepancy between the ratios of the two components found in the pheromone extract and the most attractive ratio in field trapping. At present our data are insufficient to offer a reliable explanation. There are other examples where ratio (or even presence) of components was related to the extraction method applied (Persoons et al., 1981). In the present study only one extraction method was used, so the clarification of the cause of the discrepancy should be the object of further studies.

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FIELD ATTRACTIVENESS OF (*E*)- AND (*Z*)-11-TETRADECENAL PHEROMONE BLENDS TO MALE SPRUCE BUDWORM MOTHS, *Choristoneura fumiferana* (CLEMENS)[1–3]

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Abstract—*E*:*Z* blends of (*E*)-11-tetradecenal were field tested (three experiments) for their attractiveness to male spruce budworm, *Choristoneura fumiferana* (Clemens), moths in northern Maine. Blends of 92.5–99% *E* isomer caught the most moths (three experiments); blend 95% *E* had the highest cumulative catch throughout two experiments. Rates of catch per hour for the four most attractive blends (92.5–99% *E*) showed highly variable responses among experiments; however, similarities were noted for rates of catch within the same experiment. For all experiments and observation hours, blend 95% *E* had the highest mean rate of catch.

Key Words—Spruce budworm moths, *Choristoneura fumiferana*, Lepidoptera, Tortricidae, sex pheromone, attractants, isomeric blends.

INTRODUCTION

The spruce budworm, *Choristoneura fumiferana* (Clemens), is a serious defoliating pest of spruce–fir forests in the northeastern United States and Canada. Pheromone-baited traps have potential for early detection and

[1] Lepidoptera: Tortricidae.
[2] A contribution to the Canada/United States (CANUSA) Spruce Budworms Program.
[3] Mention of commercial or proprietary products does not constitute an endorsement of those products by the USDA or the University of Maine.

monitoring populations of the spruce budworm. Before traps are routinely used for these purposes, numerous variables that may affect trap catches need to be investigated, including proper isomeric blends of synthetic pheromone.

Weatherson et al. (1971) identified (*E*)-11-tetradecenal as the primary component of the spruce budworm sex pheromone. Subsequently, Sanders and Weatherson (1976) showed that the 11-tetradecenal composition from virgin female washes was 96–96.1% *E* and 3.9–4.0% *Z*. After field trials with synthetic baits, they concluded that maximum attraction occurs with the *E* isomer containing 2–5% of the *Z* isomer. More recently Silk et al. (1980) have shown that glands from virgin female moths contain three major classes of compounds with each class consisting of an *E*11/*Z*11 unsaturated pair and an *E*:*Z* ratio of 95:5.

Because response of male moths to isomeric blends may vary from one location to another, we field-tested the attractiveness of various *E*:*Z* blends of 11-tetradecenal in a spruce-fir forest of Maine infested with spruce budworm. Similar tests had shown some locational differences in attractiveness of *E*:*Z* blends in New Brunswick, Pennsylvania, Alberta, and Ontario (Sanders and Weatherston, 1976). This paper summarizes our findings in Maine on the field attractiveness of (*E*)- and (*Z*)-11-tetradecenal blends to male spruce budworm moths, including rates of attraction and hourly cumulative catches.

METHODS AND MATERIALS

Three separate experiments with hourly observations were conducted near Telos Lake, 64 km northwest of Millinocket, Piscataquis County, Maine. The first two experiments, July 12 and 14, 1977, were near the beginning of the budworm flight period; the third experiment, July 22, 1977, was near the end of the same flight period. The flight had already begun by July 12 and had essentially ceased by July 27 (Houseweart et al., 1981).

Pherocon® 1CP traps were placed along five replicated transects. Following the methods of Sanders (1978), traps were hung ca. 1.5–2 m above ground from dead understory branches. Individual traps were spaced 20 m apart in a NW–SE line, with line transects 40 m apart. Treatments (blend ratios) were assigned randomly within each transect and positions randomized separately for each experiment.

Each Pherocon 1CP trap was baited with a 4-mm-diam. × 10-mm-long polyvinyl chloride (PVC) lure containing 3% by weight of the combined aldehydes. The following *E*:*Z* blends were tested: 60:40, 70:30, 80:20, 85:15, 90:10, 92.5:7.5, 95:5, 97:3, 99:1, and 100:0. Lures were prepared at the Great Lakes Forest Research Centre, Sault Ste. Marie, Ontario; preparation and determination of isomeric composition followed the methods described

by Sanders and Weatherston (1976). PVC formulation and release rates for similar lures are described by Sanders (1981a).

Trap catches were observed hourly for 5–7 hr. For experiment 1, traps were observed each hour from 1230 to 1630 hr (EDT); for experiment 2, from 1115 to 1715 hr (EDT); and, for experiment 3, from 1130 to 1630 hr (EDT). At each observation, counts were made of spruce budworm moths caught in the trap's sticky material. Thus, cumulative data were obtained on trap catches and rates of capture for each pheromone blend. New trap bottoms were installed at the beginning of the next succeeding experiment.

Stand measurement data were taken from two (0.04 hectare) random plots located along the transect lines. Fixed-plot samples were used, and all trees over 5 cm in diameter at breast height (DBH) were measured for basal area calculations. Budworm defoliation and population estimates were made on two nearby study sites: one 2.4 km NW and one 3.6 km SW of the blend test location. Budworm infestations had occurred in these stands for the previous 4–5 years. The area was sprayed in 1976 with Sevin-4® oil, but was not sprayed in 1977.

RESULTS AND DISCUSSION

Stand measurements indicated that the study site was a dense, mature spruce–fir stand. Average stand height was 17.3 m, average crown density was 87%, and average age at DBH was 52 years old. Total basal area averaged 53.5 m^2/hectare and was comprised of the following species by percentage: red spruce, *Picea rubens* Sargent (29.9%); balsam fir, *Abies balsamea* (L.) Miller (44.6%); northern white cedar, *Thuja occidentalis* L. (22.6%); white birch, *Betula papyrifera* Marshall (1.4%); and red maple, *Acer rubrum* L. (0.3%). Budworm defoliation averaged 56% and 51% at nearby locations in 1977; late-larval and pupal densities were 74.6 and 85.4/m^2 of foliage (Houseweart et al., 1981).

During the hourly observations, moth catches exceeded the 50-moth saturation level (Houseweart et al., 1981) in only two instances. All other catches were <50 moths per trap; hence, we conclude that trap saturation was not a significant problem during the hourly observational periods for the three experiments.

For all three experiments, blends ranging from 92.5 to 99% *E* isomer caught the most moths (Table 1). Comparing mean cumulative catches among the four most attractive blends (92.5–99% *E*) showed little change in treatment significance from the first to the final hour of observation. Although not significantly different, blend 95% *E* had the highest cumulative catch throughout experiments 1 and 2. Initially, blend 92.5% *E* was most

attractive in experiment 3, but was superseded by blend 95% *E* in the final two hours of observation. Mean cumulative catches were generally lower for blend 99% *E*, and occasionally differed from other blends in the 92.5–97% *E* range.

Our results agree with other tests designed to determine the optimum blend for attracting male *C. fumiferana* to traps baited with synthetic lures. In Ontario with 3% PVC lures and 3-M® SA-21 traps, Sanders and Weatherston (1976) found no significant differences among catches over the blend range 95–100% *E* in one experiment, whereas in a second experiment they found no significant differences over the range 90–100% *E*. Blend 92.5% *E* was not tested. In New Brunswick, Pennsylvania, and Alberta with 3% PVC lures and 3M Sectar 1 traps, there were no significant differences in catches over the range 80–100% *E*, but in all these trials highest catches occurred between 95 and 98% *E* (Sanders and Weatherston, 1976). Additional tests in Ontario with PVC lures in Pherocon 1C traps showed no significant differences in catches over the range 92.5–99% *E* (Sanders, 1981b; Cory et. al., 1982). In Michigan with rubber septa lures and Pherocon 1C traps, Ramaswamy and Cardé (1982) reported that traps baited with 1000 μg of *E* and 3–5% *Z* trapped the most moths.

By contrast the previous tests (Sanders and Weatherston, 1976; Sanders, 1981b), we detected very little attraction to the blend containing 100% *E* (Table 1). Sanders and Weatherston (1976) attributed high catches to a trace

TABLE 1. MEAN CUMULATIVE CATCHES OF MALE SPRUCE BUDWORM MOTHS IN PHEROCON 1CP TRAPS BAITED WITH VARIOUS (*E*:*Z*)-11-TETRADECENAL PHEROMONE BLENDS, TELOS LAKE, MAINE, 3 EXPERIMENTS, JULY 12, 14, AND 22, 1977

E isomer (%)	$\bar{X}$ cumulative catch		
	Experiment 1 (5 hr)	Experiment 2 (7 hr)	Experiment 3 (6 hr)
60	0.2 d[a]	0.4 d	0.0 b
70	0.6 d	1.2 cd	0.2 b
80	1.2 d	1.2 cd	0.4 b
85	1.4 d	2.0 cd	2.0 b
90	3.2 cd	6.6 bc	5.0 b
92.5	5.2 bc	12.8 a	19.4 a
95	10.6 a	15.2 a	19.8 a
97	8.4 ab	12.8 a	17.8 a
99	7.4 ab	9.4 ab	10.2 ab
100	0.2 d	0.4 d	0.0 b

[a] Column means followed by a different letter(s) are significantly different; $\alpha = 0.05$, Duncan's multiple-range test.

amount (<0.3%) of *Z* isomer in the 100% *E* blend tested. Ramaswamy and Cardé (1982) found very little attraction with the 100% *E* containing 0.2% *Z* in Michigan.

Comparing rates of catch per hour for the four most attractive blends, 92.5–99% *E*, showed highly variable responses among experiments; however, remarkable similarities were noted for mean rates of catch among blends within the same experiment, indicating that moths were attracted about equally to all blends within the same *E* range. Although individual differences were noted for certain hours, factors other than blend were probably responsible for hourly changes in capture rates.

For all experiments and hours of observation combined, blend 95% *E* had the highest mean rate of catch. The corresponding percentage, 5% *Z* isomer, compares favorably with the 4% figure found in washes from virgin female moths (Sanders and Weatherston, 1976) and even more favorably to the 5% ratio reported by Silk et al. (1980) for gland extracts.

The Maine experiments support earlier findings elsewhere concerning the optimum blend for attracting male spruce budworm moths to pheromone-baited traps. Although slight regional differences may occur, our results indicate that PVC lures containing a blend of 95–97% *E* should give maximum catches in Pherocon 1CP traps.

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(*Z*)-10-NONADECENAL: A Pheromonally Active Air Oxidation Product of (*Z,Z*)-9,19 Dienes in Yellowheaded Spruce Sawfly[1,2]

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Abstract—Each of 10 (*Z,Z*)-9,19 hydrocarbon dienes (28–37 carbons) found in female yellowheaded spruce sawflies, *Pikonema alaskensis* (Rohwer), was synthesized and found to be attractive to males by field bioassay. These dienes oxidize slowly in air, major products being aldehydes. One product common to all the dienes, (*Z*)-10-nonadecenal (*Z*10–19:Al), and one from the 29-carbon diene only, (*Z*)-10-eicosenal, initiated flight and caused upwind anemotaxis in males in a greenhouse bioassay. *Z*10–19:Al was incorporated into Hercon® controlled release formulations and was active in the field. The aldehyde could be useful for monitoring sawfly populations.

Key Words—Sawfly, pheromone, aldehyde, diene, oxidation, *Pikonema alaskensis*, Hymenoptera, Tenthredinidae.

INTRODUCTION

The yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae) is a significant defoliator of spruce in the nothern United States and Canada. Evidence for a sex pheromone in this species was presented by Bartelt et al. (1982a). Ten active (*Z,Z*)-9,19 hydrocarbon dienes, of 28–37 carbons, were identified in female sawflies, and the five more abundant, odd-chain dienes were synthesized and found to be

[1] *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae).
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active. (*Z*,*Z*)-9,19 dienes were both necessary and sufficient to elicit attraction in males, although additon of (unknown) female-derived synergists could increase the response to dienes (Bartelt et al., 1982b).

Olefins of similar, high molecular weights have been reported to have pheromonal activity in a number of dipterans, such as *Musca autumnalis*, *Fannia pusio*, and *Stomoxys calcitrans*, but these compounds did not evoke the same sort of long-range response that we reported for the sawfly; instead these olefins appeared to act only over short distances (Howard and Blomquist, 1982).

A mechanism for the activity of the (*Z*,*Z*)-9,19 dienes from *P. alaskensis* is presented as an alternative to simple evaporation. All 10 dienes oxidize slowly in air, and one common product, (*Z*)-10-nonadecenal (*Z*10–19:Al), has primary pheromonal activity. That olefin precursors are activated by oxidation has been previously reported. For example, the gypsy moth (*Lymantria dispar*) pheromone, an epoxide (2-methyl-7,8-epoxyoctadecane), is apparently produced in the females by oxidation of the olefin, 2-methyl-(*Z*)-7-octadecene (Bierl et al., 1972; Kasang et al., 1974). In the present system, however, the oxidation step is able to occur in the absence of the insect. An active compound is produced from the dienes under physical conditions which might typically cause pheromone degradation (e.g., Shani and Klug, 1980).

METHODS AND MATERIALS

Chromatography, Spectra, Bioassays. Except where otherwise indicated, the chemical separations (GLC, HPLC, column chromatography), mass spectral analysis, and field and greenhouse bioassays involved procedures and equipment described earlier (Bartelt et al., 1982a,b).

Synthetic Compounds. The 10 homologous dienes listed in Table 1 were prepared and purified essentially as outlined in Bartelt et al. (1982b). The (*Z*)-10 unsaturated aldehydes of 17, 18, and 20–26 carbons were prepared by standard methods as summarized in Figure 1. (The homologous 19-carbon aldehyde was already prepared as an intermediate in the diene synthesis). The aldehydes were purified by HPLC on $AgNO_3$–silicic acid (toluene as solvent) and by preparative GLC on Silar 5CP. The dienes and aldehydes used for bioassay were at least 97% pure (by GLC) except (*Z*)-10-pentacosenal, which was 93% pure.

Weathering of Dienes in the Laboratory. Ten-microliter aliquots of hexane solutions of purified dienes (20–26 μg/aliquot) were applied to watch glasses, four replications for each diene. (Watch glasses were also used when dienes were bioassayed). Two were placed at −15°C in tightly closed containers in the dark, while the other two were placed in a laboratory hood at

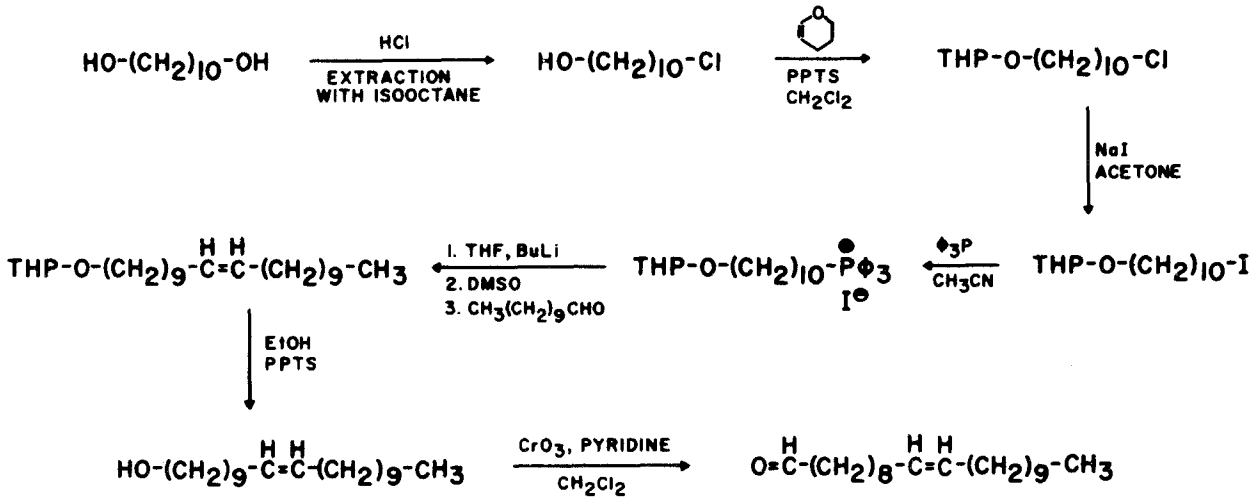


FIG. 1. Synthetic schemes for (*Z*)-10 unsaturated aldehydes. The 21-carbon aldehyde is given as an example; for other chain lengths, a different saturated aldehyde was used in the Wittig reaction. Abbreviations: PPTS = pyridinium *p*-toluenesulfonate, a catalyst; THP = the tetrahydropyranyl protective group; THF = tetrahydrofuran; BuLi = butyllithium; DMSO = dimethylsulfoxide.

ambient temperature for 24 hr. Some exposure to overhead room lights occurred. The deposits were removed from the watch glasses with hexane rinses, and quantitated by GLC. Percent loss during the 24-hr period in the hood was calculated by comparing to the plates stored in the freezer. The two samples from the hood for each diene were then combined, concentrated, and analyzed by GLC for trace oxidation products. Additional samples with 0.1 mg of 2,5-di-*tert*-pentylhydroquinone were run to test the efficiency of this antioxidant, which had been routinely added to dienes for field studies (Bartelt et al., 1982b). (It was earlier found empirically that the antioxidant extended the active life of diene baits under greenhouse conditions for several days).

Controlled Release Formulation of Z10–19:Al. Because of time constraints and the relatively large amount of material needed for commerical formulation (5 g), purification was limited to distillation and removal of nonaldehyde impurities through the use of a bisulfite addition product (Vogel, 1956, p. 331). Polymer formulations of Z10–19:Al were prepared by the Hercon Division of the Health-Chem Corp. (New York) as a bait for field bioassay. Three levels (7.5, 0.67, and 0.08 mg/cm^2) of a formulation with 16-mil polymeric film on both sides (releasing pheromone over its whole surface) and three levels (4.4, 0.68, and 0.09 mg/cm^2) of one with mylar on both sides (releasing just at the cut edges) were made, and 1.0 × 1.0-cm pieces were used as trap baits. The target release rate for the medium level of each formulation type was 50 ng/hr/cm^2. (The rate of disappearance of 100 ng of aldehyde from a watch glass under greenhouse conditions was 50 ng/hr.) The release rates of the three levels were to span two orders of magnitude. Attempts to measure actual release rates were not successful.

Field Studies with Hercon Formulations. The Hercon formulations of Z10–19:Al were compared using the randomized complete block design (1 trap/tree, five blocks). The same baits were used for the six consecutive 2-day tests (spanning virtually the entire flight season), except that one medium-level treatment was replaced every 2 days so that changes over time in the other treatments could be assessed. The study was rerandomized and trap bodies replaced every 2 days. The Hercon baits were aired in a hood for 4 days prior to beginning the field test, to stabilize the release rates. The baits to be used for replacement every 2 days were returned to the freezer until needed.

RESULTS AND DISCUSSION

Comparisons among (Z,Z)-9,19 Dienes in the Field. The study (Table 1) demonstrated that synthetic even-chain dienes of 28, 30, 32, 34, and 36 carbons were active in the field, and it reconfirmed the activities of the odd-chain dienes previously synthesized and field tested (Bartelt et al.,

TABLE 1. BIOASSAY OF SYNTHETIC DIENES[a]

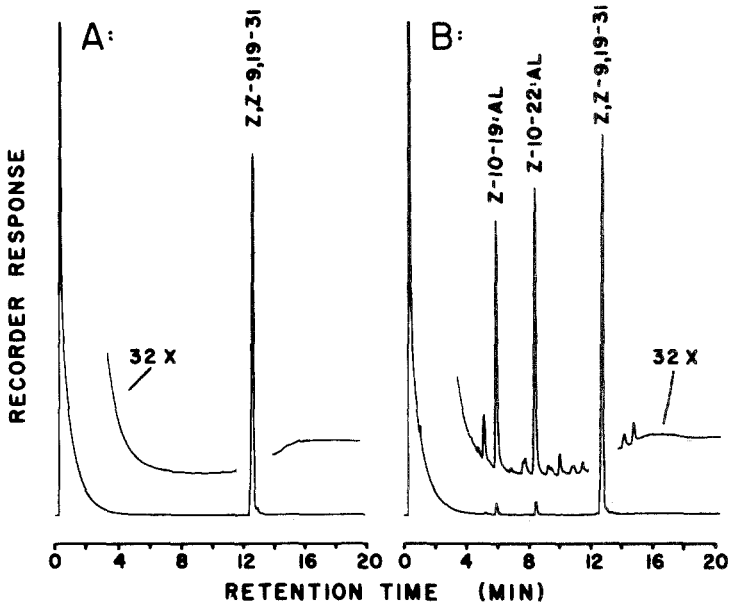
Treatment			
No. of carbons	Name	Abundance in females[b] (%)	Mean trap catch[c] (males/2 days, $N = 44$)
28	(Z,Z)-9,19-octacosadiene	0.6	23.5 e
29	(Z,Z)-9,19-nonacosadiene	22.5	11.9 c
30	(Z,Z)-9,19-triacontadiene	0.4	16.7 cde
31	(Z,Z)-9,19-hentriacontadiene	11.1	18.1 de
32	(Z,Z)-9,19-dotriacontadiene	1.1	18.1 de
33	(Z,Z)-9,19-tritriacontadiene	19.7	4.7 b
34	(Z,Z)-9,19-tetratriacontadiene	1.7	14.7 cd
35	(Z,Z)-9,19-pentatriacontadiene	35.6	6.8 b
36	(Z,Z)-9,19-hexatriacontadiene	0.5	22.9 e
37	(Z,Z)-9,19-heptatriacontadiene	5.5	17.8 de
	Mixture of all ten dienes[d]		15.6 cd
	Control		2.2 a

[a] 0.05 μmol diene/trap (20–26 μg, depending on molecular weight). Test conducted May 18–27, 1981, near Grand Rapids, Minnesota, using balanced incomplete block design (2 traps/tree).
[b] As % of total (Z,Z)-9,19 dienes in females; ca. 20 μg of total dienes can be obtained from females 2–3 days old.
[c] Means followed by the same letter are not significantly different (LSD, 0.05).
[d] Proportions as in female sawflies, 23 μg/trap.

1982b). With the exception of the 33-, 35-, and, perhaps, the 29-carbon dienes, each performed at least as well as the mixture of all 10 (proportions as in female sawflies). The relationship among the five odd-chain dienes were very similar to those reported in the earlier paper. (All dienes were freshly synthesized and purified for the present study.) In the 1982 paper, the mixture of all five odd-chain dienes were significantly more attractive than any one, but in the present study, the mixture of all 10 dienes was similar to, but not more attractive than, the best odd-chain diene. This difference might have been due to traces of five even-chain dienes in the present mixture, differences between sawfly populations, weather conditions, or other unknown factors.

The least active dienes, of 33, 35, and 29 carbons, were, curiously, the most abundant in the female sawflies, together comprising 78% of the total dienes. Although these differences will require further explanation, it was the similarity among the other seven dienes that was most striking, despite the great differences in chain length.

Weathering of Dienes in the Laboratory. After 24 hr exposure to air, recovery of the dienes initially applied to the watch glasses was 53–80%. The greatest loss occurred with the 28-carbon (smallest) diene. By GLC, a number of additional compounds were present in the weathered samples (Figure 2).



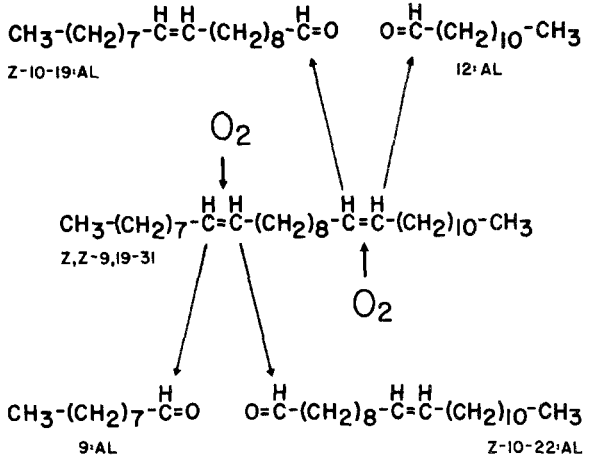


FIG. 2. Above: Gas–liquid chromatograms of the 31-carbon diene: not exposed to air in the hood (A), and after 24-hr exposure in the hood (B). Insets show scale expanded 32×. (Dexsil 300, 180° for 1 min, then 10°/min to 340°). Below: Expected aldehyde fragments from double-bond cleavage.

The major oxidation reaction apparently involved cleavage at either double bond to produce aldehydes. One product common to all 10 dienes had the same GLC retention as synthetic *Z*10–19:Al. Their mass spectra were also identical [*m*/*e*:280 (13%, M), 262 (17%), 98 (98%), 83 (100%)]. Two major GLC peaks, corresponding to the appropriate unsaturated aldehydes, were observed for each diene except the symmetrical C_{28} diene, which produced only one. The appropriate saturated aldehyde cleavage fragments were also detected for each diene (most at cooler GLC conditions than in Figure 2).

The antioxidant, 2,5-di-*tert*-pentylhydroquinone, reduced the rate of diene loss from 45% to 34% in 24 hr for the 31-carbon diene, but by GLC, *Z*10–19:Al was present in a typical amount, relative to the diene peak (1:20). Thus oxidation of dienes in field tests may have been retarded by the compund but was not prevented.

The disappearance of 30% from 20 μg (ca. 1 female equivalent) of dienes in 24 hr would represent a loss rate of 250 ng/hr. Since aldehydes appeared to be the major oxidation products and since about one fourth of the aldehyde products would be *Z*10–19:Al, a rough estimate for the maximum release rate of *Z*10–19:Al from such a plate would be ca. 60 ng/hr, similar to the amount of *Z*10–19:Al used for greenhouse bioassay.

Greenhouse Bioassay of Aldehydes. In screening tests in the greenhouse bioassay chamber, synthetic *Z*10–19:Al (in the range of 50 ng–1 μg) and *Z*10–20:Al (tested only at 100 ng) each consistently elicited initiation of flight and upwind anemotaxis. Males also landed on treated plates, but visits, when they occurred, were usually brief (less than 10 seconds) and without mating attempts. *Z*10–19:Al was produced from all 10 dienes, but *Z*10–20:Al, only from the 29-carbon diene. Of the other (*Z*)-10 unsaturated aldehydes (17, 18, 21–26 carbons), only *Z*10–21:Al caused a slight anemotactic response. None of the saturated aldehydes (9–18 carbons) caused any noticeable effect.

Female-derived (*Z,Z*)-9, 19 dienes (20 μg/plate, aged 2 hr before the tests), *Z*10–19:Al (100 ng/plate), and controls were compared by quantitative greenhouse bioassay. The mean scores were 58.2, 6.5, and 0.3 males counted, respectively ($N = 6$, 8 min/run). Thus in this 3-way choice test, the oxidation product was superior to the parent dienes.

Field Bioassay of Hercon Formulations. Table 2 shows the field bioassay results for the Hercon formulations of *Z*10–19:Al. The medium level of each formulation type [emitting from the whole surface (WS) or just the cut edges (CE)] was significantly more attractive than controls, as was the high level of the CE formulation ($P < 0.001$). The low levels of both formulations and the high level of the WS formulation were no different from controls, however. This last treatment indicated it was possible to present too much aldehyde for effective trapping.

TABLE 2. FIELD BIOASSAY OF CONTROLLED RELEASE FORMULATION OF Z10–19:Al[a]

Treatment[b]		Mean catch (males/2 days, $N = 30$)[c]
Formulation emitting from cut edges only		
4.4 mg/cm^2	(High CE)	26.0 bc
0.68 mg/cm^2	(Medium CE)	19.5 b
0.09 mg/cm^2	(Low CE)	7.5 a
Formulation emitting from whole surface		
7.5 mg/cm^2	(High WS)	8.4 a
0.67 mg/cm^2	(Medium WS)	38.9 c
0.08 mg/cm^2	(Low WS)	7.2 a
0.67 mg/cm^2 (replaced every 2 days)	(Medium WS)	31.5 bc
Control		7.2 a

[a]Test conducted May 16–29, 1982, near Grand Rapids, Minnesota; experimental design was randomized block (1 trap/tree).
[b]Except where otherwise noted, the baits were used for the entire study.
[c]Analysis done in log $(n + 1)$ scale, means converted back to numerical scale. Means followed by same letter not significantly different (LSD, 0.05).

Compared to the medium WS bait replaced every 2 days, the medium WS bait used for the entire field season showed no decrease in activity. On the other hand, the high and medium-CE formulations showed changes with time. The medium CE started out quite active but dropped to control levels by the end of the experiment, while the high-CE baits were poor initially but were at least as active as the medium-WS bait at the end of the field season. As the release rates of both baits decreased over time, the medium-CE apparently dropped below the lower threshold for activity and the high-CE entered the "window" for good trap catches.

Thus Z 10–19:Al by itself was sufficient for attraction in the field, and air oxidation of the (Z,Z)-9,19 dienes to this aldehyde is a reasonable explanation for their activity. (Further field tests comparing Z 10–19:Al, (Z,Z)-9,19 dienes, female sawflies, and other treatments will be described in another paper.)

Further Studies. The chemical reaction mechanism and controlling factors for the diene oxidation are not known. Not understanding such factors may have led to the curious results for certain diene preparations reported in Bartelt et al. (1982b). Some diene samples were active immediately upon addition to test plates, while others had to age an hour or more before becoming attractive to males.

The occurrence and production of Z10–19:Al in female sawflies remain to be thoroughly studied. In preliminary tests, no more than a trace of

*Z*10–19:Al (maximum of 1 ng/female) could be extracted from 0- to 7-day-old virgin females stored at 15° (the usual handling temperature), but when such females were transferred to a cage in the sun at 20–30° for 2–3 hr, as much as 10 ng/female was extracted (identified by GLC and mass spectrometry).

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(*Z*)-5-TETRADECEN-1-OL: A Secondary Pheromone of the Yellowheaded Spruce Sawfly,[1] and Its Relationship to (*Z*)-10-Nonadecenal[2]

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Abstract—The yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer), was known to possess a potent secondary pheromone with the polarity of an alcohol, based on earlier studies with Florisil fractions. Purification of the 25% ether–hexane Florisil fraction (polarity as with alcohols) and the hydrolyzed 5% ether–hexane fraction (polarity as with esters) from 50,000 females yielded a total of ca 3 μg of an active compound identified as (*Z*)-5-tetradecen-1-ol (*Z*5–14:OH). When added to 100 ng of (*Z*)-10-nonadecenal (*Z*10–19:Al), which behaves as the primary pheromone in this sawfly, 0.5 ng of synthetic *Z*5–14:OH caused an 8.8-fold increase in the greenhouse bioassay response, compared to *Z*10–19:Al alone. *E*5–14:OH caused a 2.8-fold increase. Only the combination of *Z*5–14:OH and *Z*10–19:Al consistently elicited mating attempts among males. When Hercon® formulations of *Z*5–14:OH and *Z*10–19:Al were used together to bait traps, catches were up to four times higher than for the *Z*10–19:Al alone, but this increase was seen only after emergence and mating activity in the field population were virtually complete. The *Z*5–14:OH was inactive alone, and at higher levels it depressed trap catches to *Z*10–19:Al. The Hercon formulations of *Z*5–14:OH and *Z*10–19:Al together were as attractive as virgin females. *Z*10–19:Al alone and the (*Z*,*Z*)-9,19 hydrocarbon dienes (found in all females and able to oxidize in air to produce *Z*10–19:Al) were similar to mated females. Thus *Z*5–14:OH accounted for the difference in attractiveness between virgin and mated females.

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Key Words—Pheromone, synergist, (*Z*)-5-tetradecen-1-ol, (*Z*)-10-nonadecenal, sawfly, *Pikonema alaskensis*, Hymenoptera, Tenthredinidae, alcohol.

INTRODUCTION

The yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae), a significant defoliator of spruce in northern forests, has a complex pheromone system. A series of (*Z*,*Z*)-9,19 hydrocarbon dienes of 28–37 carbon atoms was found to attract male sawflies (Bartelt et al. 1982b). Further work showed that an air oxidation product of these dienes, (*Z*)-10-nonadecenal (*Z*10–19:Al) by itself was sufficient to attract males (Bartelt and Jones, 1983). It was also demonstrated earlier that the sawfly possessed synergistic compounds which were inactive alone, but which caused highly significant increases in bioassay response when combined with the primary pheromone (Bartelt et al., 1982a). This paper deals with the identity and activity of one such compound, (*Z*)-5-tetradecen-1-ol (*Z*5–14:OH). Because of its relationship to the primary pheromone and its ability to elicit mating attempts; the alcohol fits the definition of "secondary pheromone" (Roelofs and Cardé, 1977).

METHODS AND MATERIALS

Female Sawflies, Extracts, and Florisil Fractions. Approximately 50,000 virgin female sawflies were reared from field-collected larvae. Handling, extraction, and initial purification of the extracts on Florisil were as described by Bartelt et al. (1982a). The 25% and 5% ether–hexane Florisil fractions (polarity as in alcohols and esters, respectively) were the sources of the synergistic compound.

Chromatography and Spectra. Equipment and methods were generally as described by Bartelt et al. (1982b). In addition, two 300 × 8.0-mm μ-Spherogel 50 Å® HPLC columns (Altex Scientific, Berkeley, California) used in series and eluted with 25% ether in hexane were employed to separate the synergist from a major contaminant, which was not accomplished by preparative GLC. The NMR hydrogen spectra were taken on a Bruker 270 MHz instrument, with samples dissolved in $DCCl_3$ and with TMS as the internal standard.

Synthetic Tetradecenols. *Z*5–14:OH and *E*5–14:OH, on hand from a previous synthesis, were further purified for greenhouse bioassay by HPLC on $AgNO_3$–silicic acid [as tetrahydropyranyl ethers, (Miyashita et al., 1977), elution with toluene], then by preparative GLC on Silar 5CP after removal of the THP group. Identities were confirmed by mass and IR spectra, ozonolysis, and GLC retention.

For field bioassay, the synthetic *Z*5–14:OH (96% pure by GLC) was incorporated, without further purification, into Hercon controlled-release formulations. One, with 0.09 mg/cm^2 and having 16-mil polymeric film on both sides, released the chemical from its whole surface, and the other, with 0.06 mg/cm^2 and having mylar on both sides, released the chemical only from cut edges. The target release rate for 1.0 × 1.0-cm pieces of both formulations was 1 ng/hr. The baits were cut into squares 0.3, 1.0, and 3.3 cm on a side so that levels of the chemical spanning roughly two orders of magnitude could be tested in the field. Actual release rates were not measured. The cut Hercon baits were aired in a hood for 4 days prior to beginning the field tests in order to stabilize release rates.

Greenhouse Bioassay. The bioassay was conducted as described by Bartelt et al. (1982a). In each trial, a watch glass treated with the test preparation and the primary pheromone and another treated only with the primary pheromone were compared simultaneously. (Usually a third watch glass with the primary pheromone and a synergistic preparation known to be active was also included to confirm the responsiveness of the males during the trial). To conclude that the test preparation was active, a significantly greater number of males had to be counted on that watch glass, compared to the primary pheromone, and mating attempts among males had to be seen on the watch glass or in the spruce foliage immediately downwind. (Such mating activity was rarely seen with the primary pheromone alone).

The primary pheromone for this work was either two female equivalents (FE) of the hexane–Florisil fraction, 0.3 FE of (*Z*,*Z*)-9,19 dienes, or 100 ng of *Z*10–19:Al. Since the first two frequently had to age for an hour or more before any male response was seen, while the last was active immediately, the aldehyde was the practical choice for bioassays when it became available.

Field Bioassay. Field tests were conducted in an infested white spruce plantation near Grand Rapids, Minnesota, May 16–29, 1982, generally as described by Bartelt et al. (1982a,b). The Hercon formulations of *Z*5–14:OH were tested in conjunction with 1.0 × 1.0-cm pieces of the 0.67 mg/cm^2 Hercon formulation of *Z*10–19:Al (Bartelt and Jones, 1983), used as the primary pheromone. The same Hercon baits were used throughout the period, although trap bodies were replaced and the study rerandomized every two days.

Single virgin females, 0–3 days old, and field-collected females were also used to bait traps. To the extent possible, the latter were captured as mating was completed. However, mating was difficult to observe in this species due to the short (15-sec) duration and the females' habit of remaining in dense foliage. Thus ovipositing females were also collected for baiting traps. Since males were never observed attempting to mate with such females and since they were captured late in the flight season, when adult emergence and mating

activity were nearing completion, these females were assumed to have mated. Comparisons with female sawflies were used to evaluate the effectiveness of Z10-19:Al and Z5-14:OH.

Emergence Traps. Sawfly emergence in the field was monitored with 15 emergence traps (Thompson and Kulman, 1976), positioned under spruce trees in the infested plantation. The traps were checked daily.

RESULTS AND DISCUSSION

Purification and Identification. Based on standards, the 25% ether-hexane Florisil fraction contained compounds with the polarity of alcohols. Acetylation with acetic anhydride and ozonolysis destroyed the synergistic activity, but hydrolysis in methanolic KOH did not (Table 1), suggesting the synergist to be an unsaturated alcohol.

Preparative GLC on Dexsil 300 yielded an active peak at 5.5 min [2 min at 150°, then 10°/min for 8 min, then 30°/min to 340°, ca. 1000 female

TABLE 1. GREENHOUSE BIOASSAY OF SYNERGISTIC FEMALE-DERIVED FLORISIL FRACTION WHEN TREATED WITH VARIOUS REAGENTS OR PURIFIED FURTHER BY PREPARATIVE GLC

	Mean scores (males counted, $N = 3$)		
Treatment	Test material[a] + primary pheromone	Primary pheromone	Active Florisil fraction + primary pheromone
Ozonolysis of 25% fraction	7.8	1.0	142.3***
Hydrolysis of 25% fraction	31.7**[c]	6.7	30.0**
Acetylation of 25% fraction	3.0	6.3	47.0**
25% fraction before GLC purification	35.0***	3.7	46.7***
Dexsil 300, all material before peak	11.3*	3.7	39.7***
Dexsil 300 peak, further purified on Silar 5CP	75.0***	7.7	32.0***
Dexsil 300, all material after peak	5.7	3.3	60.3***

[a] Test materials and the standard, active Florisil fraction were used at 5 FE/plate in the first three tests and at 2 FE/plate in the others.

[b] The primary pheromone was 2.0 FE of the hexane Florisil fraction for the ozonolysis test and 0.3 FE of (*Z*,*Z*)-9,19 dienes in all others.

[c] *, **, and *** denote significant differences from the primary pheromone at the 0.05, 0.01, and 0.001 levels, respectively (t tests in the $\sqrt{n+1}$ scale).

equivalents (FE)/run]. The peak represented ca. 4 ng/female and ca. 1% of the material in the Florisil fraction. The active peak was further purified by collection from a Silar 5CP column (isothermal at 130°, ca. 10,000 FE/run), and a subsequent bioassay verified activity (Table 1). Ten μg of the collected material were used for mass spectrometry and hydrogenation and another 70 μg for NMR analysis.

The major constituent of the GLC fraction was α-cadinol, based on the mass spectrum [*m/e*: 222 (8%, M), 204 (47%), 164 (38%), 161 (45%), 121 (81%), 95 (100%)] (e.g., Nose et al., 1971), the NMR spectrum (3H, d, 0.77δ; 3H, d, 0.92δ; 3H, s, 1.11δ; 3H, br s, 1.67δ; 1H, br s, 5.50δ) (e.g., Lin et al., 1974), and catalytic hydrogenation (Parliament, 1973) (two hydrogens added, by mass spectrometry; thus one double bond). α-Cadinol, [1*R*-(1α,4β,4aβ,8aα)] -1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-1-naphthalenol, is a sesquiterpene alcohol known to occur in spruce (von Rudloff, 1972). An extract of white spruce foliage, *Picea glauca* (Moensch) Voss, yielded a purified compound with identical GLC retentions and spectra, but it was inactive by greenhouse bioassay.

The active synergist was a very minor constituent of the GLC-collected material and was finally separated from α-cadinol by HPLC on μ-Spherogel 50 Å, the activity eluting completely before the α-cadinol even began to emerge. By mass spectrometry, the active Spherogel fraction contained 1-tetradecanol and a tetradecenol [*m/e*; 196 (3%), 168 (10%), 83 (100%); and *m/e*: 212 (0.5%, M), 194 (8%), 166 (6%), 82 (100%), respectively]. Less than 1 μg of each was obtained from the 70-μg NMR sample. That ozonolysis destroyed the activity of the crude Florisil fraction suggested the synergist to be the tetradecenol.

By comparing the mass spectrum to those of standard 3-, 5-, 7-, and 11-tetradecen-1-ols obtained on the same instrument, the double bond in the female-derived tetradecenol was believed to be at or near the 5 position, although insufficient compound remained for confirmation by ozonolysis.

The 5% ether–hexane Florisil fraction derived from females (containing compounds with the polarity of esters) was consistently inactive as a synergist in greenhouse bioassays, but it became clearly active upon hydrolysis. The purification of the hydrolyzed fraction from 50,000 females on Florisil, LiChrosorb Si60, μ-Spherogel 50 Å, and Silar 5CP was guided by bioassay and led to the recovery of an additional ca. 2 μg of tetradecenol. Ozonolysis yielded nonanal, indicating the parent compound to be 5-tetradecenol. Standard 5-tetradecen-1-ol yielded identical ozonolysis products. The *Z* and *E* isomers of 5-tetradecen-1-ol could be separated by GLC on SP-2330, and the female-derived compound had the same retention as the *Z* isomer. Thus (*Z*)-5-tetradecen-1-ol (*Z*5–14:OH) was present in females, although the absence of the *E* isomer could not be proved.

TABLE 2. GREENHOUSE BIOASSAY OF SYNTHETIC 5-TETRADECEN-1-OLS

Test	Mean scores (males counted, $N = 3$)		
	Primary pheromone[a]	$Z5$–14:OH[b] + primary pheromone	$E5$–14:OH[b] + primary pheromone
1	2.7	28.0	5.3
2	3.0	21.0	11.0
3	1.7	34.0	4.0
4	4.7	33.3	9.3
5	4.0	20.3	18.0
6	3.7	45.3	15.0
7	3.7	41.0	11.3
8	6.0	37.0	16.0
Overall means[c]	3.7	32.5	10.3

[a] Primary pheromone was 100 ng of Z10–19:Al.
[b] Used at 500 pg/plate, extended with 2 μg of tricosane (tricosane was not active as a synergist). Z10–19:Al was applied to an area of the plate separate from the other chemicals.
[c] All means were significantly different at the 0.001 level (t tests).

Greenhouse Bioassay. On the average, Z5–14:OH at 500 pg caused an 8.8-fold increase in response over the primary pheromone alone (Table 2). Mating attempts were always observed among males visiting the test plates treated with Z5–14:OH or in spruce foliage immediately downwind from them. The E isomer was not as active, although counts of males were still over those for the primary pheromone alone; mating attempts near this isomer were sporadic. Tests for synergism with the other compound from females, 1-tetradecanol, were negative, consistent with the destruction of activity upon ozonolysis of the crude synergist.

Field Tests with Synthetic Z5–14:OH. Synthetic Z5–14:OH was incorporated into a controlled release formulation because it promised to be a useful addition to a bait for monitoring sawfly populations. Overall, the 0.3 × 0.3-cm Z5–14:OH baits caused a significant increase in trap catch over the Z10–19:Al alone (Table 3), but the 1.0 × 1.0-cm baits caused no positive effect, and the 3.3 × 3.3-cm baits were actually inhibitory. Both the whole-surface and cut-edge-only formulations performed similarly. Thus, the effective dose of the synergist was very low, probably less than 1 ng/hr, and the optimum may have been even lower than released by the 0.3 × 0.3-cm baits. The Z5–14:OH by itself was no different from the controls.

The effectiveness of the synergist changed during the flight season. During the first three trapping periods (May 16–22), the 0.3 × 0.3-cm baits caused no significant effect, but during the last 3 (May 23–28) these baits

TABLE 3. FIELD BIOASSAY OF HERCON FORMULATIONS OF *Z*5-14:OH

Treatment	Mean trap catches (males/2 days)		
	Overall ($N = 30$)	May 16-22 ($N = 15$)	May 23-28 ($N = 15$)
Primary pheromone[a]	25.8	42.9	15.3
0.3 × 0.3-cm WS[b] + primary pheromone	52.8**(+)[c]	50.4 NS	55.4***(+)
0.3 × 0.3-cm CE[b] + primary pheromone	43.1*(+)	27.6 NS	66.8***(+)
1.0 × 1.0-cm WS + primary pheromone	19.1 NS	17.3**(−)	21.2 NS
1.0 × 1.0-cm CE + primary pheromone	16.1 NS	15.0**(−)	17.3 NS
3.3 × 3.3-cm WS + primary pheromone	9.7***(−)	10.0***(−)	9.4 NS
3.3 × 3.3-cm CE + primary pheromone	9.1***(−)	13.9***(−)	5.9**(−)
1.0 × 1.0-cm WS	4.5***(−)	6.8***(−)	2.9***(−)
Control	4.0***(−)	3.6***(−)	4.4***(−)

[a] The primary pheromone was the Hercon formulation of *Z*10-19:Al.
[b] WS and CE represent the Hercon formulations of *Z*5-14:OH emitting from the whole surface and cut edge only, respectively.
[c] *, **, and *** denote significant differences from the "primary pheromone" treatment at the 0.05, 0.01, and 0.001 levels, respectively (*t* tests). Signs denote "greater than" or "less than" the primary pheromone. In no case was the "1.0 × 1.0-cm WS" treatment significantly different from controls.

caused almost a four-fold increase. When only the data for the last three periods were analyzed, the increases for both 0.3 × 0.3-cm baits were significant at the 0.001 level. Based on emergence trap data, the rise in the synergist's effectiveness coincided with the end of the emergence of female sawflies (Figure 1). Competition from virgin females was believed at least partially responsible for the poor early results for the synergist. This effect with time had not been noted before, but all previously reported synergist tests were conducted late in the flight season.

Field Tests with Female Sawflies and Synthetic Compounds. Three synthetic treatments and the female sawflies were compared (Table 4). In the early portion of the flight season (May 17-22), virgin female sawflies were not significantly different from *Z*10-19:Al, *Z*10-19:Al + *Z*5-14:OH, or (*Z*,*Z*)-9,19 dienes, although all four were over control levels. The failure of *Z*5-14:OH to synergize *Z*10-19:Al during this time period was in agreement with the previous study (Table 3, Figure 1). Later in the flight season (May 23-26),

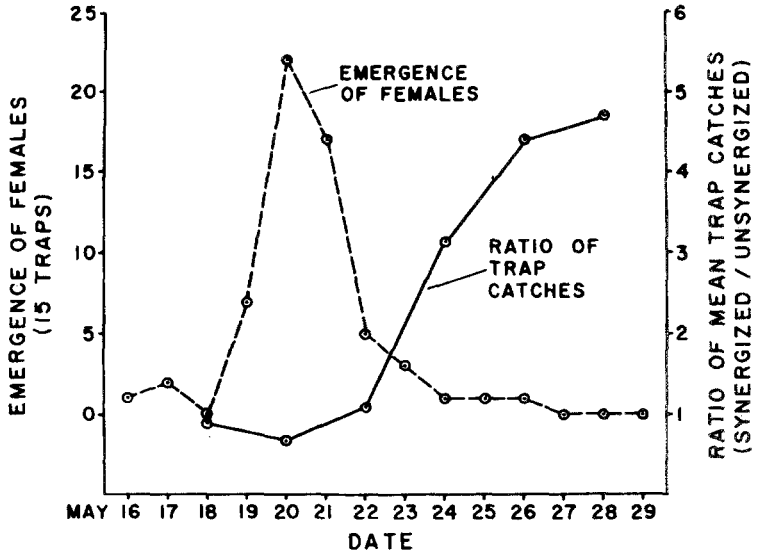

FIG. 1. Relationship between emergence of female sawflies in the field and the effectiveness of Z5-14:OH. "Synergized" refers to traps with the 0.3 × 0.3-cm Hercon whole-surface or cut-edge-only formulations, plus the primary pheromone. "Unsynergized" refers to the primary pheromone only. A ratio of 1.0 indicates no synergistic effect.

however, the effect of Z5-14:OH became clear, causing over a three-fold increase over the aldehyde alone, again much as in the previous study. Virgin females were very similar to this synergized aldehyde. Field-collected (presumably mated) females were also used in the latter half of the study and were similar in attractiveness to Z10-19:Al and (Z,Z)-9,19 dienes. The effect of Z5-14:OH on Z10-19:Al paralleled the difference between virgin and field-collected females. The mating behavior invariably associated with Z5-14:OH in greenhouse tests suggested males use it to locate virgin females at close range.

Z10-19:Al was at least as effective as the (Z,Z)-9,19 dienes throughout the study, although the difference between the treatments in the field was less dramatic than in greenhouse tests (Bartelt and Jones, 1983).

Ovipositing females have not been observed to elicit mating attempts or close approaches from males in the field, yet they had definite attractiveness when used to bait traps. Such females possess (Z,Z)-9,19 dienes in amounts and proportions similar to virgin females (unpublished data). That these dienes would oxidize (producing Z10-19:Al) under field conditions would not be surprising.

TABLE 4. COMPARISONS AMONG SYNTHETIC PREPARATIONS AND FEMALE SAWFLIES IN THE FIELD

Treatment	May 17–22, mean 2-day trap catch[a] ($N = 15$)	May 23–26, mean 1-day trap catch[a] ($N = 20$)
Hercon *Z*10–19:Al	37.4 b	10.5 b
(*Z*,*Z*)-9,19 dienes[b]	19.6 b	8.7 b
Field-collected female[c]		15.0 b
Hercon *Z*5–14:OH[d] + Hercon *Z*10–19:Al	38.5 b	35.0 c
Virgin female	26.7 b	42.7 c
Control	4.0 a	2.9 a

[a]Experimental design for May 17–22 was randomized block with 1 trap/tree, for May 23–26, balanced incomplete block with 2 traps/tree. The latter design compensated for the considerable tree-to-tree variability encountered. In earlier studies (Bartelt et al., 1982b), both designs gave comparable means, but the latter gave better precision. In each column, means followed by different letters were significantly different [LSD, 0.05, analysis done in log ($n + 1$) scale].
[b]20 μg of synthetic dienes, proportions as in female sawflies.
[c]Assumed to have mated, see text.
[d]0.3 × 0.3-cm bait, emitting from whole surface.

CONCLUSION

Synthetic *Z*5–14:OH is like the female-derived 25% ether–hexane Florisil fraction in being able to induce mating activity and to increase the numbers of males responding in bioassays when added to the primary pheromone. Although the combination of *Z*5–14:OH and *Z*10–19:Al always compared favorably with virgin females, it is likely that still other compounds have activity in this insect. In particular, the 5% ether–hexane Florisil fraction from females (with the polarity of esters) invariably became highly synergistic in the field upon aging an hour or more (Bartelt et al., 1982a,b). It is unknown if this activity was due to hydrolysis (or another reaction) slowly releasing the *Z*5–14:OH contained therein or if yet another chemical was involved. In any case, *Z*5–14:OH and *Z*10–19:Al should prove useful in monitoring sawfly populations.

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AVOIDANCE OF NONPROTEIN AMINO ACID INCORPORATION INTO PROTEIN BY THE SEED PREDATOR, *Caryedes brasiliensis* (BRUCHIDAE)

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Abstract—Larvae of the bruchid beetle, *Caryedes brasiliensis* (Bruchidae) have the ability to avoid significant incorporation of L-canavanine, the guanidinooxy structural analog of L-arginine, into de novo synthesized proteins. This ability is related to a highly discriminatory protein-synthesizing system which exhibits marked ability to avoid processing an array of nonprotein amino acids structurally related to arginine.

Key Words—Canavanine, *Caryedes brasiliensis*, Coleoptera, Bruchidae, *Dioclea megacarpa*, plant–insect interactions, amino acids.

INTRODUCTION

The seed of the neotropical legume, *Dioclea megacarpa* Rolfe is remarkably free of predation by insects (Janzen, 1971). This freedom from attack results in part from massive seed storage of L-canavanine, a highly toxic allelochemical and structurally analog of L-arginine that accounts for more than 95% of the nitrogen allocated to seed free amino acids (Rosenthal, 1977). Formation of structurally aberrant, canavanine-containing proteins is an important biochemical basis for the antimetabolic properties of this anginine antagonist (Rosenthal, 1982a). Bruchid beetle larvae, *Caryedes brasiliensis* (Bruchidae) develop entirely within the seed of this usually toxic legume (Figure 1). This ability is due to their capacity to avoid production of canavanyl proteins (Rosenthal et al., 1976). In this report we provide a direct

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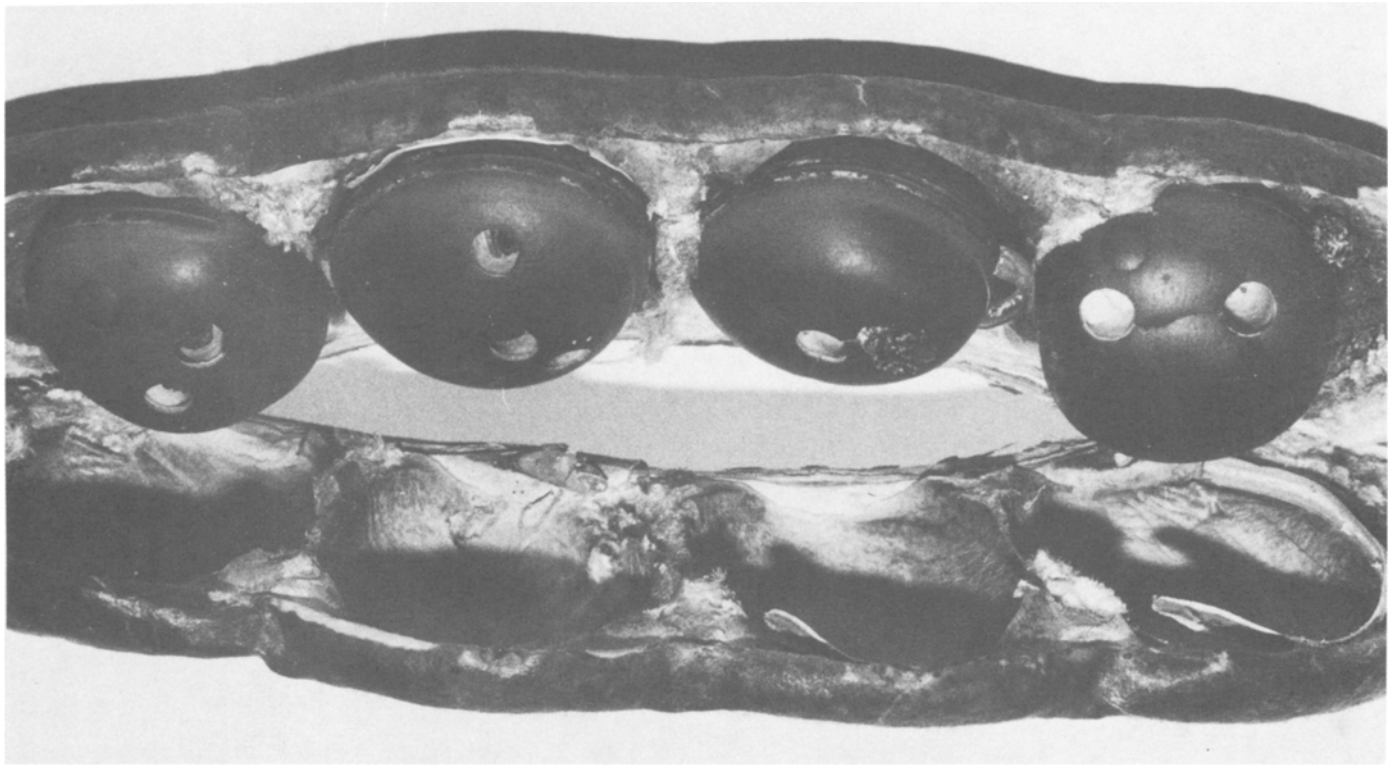


evaluation of the discriminatory capacity of the protein-synthesizing system of this insect and address the question of whether it provides broad-spectrum resistance to nonprotein amino acid incorporation into protein.

METHODS AND MATERIALS

Insects. The bruchid beetles used in this study were obtained from infected *Dioclea megacarpa* seeds collected in December, 1981 in Santa Rosa National Park, northwestern Guanacaste Province, Costa Rica. Tobacco hornworms, *Manduca sexta (L.)*(*Sphingidae*), were obtained from a colony maintained at the University of Kentucky.

Radioactive Amino Acids. The radioactive arginine analogs were prepared from ornithine, 5-hydroxylysine, lysine, canaline, 2,3-diaminopropionic acid, and 2-4-diaminobutyric acid by reaction with [^{14}C]*O*-methylisourea. This incorporated a radioactive ω-carbon atom into these compounds to create: [*guanidino*-^{14}C]arginine, [*guanidino*-^{14}C]5-hydroxyhomoarginine, [*guanidino*-^{14}C]homoarginine, [*guanidinooxy*-^{14}C]canavanine, [*guanidino*-^{14}C]2-amino-3-guanidinopropionic acid, and [*guanidino*-^{14}C-amino-4-guanidinobutyric acid, respectively (Figure 2).

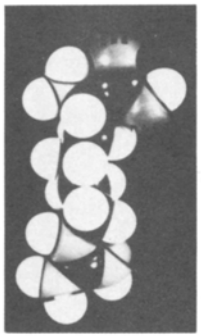
The free amino acids were reacted with excess CuO to form a copper salt protecting the α-NH_2 group from guanidination. Full details on the preparation of both [^{14}C]*O*-methylisourea from [^{14}C]$BaCO_3$ via labeled cyanamide and the various labeled amino acids have been published (Rosenthal *et al.*, 1983).

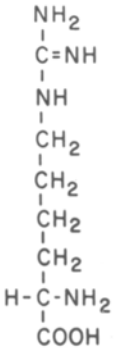
Amino Acid Administration. The amount of labeled canavanine or arginine injected into each bruchid beetle was adjusted to equalize the specific activity of each amino acid. Measurement of the free canavanine and arginine of these insects over the course of the experimental protocol revealed a lack of appreciable alteration in the relative pool size of these free amino acids.

Unlike the bruchid beetle, terminal instar tobacco hornworm larvae grow significantly during 18 hr. In order to avoid dilution of the radioactive arginine provided to these larvae, the time from injection of the amino acid to insect grinding was reduced to 3 hr. Since terminal instar tobacco hornworm larvae do not synthesize canavanine, comparable initial specific activity for arginine and canavanine was achieved by providing carrier canavanine; this compound does not inhibit protein synthesis (Rosenthal, 1982a).

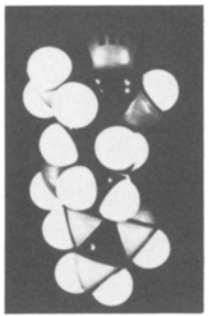
Analysis of Radioactive Proteins. Incorporation of radioactive amino acid into de novo synthesized insect protein was evaluated by injecting the

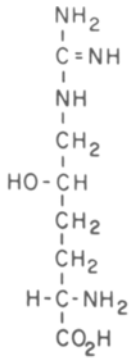
FIG. 1. Infected *Dioclea megacarpa* seeds. The matured fruit was opened to reveal the heavily infected seeds. The exit holes as well as several adult *Caryedes brasiliensis* can also be seen.



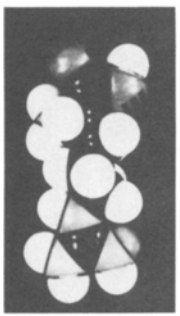


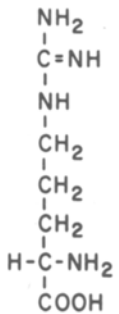
HOMOARGININE



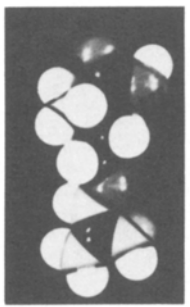


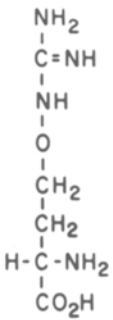
5-HYDROXYHOMOARGININE



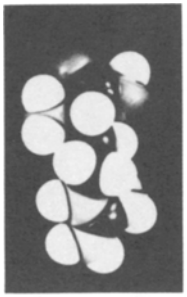


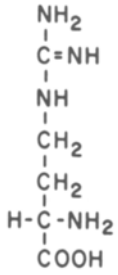
ARGININE



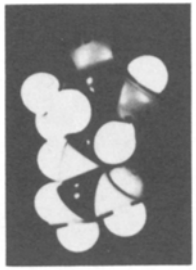


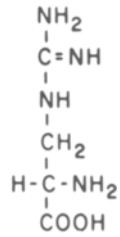
CANAVANINE





2-AMINO-4-GUANIDINOBUTYRIC ACID





2-AMINO-3-GUANIDINOPROPIONIC ACID

FIG. 2. CPK space-filling model of L-arginine and some of its structural analogs.

appropriate radioactive compound into the body of final instar larvae. The treated larvae were ground with 25 ml of 50 mM tris HCl buffer (pH 9.5) and kept at 37°C for 30 min to discharge amino acid-bound tRNA. After centrifugation at 13,000 *g* for 3 min to remove cellular debris, the supernatant solution was decanted into 6 ml of 50% (w/v) trichloroacetic acid. The insoluble materials were allowed to precipitate for 30 min at 3°C and then collected by centrifugation at 19,000 *g* for 15 min.

Trichloroacetic acid-precipitated materials were freed of unincorporated radioactive amino acids by four successive extractions with 25 ml of 5% (w/v) trichloroacetic acid and then processed once each with absolute ethanol-anhydrous ether (1:1, v/v) and anhydrous ether. After each extraction, the acid-insoluble materials were collected by centrifugation as above. The final pellet was air-dried, ground very finely, and dried in vacuo at 80°C. Protein incorporation values for the various amino acids were secured by acid hydrolysis of the radioactive protein and automated amino acid analysis coupled with liquid scintillation spectroscopy (Bray, 1960).

Procedures for automated amino acid analysis of the protein hydrolysate and evaluation of the spent ninhydrin-amino acid complex for its labeled carbon content have been thoroughly described (Rosenthal, 1982b).

Analysis of Acid Hydrolysate. Canavanine or arginine in the acid hydrolysate was isolated by ion-exchange chromatography. These radioactive amino acids were then subjected to combined enzymic cleavage with arginase and urease and the subsequent capture of the labeled, ω-carbon as $^{14}CO_2$. This procedure results in a stoichiometric conversion of L-[*guanidino*-^{14}C]arginine or L-[*guanidinooxy*-^{14}C]canavanine to $^{14}CO_2$ and unequivocally establishes the level of these amino acids (Rosenthal, 1982b).

RESULTS AND DISCUSSION

A comparative evaluation was made of the placement of L-[*guanidino*-^{14}C]arginine and L-[*guanidinooxy*-^{14}C]canavanine into the proteins of the bruchid beetle and the tobacco hornworm, *Manduca sexta* (Sphingidae). Of the labeled amino acids administered to the bruchid beetle, a considerable portion was diverted into respiratory $^{14}CO_2$, movement into non-amino acid pools, or fixation into various amino acids (Figure 3). Enzymatic evalutation of arginine and canavanine from the acid-hydrolyzed proteins of the bruchid beetle revealed incorporation of 347 nCi of arginine, from the 445 nCi of labeled material found in all protein amino acids, but only 0.95 nCi of canavanine (Figure 3). Thus, the ratio of labeled arginine to canavanine incorporated into the proteins of the bruchid beetle larvae was 347:0.95 or 365:1. This value provides a quantitative measure of the relatively poor ability

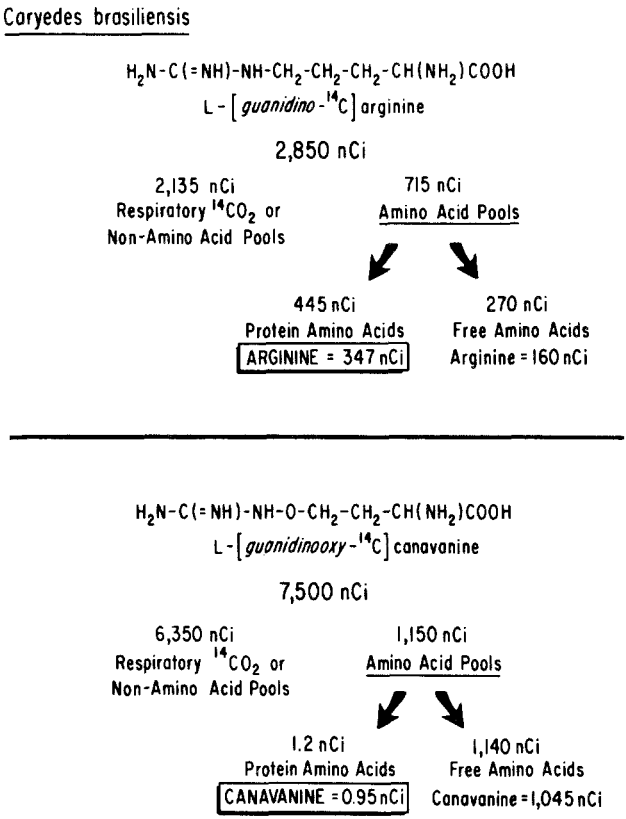


FIG. 3. Incorporation of labeled amino acid into soluble insect protein. Each terminal instar bruchid beetle larva (3.98 g fresh weight, $N = 30$) was administered 95 nCi arginine or 250 nCi canavanine and left for 18 hr. Newly ecdysed fifth stadium larvae of the tobacco hornworm (9.2 g fresh weight, $N = 3$) received 2500 nCi of L-[*guanidino*-^{14}C]arginine or L-[*guanidinooxy*-^{14}C]canavanine for 3 hr.

of canavanine as compared to arginine to support protein formation in this canavanine-resistant insect.

Comparable evaluations were made of the radioactive proteins of the larvae of the tobacco hornworm, an insect that does not feed on any canavanine-containing plant and which is highly susceptible to the deleterious action of canavanine. These determinations revealed an arginine to canavanine ratio of 5.6:1. These values provide a dramatic indication of bruchid beetle ability relative to that of the tobacco hornworm to avoid canavanyl protein formation. Such indirect evaluations are necessary since direct determinations with canavanine of the appropriate kinetic parameters

TABLE 1. INCORPORATION OF ARGININE AND CERTAIN OF ITS STRUCTURAL ANALOGS INTO SOLUBLE INSECT PROTEIN

Substrate[a]	^{14}C incorporation (pCi/mg soluble protein)	
	Manduca sexta	*Caryedes brasiliensis*
Arginine	20,278	12,393
Canavanine	3,611	34
Homoarginine	264	<5
5-Hydroxyhomoarginine	480	<5
2-Amino-4-guanidinobutyric acid	262	<5
2-Amino-3-guanidinopropionic acid	112	<5

[a]Arginine and canavanine were administered to the tested larvae as described in Figure 3; the remaining substrates were provided as indicated for canavanine.

of bruchid beetle arginyl tRNA synthetase is not feasible due to the minimal activity of canavanine as a substrate (Rosenthal et al., 1976).

Protein incorporation values for a series of structural analogs of arginine (Figure 2) were also tested with these insects. Tobacco hornworm larvae fixed each of the tested compounds into de novo synthesized proteins (Table 1). Both the immediately higher and lower arginine homolog, namely homoarginine and 2-amino-4-guanidinobutyric acid, were incorporated to an equivalent degree while the smallest of the tested molecules, 2-amino-3-guanidinopropioninc acid, was assimilated to a lesser extent. The presence of a hydroxyl group on the penultimate carbon of homoarginine increased its presence in protein relative to that of homoarginine (Table 1). Canavanine, which is the most toxic of the tested arginine analogs, is also most effectively assimilated into protein; this finding is consistent with existing evidence that canavanine toxicity is related to abnormal protein formation (Rosenthal, 1982a). In systems that fail to aminoacylate canavanine, this nonprotein amino acid is not demonstrably toxic (Rosenthal, 1982a).

Evaluation of the tested arginine analogs for their assimilation into bruchid beetle protein disclosed the acutely discriminatory nature of this insect's protein-synthesizing system (Table 1). The ability of this bruchid beetle to distinguish molecules structurally akin to arginine confers the biological benefit of a general resistance to the incorporation of such nonprotein amino acids into newly synthesized proteins.

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IDENTIFICATION OF A FEMALE-PRODUCED SEX PHEROMONE FROM THE SOUTHERN CORN ROOTWORM, *Diabrotica undecimpunctata howardi* BARBER[1,2]

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Abstract—A sex pheromone has been isolated and identified from virgin females of the southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi* Barber. The synthesized compound, 10-methyl-2-tridecanone was shown to be attractive to males of the SCR, and also to males of *D. u. undecimpunctata* Mannerheim, the western spotted cucumber beetle (WSCB), and of *D. u. duodecimnotata* in Mexico. Males of both the SCR and the WSCB strongly preferred the *R* over the *S* enantiomer. The resolved enantiomers were not tested against *D. u. duodecimnotata*.

Key Words—Coleoptera, Chrysomelidae, *Diabrotica*, southern corn rootworm, spotted cucumber beetle, western spotted cucumber beetle, sex pheromone, 10-methyl-2-tridecanone, ketone.

INTRODUCTION

The southern corn rootworm (SCR), *Diabrotica undecimpunctata howardi* Barber (Coleoptera: Chrysomelidae), is a widely distributed polyphagous insect of particular economic importance on peanuts, domestic cucurbits, and corn. Often called the spotted cucumber beetle, the SCR is

[1] Coleoptera: Chrysomelidae.
[2] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

included in a taxon containing two other subspecies; the western spotted cucumber beetle (WSCB), *D. u. undecimpunctata* Mannerheim, whose range is restricted to the far western United States and the upper Baja peninsula, and *D. u. duodecimnotata* Harold, which is found in Mexico (Smith, 1966).

Larvae of the SCR are most damaging to corn in the southeastern U.S. where those hatched from eggs of overwintering adults either feed on seedling corn roots or bore into the base of the stem (Arant, 1929). SCR larvae are also serious pests of peanuts, causing damage by penetrating the developing peanut—either consuming it or providing entry for pathogenic microorganisms (Campbell and Emery, 1967). In addition to corn and peanuts, larvae of the SCR are known to attack cucurbits and most legumes (Campbell and Emery, 1967).

Adults of the SCR have been collected from some 280 species of plants (Sell, 1916) but are of primary concern on cucurbits and peanuts. Adults of the WSCB have been known to cause severe damage to forage corps in the Pacific Northwest (Rockwood and Chamberlin, 1943). In addition to feeding damage, *D. undecimpunctata* spp. have been implicated as vectors of a number of plant diseases (see e.g., Doolittle and Beecher, 1950; Porter and Smith, 1974; Coudriet et la., 1979).

A sex pheromone has been demonstrated for the SCR, and Branson et al. (1978) showed that males of *D. u. duodecimnotata* in Mexico are attracted to traps baited with unfractionated volatiles from female SCR originating in South Dakota. This paper reports the isolation, identification, and synthesis of the major pheromonal component produced by female SCR.

METHODS AND MATERIALS

Pheromone Collection and Bioassay. Insects used in this study came from a laboratory colony reared according to Branson et al. (1975). Virgin females were isolated from males within 24 hr of emergence and held in screened cages (30 × 30 × 30 cm) for 3 days before being placed in a pheromone collection chamber. Adults were provided water and a dry diet mixed with a small amount of honey (Guss and Krysan, 1973).

For pheromone collection, about 15,000 virgin females were placed in a metal screened cage (29 × 29 × 89 cm) which was subsequently enclosed in a Plexiglas box (31 × 31 × 91 cm) containing a hole (35 mm diam) on either end. A glass tube (130 × 18 mm ID) filled with about 15 g of Porapak Q® for pheromone absorption (Byrne et al., 1975) was attached to one end of the Plexiglas box with a rubber stopper. The other end of the tube was attached to a vacuum source. The chamber was located in an isolation room at 24° C with a 12-hr photophase. Unpurified air at a rate of 6.5 liters/min was passed over the insects and through the Porapak Q filter. A complete exchange of air in the

chamber took about 13.5 min. Twice each week the apparatus was disassembled, and dead insects were removed and replaced with fresh females. Food and water were also changed at these times. Once each week the Porapak Q filter was removed and replaced with a fresh one. At this time the live insects were transferred to a clean screened cage, and the Plexiglas box was thoroughly cleaned before reassembly.

Laboratory bioassay techniques developed for the western corn rootworm (Guss et al., 1982) were directly adaptable to the SCR. Four or five male SCR beetles were placed in a disposable Petri dish (150 × 15 mm) and allowed to acclimate for 15 min. Then a test compound in 1–5 μl of hexane was applied to a filter paper chip (5 mm^2) and, after the solvent evaporated (about 10 min), the treated chip was placed in the Petri dish. Positive responses consisted of orientation of the beetles toward the chip, distinctive antennal waving, and copulatory behavior toward the other males.

Field bioassy of extracts and chromatographic functions during purification of the pheromone were carried out in a small domestic cucurbit patch (about 85 m^2) near Brookings, South Dakota. Because native populations were low, the patch was periodically infested with laboratory-reared SCR males. The traps consisted of 360-ml inverted drink cartons coated with Stickem Special®. Pheromone was dispensed from cotton wicks (30 × 10 mm diam) placed on top of the traps. Trap height was slightly above canopy height or about 0.4 m. This same type of trap was used for field testing synthetic pheromone against the WSCB near Riverside, California, and *D. u. duodecimnotata* in the state of Jalisco, Mexico; rubber septa were used as dispensers in those studies.

For field studies comparing natural and synthetic pheromone, sticky liners from Zoecon 1C® traps were used. Wooden dowels (0.63 cm diam) were run through the liner mounting holes so that the trap consisted of a vertical white cardboard panel with Stickem on one face. When the dowel was inserted in the ground, the top of the trap was just above the height of the vegetation. For each test the traps were placed in a randomized complete block and treatment positions were rerandomized each time traps were checked. Traps were about 3 m apart in each block, and blocks were 30–45 m apart. The tests were run in plots of mixed cucurbits (squash, melon, cucumber) in Gainesville, Florida, in June–July 1981. Pheromone was dissolved in hexane and appropriate quantities of each solution were pipetted onto a 4.5-cm-diam Whatman No. 1 filter paper disk just prior to baiting the traps. The filter paper bait was fastened just below the top edge of the sticky surface of the panel at ca. 3:20 to 4:30 PM each day.

A dose–response curve was obtained with traps baited as above with half of a rubber septum (A.H. Thomas No. 8753-D22, Philadelphia, Pennsylvania, 5 × 9 mm, split lengthwise and extracted for 1 hr with

methylene chloride) impregnated with 0.3–100 μg of synthetic pheromone in 25 μl of hexane. These traps were inspected and rerandomized within blocks each day.

Pheromone Purification. Pheromone was extracted from the Porapak Q of each filter with 50 ml ether–hexane (60:40) in a 125-ml Erlenmeyer flask. The mixture was allowed to steep with occasional agitation for 24 hr after which the Porapak Q was removed by filtration. The remaining solvent containing the pheromone was reduced in volume under vacuum at 0° C to about 1 ml, and the concentrated solution was subjected to gas–liquid chromatography (GLC) without further treatment.

Micropreparative GLC for isolation of the pheromone was performed with a Varian model 1400 gas chromatograph equipped with a flame ionization detector. Stainless-steel columns were packed with 30% DEGS on 60/80 mesh Chromosorb-W® (2 mm ID × 9.1 m) or 1.5% OV-101 on 100/120 mash Chromosorb G-HP® (2 mm ID × 1.5 m). The injection port temperatures were 185° C and 160° C with each column type, respectively; the detector temperature was 250° C. The DEGS and the OV-101 columns were maintained at 165° and 130° C, respectively. The carrier gas (N_2) flow rate through all columns was 20 ml/min.

The chromatograph was modified to accommodate a 90:10 effluent splitter and an external, dry ice-cooled fraction collector (Brownlee and Silverstein, 1968). Fractions were collected in 1.5 × 305-mm capillary tubes and were subsequently eluted with a minimal volume of hexane.

Synthesized pheromone was purified by preparative, high-pressure liquid chromatography (HPLC) on a 1.27-cm OD × 25-cm stainless-steel column packed with 5 μm Lichrosorb SI60® (Heath et al., 1978). A Lab Data Control Constametric II G® pump delivered the hexane–ether (97:3) mobile phase at 4 ml/min, and the eluting components were detected with a Waters model R401 differential refractometer.

Pheromone Analysis and Identification. Natural and synthesized pheromone was analyzed on 0.25 mm (ID) glass capillary columns prepared in the manner described by Heath et al. (1980). An SP2340® column, 60 m long, was maintained at 60° C for 2 min after injection, programed at 32°/min to 150° C, and then operated isothermally. An OV-101 column, 52 m long, was maintained at 50° C for 2 min after injection, programed at 32°/min to 170° C, and then operated isothermally. Both columns were operated in the splitless mode with an N_2 carrier gas flow of 9.5 cm/sec in a Hewlett-Packard model 5710A gas chromatograph equipped with a model of 18740B split–splitless injector system.

Mass spectral data were obtained with a Finnigan model 3200 mass spectrometer equipped with both chemical ionization (CI) and electron impact (EI) sources. A Varian model 1400 gas chromatograph equipped with

a 5% OV-1 column, 2.2 mm (ID) × 2 m, served to introduce samples to the CI source. Methane was employed as reagent gas and GLC carrier gas. The EI source was served by a Finnigan model 9500 gas chromatograph equipped with an OV-1 column of the same dimensions. Helium was normally used as the carrier gas.

Hydrogenolysis was carried out in the injector gas chromatograph that served the EI source by the method of Beroza and Sarmiento (1963, 1964). About 6 cm of a 3.2-mm-ID stainless-steel tube was filled with neutral Pd catalyst and placed in the injector port ahead of the OV-1 column. The catalyst was maintained at 260° C for the hydrogenolysis, and H_2 was used as the carrier gas at 30 ml/min.

For NMR, a 25-μg sample of the purified natural pheromone was chromatographed on a 1.2-m × 2-mm (ID) stainless-steel column packed with 10% Carbowax 20 M terephthalate on 120/140 mesh Chromosorb W operated at 150° C in a Varian model 1400 gas chromatograph equipped with a 95:5 effluent splitter and external fraction collector as described by Brownlee and Silverstein (1968). The pheromone was collected in a glass capillary cooled with solid CO_2 and then rinsed with 22 μl of benzene-D6 into an NMR tube, the top of which was 5 mm (OD), with a 50 × 2-mm (OD) coaxial extension on the bottom (Wilmad Glass Co., catalog No. 507 with WGS-5BL stem). This solution filled the 2-mm (OD) extension of the NMR tube to a height of 17 mm. The NMR spectrum was obtained with 500 scans (16 K data points, 5-μsec pulse, total experimental time 23.5 min) on a Nicolet® 300 MHz Fourier transform NMR spectrometer interfaced to a Nicolet 1280 data system.

RESULTS AND DISCUSSION

Hexane–ether extracts of Porapak Q filters from the collection chamber were tested for pheromone activity in the laboratory and in the field. In the laboratory bioassay, SCR males exhibited behavior typical of other *Diabrotica* spp. in the presence of their pheromones, i.e., orientation toward the pheromone source, distinctive antennal waving, and copulatory behavior toward other males. In the field, SCR males were captured on sticky traps baited with these same volatiles.

Laboratory bioassay of gas chromatographic fractions obtained from the Porapak-collected volatiles indicated that the pheromonal activity was confined to a single area of the chromatogram eluting after 42–46 min on DEGS. Fractionation of total volatiles, as well as rechromatography of the active area from DEGS, on OV-101 also produced a single area of activity eluting after 20–24 min. Field bioassay of these same fractions confirmed the above results. Comparison of the active peak with paraffin hydrocarbons

yielded a retention index of 2020 on DEGS and 1540 on OV-101 (Kovats, 1965).

Pheromone was purified for identification by fractionation of the Porapak extract on DEGS and subsequent refractionation of the eluate from the active area on OV-101. This produced a single, well-resolved peak of active material. Analysis of the purified pheromone from the packed OV-101 column on OV-101 and SP2340 capillary columns indicated that it was about 99% pure. About 60 μg of pure pheromone was obtained from about 50,000 virgin females over a 3-month period.

The methane CI mass spectrum (Figure 1A) established that the molecular weight of the pheromone was 212 with diagnostic peaks at *m/e* 211 (M − 1), 213 (M + 1), 241 (M + 29), and 253 (M + 41). The facts that the M + 1 peak is the base peak and that there is almost no fragmentation suggest a fairly stable parent molecule with an oxygen function. In the EI mass spectrum (Figure 1B), the base peak at *m/e* 43, the strong peak at *m/e* 58, and the weak peak at *m/e* 57, plus the moderately strong peak at *m/e* 71 strongly suggest a methyl ketone with no substitution on the carbons α or β to the carbonyl (Budzikiewicz et al., 1967).

A comparison of the EI mass spectra of authentic 2-tetradecanone with the natural pheromone at this point indicated that there were several similarities in the low mass region of the spectra but distinct differences in the high mass region. Additionally, the pheromone eluted prior to 2-tetradecanone on both capillary columns, suggesting the possibility of a branched-chain 14-carbon methyl ketone.

The NMR spectrum of the isolated pheromone (Figure 2) confirmed the presence of a methyl ketone with unsubstituted methylenes α and β to the carbonyl with the following signals: δ 1.93 [t, 2H, —(C=O)—$\underline{CH_2}$—CH_2], 1.64 [S, 3H, CH_3—(C=O)—], and 1.48 ppm [m, 2H, —(C=O)—CH_2—$\underline{CH_2}$—]. Additionally, two overlapping signals in the methyl region appear to be a triplet centered at δ 0.91 and a doublet centered at δ 0.90, indicative of $\underline{CH_3}$—CH_2— and $\underline{CH_3}$—CH—, respectively. Thus the spectroscopic data support a 14-carbon ketone with the following structure:

$$CH_3-\overset{\overset{O}{\|}}{C}-CH_2-CH_2(CH_2)_n-\overset{\overset{CH_3}{|}}{CH}-(CH_2)_mCH_2CH_3$$

Hydrogenolysis of the pheromone in the gas chromatographic injector leading to the EI source produced a product with the mass spectrum shown in Figure 3. The peak at *m/e* 198(M+) confirms the 14-carbon skeleton indicated by mass spectra of the parent compound. The peaks at *m/e* 70, 71, and 154, 155, are of greater intensity than would be expected in a normal hydrocarbon and represent preferred cleavage on either side of a methyl branch located at the 4-position in the chain. Thus, the structure of the

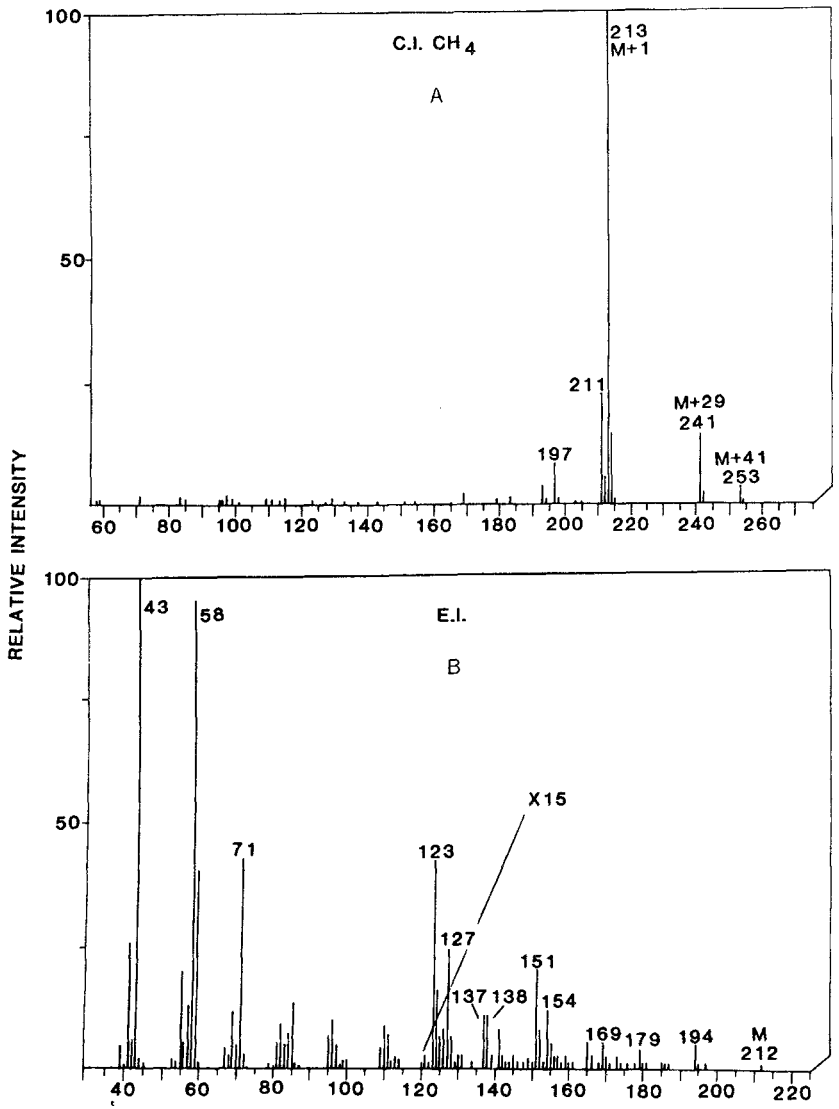

FIG. 1. (A) Chemical ionization (CH_4) and (B) electron impact mass spectra of *D. undecimpunctata* pheromone.

hydrogenolysis product is established as 4-methyl tridecane, and the pheromone of the southern corn rootworm is therefore 10-methyl 2-tridecanone (I, Figure 4).

Synthesis of I. The synthesis of racemic 10-methyl-2-tridecanone was initiated in a manner analogous to the preparation of the sex pheromone of

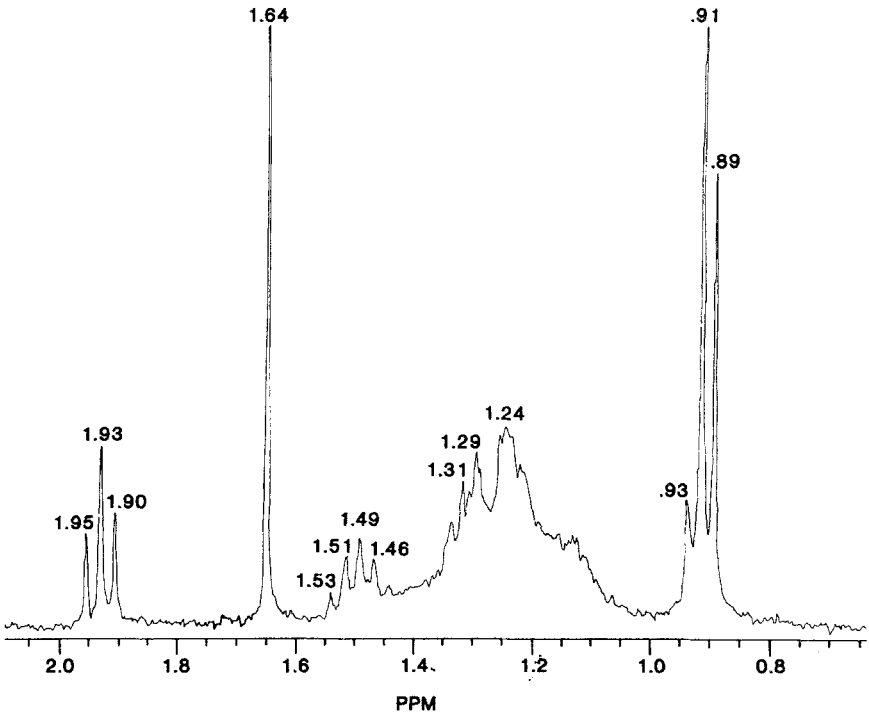

FIG. 2. 300-MHz proton magnetic resonance spectrum of *D. undecimpunctata* pheromone.

the western corn rootworm (Guss et al., 1982). Methyl cyclopropyl ketone (Figure 4) was allowed to react with *n*-propyl magnesium chloride. The intermediate tertiary carbinol was isomerized to the homoallylic bromide, II (bp 91–93° C at 27 mm, yield: 58%) using cold 48% hydrobromic acid. This reaction was first described by Julia (1961), and the halide II has been previously prepared in this fashion by Kulesza et al. (1969) who reported bp 51–52° C at 2 mm. Compound II was reduced to 1-bromo-4-methylheptane, III, by hydrogenation with platinum oxide in propionic acid. The alkyl bromide was obtained in ca. 91% crude yield; apparently hydrogenolysis of the C–Br bond under these conditions is insignificant [bp not determined because the halide foamed badly; NMR ($CDCl_3$, δ) 0.86 (t, $\underline{CH_3}CH_2$), 0.87 (d, $\underline{CH_3}CH$), and 3.40 ppm (m, 2H, $CH_2\underline{CH_2}Br$)]. Bromide III was converted first to a Grignard reagent and then to a cuprate with methyl copper (Bergbreiter and Whitesides, 1975). The organocuprate was coupled to the propionate ester of 6-iodo-2-hexanol, IV (the synthesis of this compound will be reported by us in connection with other research) to give the required carbon skeleton in the form of propionate ester V [IR (CCl_4) 1740 cm^{-1};

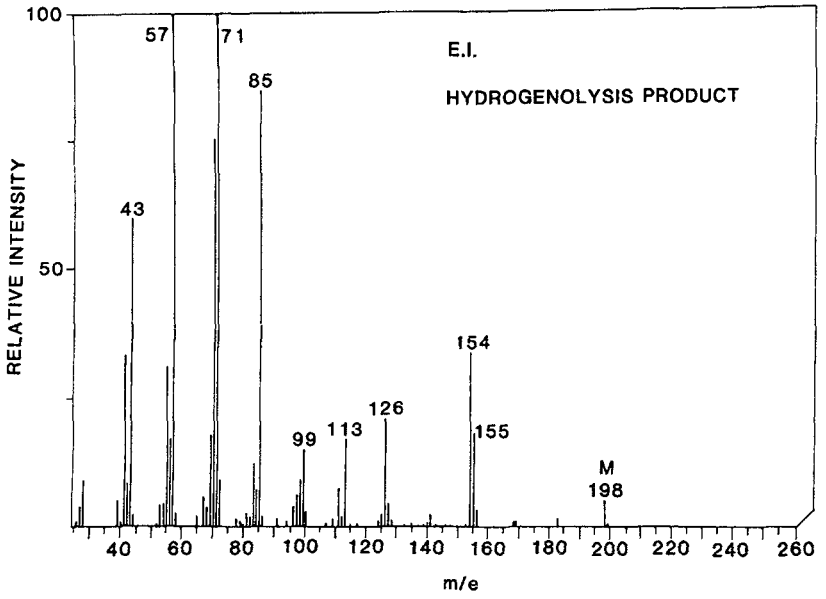

FIG. 3. Electron impact mass spectrum of product of hydrogenolysis of *D. undecimpunctata* pheromone.

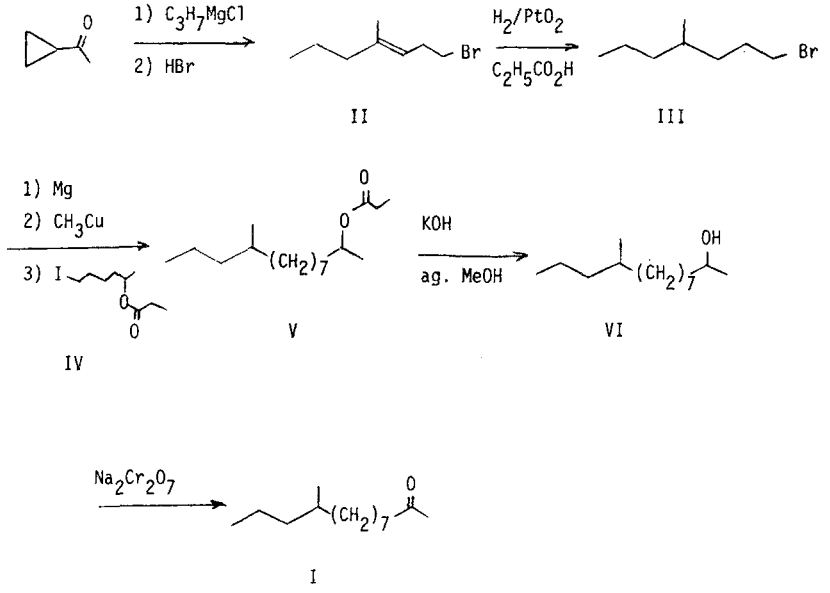

FIG. 4. Synthesis of racemic 10-methyl-2-tridecanone.

CIMS: $(M + 1)^+$ 271, $(M + 1—HO_2CC_2H_5)^+$ 197]. Saponification of V produced alcohol VI in 68% overall yield from III [IR (CCl_4) 3640 cm^{-1}; NMR ($CDCl_3$, δ) 0.85 (d, 3H, $J = 7$ Hz, $\underline{CH_3}CH$), 0.88 (t, 3H, $J = 7$ Hz, $\underline{CH_3}CH_2$), and 3.64 ppm (t, 2H, $J = 6$ Hz, $\underline{CH_2}OH$); CIMS: $(M)^+214$, $(M - 1)^+$ 213, $(M + 1\ 18)^+$ 197]. Oxidation of VI to I was accomplished with aqueous acidic dichromate in near quantitative yield [IR (CCl_4) 1720 cm^{-1}; NMR ($CDCl_3$) δ 2.13 (s, 3H, $\underline{CH_3}C{=}O$), 2.42 (t, 2H, $J = 7.4$ Hz, $CH_2\underline{CH_2}C{=}O$) ppm; CIMS: $(M + 1)^+$ 213].

The synthesized 10-methyl-2-tridecanone was purified by HPLC on Lichrosorb SI60. GLC analysis of the material collected from HPLC on the SP2340 and OV101 capillary columns indicated that it was greater than 99% pure. The pure natural pheromone and the synthesized 10-methyl-2-tridecanone cochromatographed on both capillary columns, and the EI and CI mass spectra of the synthesized compound were identical to the respective spectra of the natural pheromone.

Field Tests. The racemic synthetic 10-methyl-2-tridecanone (I) applied at 1 μg in 25 μl of hexane on filter paper was compared to natural, pure pheromone at the same rate and to a hexane blank. The test was run from June 13–15, 1981, with two randomized blocks. Traps baited with I captured a mean of 11.5 ± 1.8 (SE) males per trap per day while traps baited with natural pheromone captured 22.0 ± 3.9 (SE) males. These means are significantly different (ANOVA, $P \leqslant 0.01$). No males were captured in the hexane-baited traps (control). Thus, the racemic I appeared less attractive than the natural pheromone. Attraction of both natural and synthetic pheromones lasted only about 20 min with the filter paper formulation.

When adjusted for pheromone content, filter paper formulations of total volatiles collected on Porapak were equally attractive to males as those baited with purified natural pheromone (Table 1). The presence of another

TABLE 1. FIELD RESPONSES OF SCR MALES TO NATURAL SCR PHEROMONE AND 10-METHYL-2-TRIDECANONE (I) DISPENSED FROM FILTER PAPER, JULY, 10–13, 1981, GAINESVILLE, FLORIDA

Pheromone source	Amount[a] (μg)	$\bar{X} \pm SE^b$ males/trap/replicate
Unpurified volatiles	1	21.8 ± 1.5 a
	3	42.8 ± 5.1 b
Natural pure pheromone	1	22.4 ± 2.0 a
Synthetic racemic (I)	2	29.9 ± 4.2 a

[a] Amount of active material based on GLC analysis.
[b] Mean with a different letter is significantly different from the other means, NMR, $P \leqslant 0.01$. Randomized complete block (2) experiment ($N = 6$).

TABLE 2. FIELD RESPONSE[a] OF SCR MALES TO ENANTIOMERS OF 10-METHYL-2-TRIDECANONE DISPENSED (100 μg) FROM SPLIT 5 × 9-mm RUBBER SEPTUM, JULY 23–25, 1981, GAINESVILLE, FLORIDA

Synthetic material	$\bar{X} \pm$ SE[b] males/trap/replicate
R enantiomer (≤0.4% S)	73.5 ± 19.6 a
S enantiomer (≤0.1% R)	7.0 ± 2.3 b
1:1 $R + S$ (50 μg each)	53.9 ± 17.8 c
Racemic I	51.9 ± 12.6 c

[a] Randomized complete block experiment. One block in each of 3 widely separated peanut fields ($N = 9$).
[b] Means followed by different letters differ significantly, Duncan's NMR using 2-way ANOVA, $P \leq 0.05$.

pheromone component is therefore not indicated. Twice the dose of I was required to obtain attraction equal to the insect-produced material, suggesting that only one enantiomer of I is the active component. This contention is supported by results obtained with preparations of the separate enantiomers (Sonnet, 1982) evaporated from rubber septa. The R-enantiomer (Table 2) exhibited greater activity than I or a 1:1 mixture of $R + S$ enantiomiers. The S enantiomer had virtually no activity.

A dose–response effect was demonstrated when I was evaporated from rubber septa (Table 3). The data were best characterized by the equation $y = 0.57 + 1.83 \ln x$ ($R^2 = 0.95$) over a dose range of 1–100 μg.

The high degree of laboratory and field activity, dose–response effect, and differential activity of enantiomers leads us to the conclusion that (R)-10-

TABLE 3. DOSE-RESPONSE EFFECTS OF RACEMIC 10-METHYL-2-TRIDECANONE DISPENSED FROM RUBBER SEPTA ON TRAP CATCHES OF SCR MALES, MAY 28–JUNE 1, 1981, GAINESVILLE, FLORIDA

Dose (μg)	$\bar{X} \pm$ SE males/trap/replicate ($N = 12$)
100	8.7 ± 2.6
30	5.1 ± 1.5
10	2.8 ± 0.9
3	1.3 ± 0.7
1	0.1 ± 0.03
0.3	0 ± 0
Blank	0.1 ± 0.7

methyl-2-tridecanone is the major natural product produced by SCR females that is responsible for the attraction of males. The total sex pheromone system of the SCR may contain other components responsible for untested aspects of its mating behavior.

In addition to the SCR, 10-methyl-2-tridecanone (I) was tested for activity toward the other two known subspecies in this taxa. Near Riverside, California, wild males of the western spotted cucumber beetle, *D. u. undecimpunctata* Mannerheim (WSCB), were highly attracted to traps baited with 100 μg of racemic I on rubber septa. Subsequent tests indicated that the WSCB also strongly preferred the *R* over the *S* enantiomer (A.N. Kishaba, personal communication). Males of *D. u. duodecimnotata* also were captured in traps baited with racemic I in the state of Jalisco, Mexico (H. Valdés M. personal communication).

Acknowledgments—We thank Dr. A.N. Kishaba for field tests involving the WSCB and Ing. H. Valdés M. for field tests involving *D. u. duodecimnotata*. We also thank A.J. Proveaux for mass spectral analyses, J.R. Rocca for obtaining NMR spectra of the natural and synthetic pheromones, and M.A. Williams for technical assistance.

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Book Review

Les médiateurs chimiques agissant sur le comportement des insectes. C. Descoins (ed.). Institut National de la Recherche Agronomique, Versailles, 1982, 150 F, 414 pp.

Several volumes have appeared in recent years dealing with insect pheromones. They have generally emphasized the potential applications of pheromones in insect pest management. However, it is becoming clear that the successful use in pest control techology of chemicals that modify insect behavior will depend greatly on an understanding of the subtleties involved in individual species. Successes achieved by a largely empirical approach to air permeation may have given rise to overly optimistic expectations when this technique was used without a proper understanding of the ethology, ecology, and physiology of the species in question.

The important role of pheromones in detection and monitoring is well established; to establish their role in managing major insect pests will require considerable research across a number of disciplines. Therefore books such as "Les médiateurs chimiques agissant sur le comportement des insectes" are welcome because they represent contributions from a variety of fields. This volume contains the proceeding of an international symposium held at Versailles in November 1981 at which chemists and laboratory and field entomologists met to discuss research on the influence of chemicals on insect behavior. This symposium was a successor to previous meetings of the European pheromone working group of the OILB and was sponsored by the French Comité National de la Recherche Scientifique and the Institut National de la Recherche Agronomique.

The book provides a good indication of current interests and status of this research field, particularly where European interests are involved. There is some unevenness in the depth of coverage among the papers, and poster presentations are included; nevertheless the book provides a broad selection of topics: synthetic and analytical methods for semiochemicals, antifeeding agents and insect chemistry, semiochemicals and insect behavior, isolation and identification of sex pheromones, and practical uses of sex pheromones.

Discussion of synthetic methods is well represented. There are a number of papers on approaches to the synthesis of specific isomers of derivatives of long-chain unsaturated hydrocarbons, and there is also a good account by Professor K. Mori of his impressive progress in preparing optically active pheromones. Also of interest are reports on pheromones of novel structural types, particularly the spiroacetals and cyclic enol ethers.

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Dr. Kubo's investigations of limonoids are summarized, and three brief reports deal with antifeedant properties and chemistry of phenylpropanoids, polygodial and cyanogenic glucosides.

In the section on insect behavior, Professor Blum provides discussion of the natural products synthesized by social insects, their structural variety, and functional diversity, and speculation about the regulatory primer pheromones that drive the system. The question of orientation to and location of pheromone sources is the major topic of papers by Kennedy, Sanders, Wall, and Perry, while Tumlinson reports details of the behavior of *Heliothis virescens* and other Lepidoptera in the presence of pheromonal components and conspecific females. The chapter by Sanders is relevant to the mechanisms involved in mating disruption of spruce budworm by aerial application of synthetic pheromone, based on experiments conducted in a wind tunnel. The revelation that, among chemical mediators from a variety of sources, pheromonal components released from the ovipositor gland of female *H. zea* adults are involved in the host-searching behavior of *Trichogramma* species has far-reaching implications that are being further explored by Lewis and his coworkers.

A modification of the aphid alarm pheromone, farnesene, gives rise to compounds that may be of practical value in the field, and Pickett et al. provide a further contribution to this topic.

The complexity of insect pheromones is being revealed, and precise knowledge concerning the volatile stimuli is slowly accumulating. Re-evaluations of past information are often called for, and the section on isolation and identification of sex pheromones emphasizes this aspect of the problem.

Practical application of pheromones for trapping and mating disruption is discussed in a number of papers relating to the processionary moth (*Thaumetopoea pityocampa*), the turnip moth (*Agrotis segetum*), the European corn borer (*Ostrinia nubilalis*), the Egyptian cotton leafworm (*Spodoptera littoralis*), the pink bollworm (*Pectinophora gossypiella*), the artichoke plume moth (*Platyptilia carduidactyla*), the summerfruit tortrix (*Adoxophyes orana*), the grape vine moth (*Lobesia botrana*) and the leek moth (*Acrolepiopis assectella*).

Thirteen of the 45 contributions to this text are in French (the remainder are in English); however, this should not deter the potential reader. Of greater concern is the workmanship involved in the production of the book itself; the outer binding of the reviewer's copy was detached on receipt and individual sections gradually separated during handling. A more durable edition is needed if the volume is to survive.

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Journal of Chemical Ecology, Vol. 9, No. 9, 1983

Book Review

Insect Suppression with Controlled Release Pheromone Systems. Agis F. Kydonieus and Morton Beroza (eds.). CRC Press, Volume I, $76 in USA, $87 outside USA, 274 pp., Volume II, $89.50 in USA, $103.00 outside USA, 312 pp.

Public alarm about toxic pesticides and the development of technology elegant enough to decipher the molecular structures of microgram quantities of odorants coincided in the 1960s to produce a wave of research on insect pheromones. In two decades that wave cast forth about 5000 scientific papers, 39 books, and myriad popular accounts of the promise of pheromones, but very few operational uses of pheromones in pest control programs. The two-volume series, *Insect Suppression with Controlled Release Pheromone Systems*, convincingly demonstrates that the application of pheromones in pest control programs is no longer a prospect; it is a reality.

This is a two-volume work in a series of CRC technical reviews of pesticide chemistry for which Gunter Zweig is editor-in-chief. Kydoneius and Beroza coordinated the contributions of 36 authors who cover subject areas from the tabulation of arthropod species for which reports of chemical ecology have appeared, to detailed accounts of the state of the art of applications of pheromones for the management of specific insect pests. The chapters are quite uniformly well written and free of blemishes. These volumes are essential references for researchers in chemical ecology of arthropods, and they are excellent sources of examples for students of pest management.

Volume I is divided into two sections: I, Fundamental Concepts; and II, Formulations, Methods and Applications, and Insect Monitoring. Volume II includes sections III, Mass Trapping and Trap Cropping; IV, Disruption of Mating; and V, The Future Outlook. The initial chapter, Pheromones and their Use, is an introduction and overview. Chapter 2, Chemical Attractants in Integrated Pest Management Programs, provides an enormous 53-page tabulation of insects, mites, and ticks about which reports of semiochemicals have appeared in the literature. The species are listed alphabetically by genus under orders (Lepidoptera, etc.); curiously, there is no indication of family (Geometridae, etc.). An impressive 889 works are referenced. Chapter 3 reviews controlled-release technologies. Chapter 4 presents modes and theories of measuring pheromone vapors in the atmosphere. Finally, chapter 5 gives the latest word on the requirements of the Environmental Protection

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Agency (EPA) for registration of semiochemicals for pest control in the United States.

Section II includes chapters 6–10 which review technologies for monitoring insect populations, controlled-release dispensers, and methods of measuring release rates. The chapters on controlled release are essentially extractions of matieral from the earlier two-volume CRC series, *Controlled Release Technologies: Methods, Theory and Applications*, edited by Kydonieus.

Volume II begins with section III, Mass Trapping and Trap Cropping, which includes five chapters that are case studies of the spruce bark beetle in Norway, the gypsy moth, Japanese beetle, and cotton boll weevil. Section IV, Disruption of Mating, incudes eight Chapters reporting research programs and/or operational results on suppression of pink bollworm and tobacco budworm on cotton, the peachtree borer, and Oriental fruit moth, and codling moth, the gypsy moth, the western pine shoot borer, the Douglas-fir tussock moth, and the spruce budworm.

Section V gives a detailed analysis, Marketing and Economic Considerations in the Use of Pheromones for Suppression of Insect Populations. Unfortunately the analysis is out of step with the new EPA guidelines for registration of pheromones, outlined in Chapter 5 of Volume I. Because costs of registration are now much reduced, the profitability of commercial use of pheromones should be greater than in the case presented. The final chapter lists known semiochemicals organized by compound, by insect species, and by trivial names of lures and compounds. This chapter cites the same 899 references given in Chapter 2 of Volume 1.

These volumes parallel, in part, *Management of Insect Pests with Semiochemicals, Concepts and Practice*, (E.E. Mitchell, editor, Plenum Press). Several authors contribute almost the same information in both works. As a source of examples of applications of semiochemicals, the works are interchangeable. However, the Mitchell book does not include some of the material in which potential users of the CRC volumes may have particular interest. The work reviewed here can serve as a detailed guide for applications of semiochemicals, design of controlled-release systems, methods for field experiments, EPA registration, and marketing. It also provides the most detailed referenced list of available semiochemicals of arthropod species.

I could not help but wonder why the volumes were organized as they were. Insect monitoring topics, Chapters 7 and 8 of Volume I, might have been accorded a separate section parallel to those on mass trapping and mating disruption, rather than being grouped with chapters dealing with the mechanics of pheromone dispensers. The final chapter of Volume II, Insect Attractants, Attractant Pheromones, and Related Compounds, clearly does not belong under Section V, Future Outlook. If it were part of Chapter 2,

Volume I, Chemical Attractants in Integrated Pest Management Programs, all of the tabulations of insects and pheromones would be together, the duplication of 30 pages of references could have been avoided and, perhaps, the price of the works could have been lower.

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ANTENNAL OLFACTORY RESPONSIVENESS OF DOUGLAS-FIR BEETLE, *Dendroctonus pseudotsugae* HOPKINS (COLEOPTERA: SCOLYTIDAE) TO PHEROMONES AND HOST ODORS[1]

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Abstract—Electroantennograms were obtained from *D. pseudotsugae* in response to the pheromones 3-methylcyclohex-2-en-1-one (3,2-MCHone), 3-methylcyclohex-2-en-1-ol (3,2-MCHol), frontalin, *trans*-verbenol, verbenone, and the host terpene hydrocarbons limonene and camphene. Male and female beetles were 10 and 100 times more sensitive to 3,2-MCH-one and 3,2-MCHol than to the other compounds. Of the other compounds, males were most sensitive to *trans*-verbenol, verbenone, and camphene, while females were most sensitive to frontalin, limonene, and camphene. The results parallel and help explain behavior of individual males and females during host tree selection, aggregation, and colonization.

Key Words—Douglas-fir beetle, *Dendroctonus pseudotsugae*, Coleoptera, Scolytidae, electroantennograms, host selection, attractants, aggregation, 3-methylcyclohex-2-en-1-one, 3-methylcyclohex-2-en-1-ol, frontalin, *trans*-verbenol, verbenone, limonene, camphene.

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INTRODUCTION

In recent years numerous chemicals that influence the behavior of beetles (Vité and Francke, 1976; Borden, 1977) have been identified from bark beetles and their hosts. Aggregation, host colonization, and mating of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopkins (Coleoptera: Scolytidae), on suitable host trees involves a hierarchy of successive behavioral events, each largely dependent on interacting chemical and acoustical signals and responses (Rudinsky, 1973; Rudinsky and Ryker, 1977).

Pioneering *D. pseudotsugae* females are attracted to prospective hosts by certain terpenes (Rudinsky 1966) and, upon host attack, release at least seven pheromones (Ryker et al., 1979). Identified pheromones include: 3,2-MCHone (3-methylcyclohex-2-en-1-one) (Kinzer et al., 1971), 3,2-MCHol (3-methylcyclohex-2-en-1-ol) (Vité et al., 1972; Rudinsky et al., 1974), frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1.]octane) (Kinzer et al., 1969; Pitman and Vité, 1970; Rudinsky et al., 1974), verbenone (Rudinsky et al., 1974) and *trans*-verbenol (Rudinsky et al., 1972b). Furthermore, boring into the host tree by the female releases additional quantities of volatiles from the host, including alpha-pinene, camphene, and limonene, which function as attractive kairomones (Rudinsky, 1966) and/or pheromonal synergists (Furniss et al., 1972; Rudinsky et al., 1972a).

Predominantly male beetles respond to the attractive bouquet produced by pioneering beetles (Rudinsky, 1969). After landing on the host tree and locating the female gallery entrance, the male is induced to stridulate by the female attractant (Rudinsky, 1969; Rudinsky and Michael, 1972). Stridulation by the male leads to both increased release of the pheromone by the female and female stridulation. The increased release by the female of 3,2-MCHone, a multifunctional pheromone (i.e., at low concentrations an attractant component, but in high concentrations an attractant inhibitor), inhibits further attraction of both sexes (Rudinsky, 1973). Female stridulation stimulates the male to release his pheromones 3,2-MCHone, frontalin, and 3,3-MCHone (Rudinsky et al., 1976a,b; Libbey et al., 1976). The male contribution of 3,2-MCHone further inhibits aggregation which benefits both resident and dispersing females and males by providing information about the rapidly changing colonization density and risk of attack (Alcock, 1981). The source and function of the major identified pheromones of *D. pseudotsugae* and two important host terpenes are summarized in Table 1.

The purpose of this study was to investigate the peripheral olfactory responsiveness of *D. pseudotsugae* males and females to their identified pheromones and kairomones and, further, to provide the basis for single cell studies.

METHODS AND MATERIALS

Adult *D. pseudotsugae* used in this study emerged from naturally infested bolts of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, from McDonald Forest of Oregon State University, Corvallis, Oregon. Following emergence, beetles were sexed (Jantz and Johnsey, 1964) and maintained on moist filter paper in Petri dishes at 6° C until use 1–3 weeks after emergence.

Electroantennogram (EAG) techniques were previously described in detail (Dickens, 1979) and were a modification of earlier techniques (Schneider, 1957; Payne, 1975). Ag–AgCl capillary electrodes filled with 3 M KCl or Beadle-Ephrussi's solution were used. The recording electrode was inserted into the distal end of the antennal club following prepuncture with a sharpened tungsten needle. The indifferent electrode was implanted either in the mouth or head capsule. EAGs were recorded on Polaroid film with a Tektronix 405 oscilloscope camera.

Pheromones and host odors used as test stimuli are listed in Table 1. Dosage–response curves were constructed from serial dilutions of test stimuli prepared in nanograde pentane. Stimuli were delivered as 10-μl samples placed on filter paper (20 × 7 mm) inserted into glass cartridges (75 mm long; 5 mm ID) oriented toward the preparation from ca. 1 cm. Stimulus duration was 1–2 sec. Air flow was ca. 2 liters/min.

Stimulus dilutions were standardly presented in order from the lowest to the highest concentration of a given compound. At least 3 min were allowed between each stimulus except at higher concentrations when 5 min were allowed between successive stimuli. These intervals were found to be adequate for complete recovery of the EAG. Five replicates were recorded from each sex to each stimulus. Each preparation was exposed to *n*-pentane as a control. Any response to the control was subtracted from subsequent responses to the compounds for a given preparation.

Due to the number of compounds and dilutions tested, generally the longevity of a single preparation did not allow for stimulation by all compounds and dilutions. The use of common standards for all preparations allowed for normalization of the data and subsequent comparisons between compounds and sexes. Therefore, to compare responses from different preparations, stimulation with a racemic frontalin standard at 10 μg either preceded or followed each stimulus by 4 min. Racemic frontalin at 100 μg was used as a standard for 3,2-MCHone and 3,2-MCHol. Response at higher concentrations to these two compounds was very large and hence the need for a standard giving a relatively large response. Small changes in a standard giving a relatively small response would lead to large variations in values computed as percent responses. No significant differences were found in the

TABLE 1. BIOLOGICAL SOURCE AND FUNCTION OF MAJOR PHEROMONES AND KAIROMONES IDENTIFIED FOR *D. pseudotsugae*

Compound	Biological Source	Function	Source of supply	Purity (%)[b]
3-Methylcyclohex-2-en-1-one	Fed and unfed males and females (Kinzer et al., 1971; Ryker et al., 1979)	Multifunctional (Rudinsky, 1973; Rudinsky and Ryker, 1976) low concentration: attractant; high concentration: inhibitor	A[a]	98
3-Methylcyclohex-2-en-1-ol	Fed females (Vité et al., 1972; Rudinsky et al., 1974; Ryker et al., 1979)	Attractant, esp. for males (Rudinsky et al., 1974)	B	97
Frontalin	Fed and unfed males and esp. females (Kinzer et al., 1971; Rudinsky et al., 1976b; Ryker et al., 1979)	Attractant (Pitman and Vité, 1970)	B	99
trans-Verbenol	Female hindguts (Rudinsky et al., 1972b; Rudinsky, 1973)	Attractant synergist in combination with frontalin (Rudinsky et al., 1972b)	B	99
Verbenone	Fed females (Rudinsky et al., 1974)	Unknown	B	99
Limonene	Host tree, Douglas-fir, *Pseudotsuga menziesii* (Mirb) Franco (Rudinsky, 1966); released by acoustically stimulated males and females (Rudinsky et al., 1977)	Attractant synergist in combination with aggregation pheromone (Rudinsky and Ryker, 1977; Ryker et al., 1979)	C	98
Camphene	Host tree, Douglas-fir, *Pseudotsuga menziessi* (Mirb) Franco (Rudinsky, 1966)	Attractant synergist in combination with aggregation pheromone (Pitman and Vité, 1970)	C	98

[a]A, Aldrich Chemical Co., Milwaukee, Wisconsin; B, Chem Samp Co., Colombus, Ohio; C, K & K Laboratories
[b]Purity of compounds used in the electrophysiological experiments.

responses of sexes to either the 10 μg or 100 μg frontalin standards [$N = 40, 20$ males ($\overline{X} = 1.20$ mV; SE = 0.11 mV) and 20 females ($\overline{X} = 1.41$ mV; SE = 0.59 mV); and $N = 20$, 10 males ($\overline{X} = 1.76$ mV; SE = 0.21 mV) and 10 females ($X = 1.88$ mV; SE = 0.17 mV), respectively, $P \geq 0.95$].

Responses to intervening test stimuli were represented as a percent of the mean average of the two responses to the standard closest in time of occurrence. The size of the EAG depolarization was considered a measure of the relative number of responding receptor sites (acceptors) (Payne, 1975; Dickens and Payne, 1977). The threshold of response was considered the stimulus concentration at which the lower limit of the standard error did not overlap with the upper limit of the standard error for response, if any, at the lowest concentration tested (Dickens, 1978). Responses were compared for significant differences using a t test (Ostle, 1969).

RESULTS AND DISCUSSION

EAGs recorded to stimulus dilutions of attractant components increased with increasing stimulus concentrations above threshold (Figures 1–4). Saturation did not appear to be reached for any odorant at the highest concentration tested. Both threshold responses and maximal responses to the highest concentrations were significantly different for various odors. However, the shapes of dosage–response curves were similar for a given compound for each sex. For example, even though males had a 10-fold lower threshold than female beetles for both 3,2-MCHone and 3,2-MCHol and a significantly greater response at 100 μg ($P \geq 0.90$ and 0.95, respectively), dosage–response curves for the female were parallel to those for the male, although shifted closer to the abscissa (Figure 1A,B).

Similarities in shapes of dosage–response curves for each of the odorants tested for both male and female *D. pseudotsugae* indicate that receptor mechanisms may be similar for each compound in both sexes. The fact that male and female beetles had different thresholds and greatest responses to particular odorants (e.g., 3,2-MCHol and 3,2-MCHone) at the highest concentrations tested is probably indicative of differences in the size of receptor site populations of each sex responsive to the respective compounds. Thus at low concentrations, molecules of an odorant would be more likely to stimulate membrane receptors in the sex with the larger population of receptor sites for the odorant being tested.

Female and male *D. pseudotsugae* were 10 and 100 times, respectively, more sensitive to 3,2-MCHone and 3,2-MCHol than any other compound tested (Figure 5). EAGs to each of these compounds at 100 μg were significantly greater than for all other odorous stimuli. Among the other

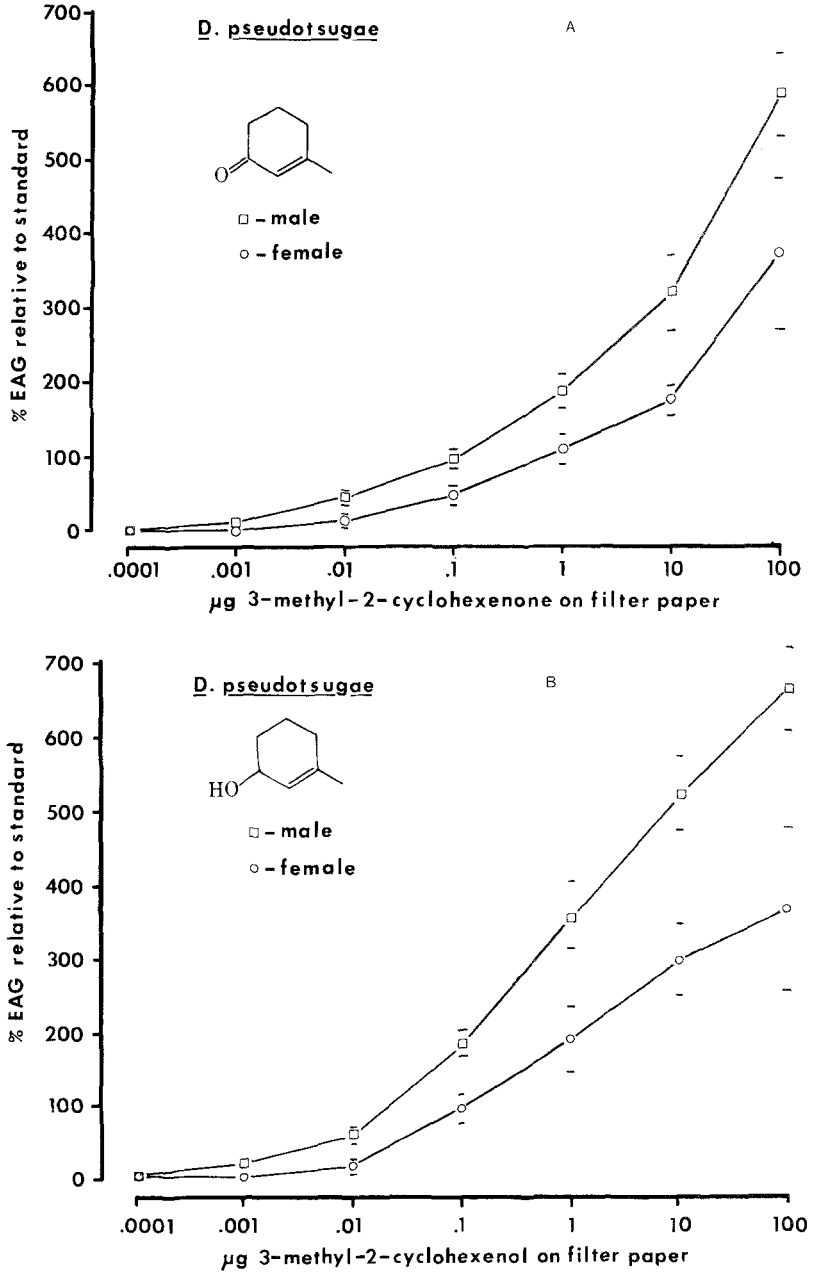

FIG. 1. Dosage–response curves constructed from EAGs of *D. pseudotsugae* males (blocks) and females (circles) to serial dilutions of 3,2-MCHone (A) and 3,2-MCHol (B). $N = 5$; horizontal lines represent $\pm$ $SE_{\bar{x}}$.

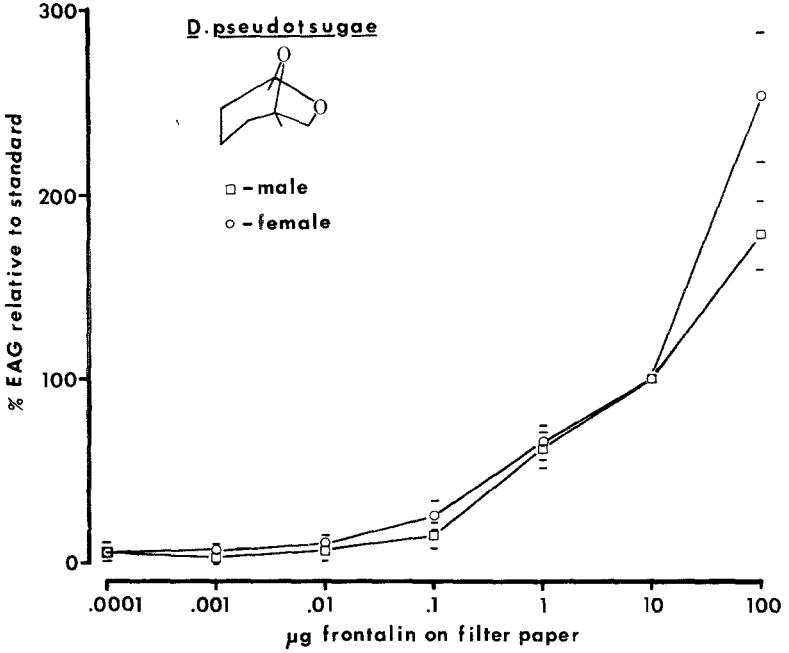

FIG. 2. Dosage-response curves constructed form EAGs of *D. pseudotsugae* males and females to serial dilutions of racemic frontalin. $N = 5$; horizontal lines represent $\pm$ SE_x.

odors tested, males were most sensitive to *trans*-verbenol, verbenone, and camphene, while females were most sensitive to frontalin, limonene, and camphene.

Based on low thresholds and large EAGs at the highest concentration tested, the importance of 3,2-MCHone and 3,2-MCHol to both sexes is apparent (Figure 5). This supports results from behavioral studies which showed that males are more sensitive than females to the multifunctional effects of 3,2-MCHone (Rudinsky, 1973; Rudinsky et al., 1972a). The same thing is true for 3,2-MCHol, which is produced only by *D. pseudotsugae* females (Vité et al., 1972; Rudinsky et al., 1974; Ryker et al., 1979) and predominantly attracts males (Rudinsky et al. 1974).

Of the two host terpenes tested, males and females had similar thresholds and greatest responses to camphene; however, females had a 10-fold lower threshold for limonene than did males (Figure 5). Females initiate attack on prospective host trees and have been shown to be more attracted by host odors, e.g., alpha-pinene, camphene, and limonene, than male beetles (Rudinsky, 1966). In addition, these terpenes are actively released by males

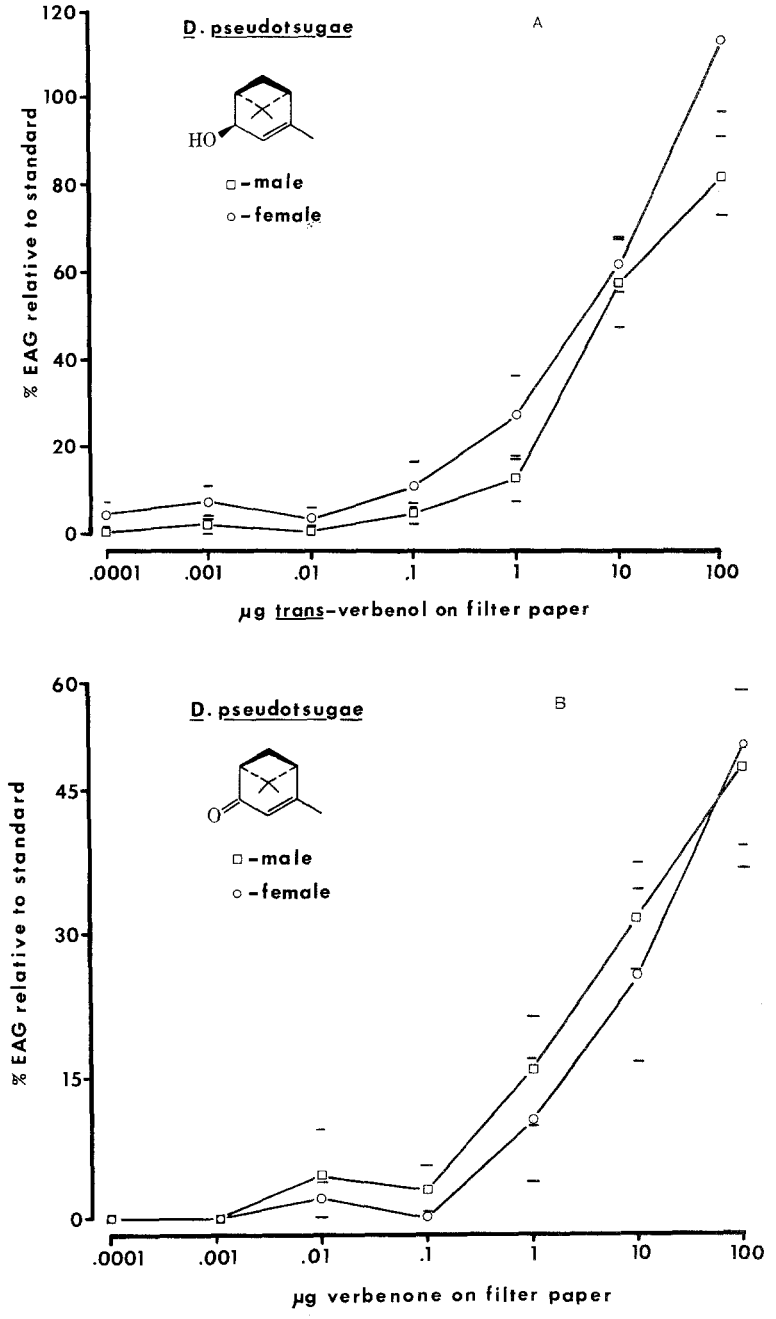

FIG. 3. Dosage–response curves constructed from EAGs of *D. pseudotsugae* males and females to serial dilutions of the pheromones, *trans*-verbenol (A) and verbenone (B). $N = 5$; horizontal lines represent $\pm \overline{SE}_{\bar{x}}$.

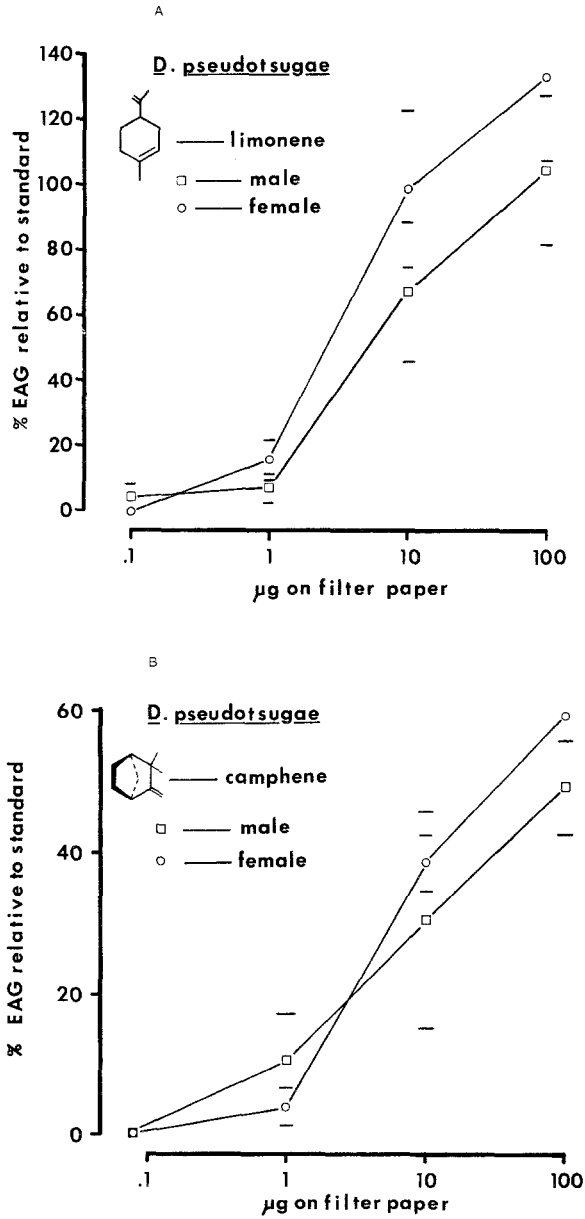

FIG. 4. Dosage–response curves constructed from EAGs of *D. pseudotsugae* males and females to serial dilutions of the host tree terpenes, limonene (A) and camphene (B). $N = 5$; horizontal lines represent $\pm$ $SE_{\bar{x}}$.

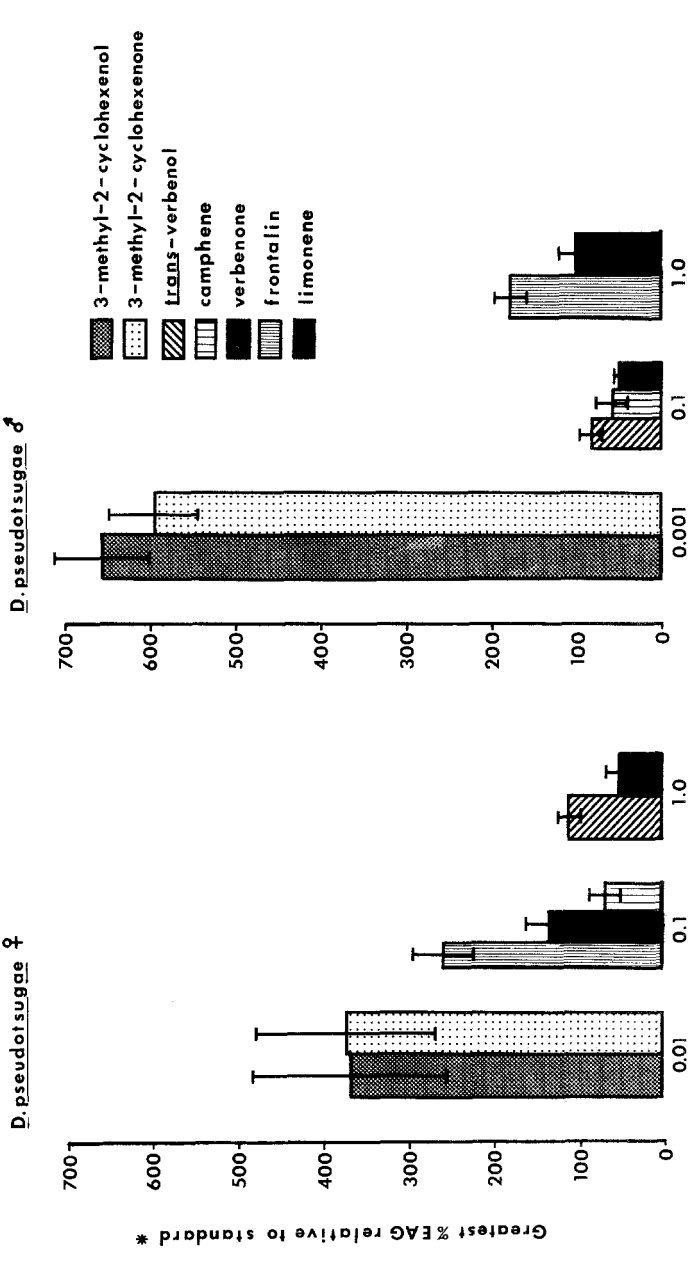

FIG. 5. Threshold for significant EAG as related to maximal EAGs to pheromones and host odors recorded from *D. pseudotsugae* females and males. $N = 5$; vertical bars represent $\pm$ SE$_{\bar{x}}$. Inserted values are threshold for significant response in μg on filter paper. *Standard = 100 μg racemic frontalin for 3,2-MCHol and 3,2-MCHone; 10 μg racemic frontalin for all other compounds tested.

and females upon intersexual sonic stimulation (Rudinsky et al., 1977; Ryker et al., 1979). Thus orientation and host selection by the pioneering sex (in this case females) would be facilitated by heightened sensitivity to a particular host odor. A similar example is found in *Ips typographus* for which a greater number of receptor sites responsive to the host terpene, (−)-alpha-pinene, were found in pioneering males than in females (Dickens, 1981).

Thus, olfactory response of *D. pseudotsugae* on the electrophysiological level parallels and helps to explain the behavior of individual males and females during host tree selection, aggregation, and colonization. The greater sensitivity of females to host terpenes and males to 3,2-MCHol and 3,2-MCHone reveals the underlying mechanism for primary attraction of pioneering females to the host and the preponderance of males that respond to secondary attractants. It would be interesting to compare the electrophysiological response to terpenes of *D. pseudotsugae* to that of a bark beetle like *D. ponderosae* Hopkins, which purportedly exhibits no primary attraction to its host trees (Hynum and Berryman, 1980; Moeck et al., 1981).

Although similarities in dosage–response curves might be indicative of similar molecule–receptor site interactions on responsive receptor cells, this cannot be fully understood based on EAGs alone. However, differing thresholds and slopes of dosage–response curves constructed from EAGs to the various odorants tested are indicative of at least four different receptor types: (1) two receptor types responsive to 3,2-MCHone and 3,2-MCHol showing dosage–response curves with the lowest thresholds and flattest slopes, (2) a third receptor type responsive to frontalin with an intermediate threshold and steepness of slope, and (3) a fourth receptor type responsive to *trans*-verbenol, verbenone, limonene, and camphene with the highest threshold and steepest slope.

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ISOPRENOIDS FROM METATHORACIC SCENT GLAND OF COTTON SEED BUG, *Oxycarenus hyalinipennis* (COSTA) (HETEROPTERA: LYGAEIDAE)

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Abstract—This paper gives the results of a gas chromatographic–mass spectrometric investigation on the composition of the scent oils from the metathoracic scent gland of *Oxycarenus hyalinipennis* (Costa). It reports the presence of at least 18 different compounds in the scent oils: fatty aliphatic materials comprising the C_6 and C_8 alk-2-enals, 4-oxo-alk-2-enals, and alk-2-enyl acetates; monoterpenoids comprising α-pinene, limonene, and 1,8-cineole (eucalyptol); and several sequiterpenoids. Observations made during gland reservoir filling indicate that the secretion from the gland tubules undergoes a dramatic change in composition within the first day or so after adult emergence. The newly formed secretion is dominated by fatty aliphatic materials while that formed later is dominated by the isoprenoids. When fully distended with secretion, the lateral scent reservoir contains isoprenoids and little else. It is an interesting feature of the metathoracic system in *Oxycarenus* that oct-2-enyl acetate persists together with oct-2-enal as a major component in the scent oil in the median reservoir. An assessment of the possible biological significance of the chemical findings is attempted.

Key Words—Scent gland, defensive secretion, pheromone, *Oxycarenus hyalinipennis*, Heteroptera, Lygaeidae, cotton seed bug, monoterpenoids, sesquiterpenoids, α-pinene, oct-2-enal, oct-2-enyl actate, sexual maturation.

INTRODUCTION

Scent glands supply many land bugs with a means of chemical defense against predators (Remold, 1962). The scent oils consist as a rule of fatty

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aliphatic aldehydes, ketoaldehydes, esters, and other biosynthetically related materials (Blum, 1981). Exceptionally, the presence of monoterpenoids alone or admixed with fatty aliphatic materials has been reported (Aldrich et al., 1978a, 1979; Everton et al., 1979; Daroogheh and Olagbemiro, 1981). In two studies an additional role for certain fatty aldehydes in defense as alarm pheromones has been demonstrated (Calam and Youdeowei, 1968; Levinson et al., 1974). Other suggestions on function in Heteroptera scent glands have been made but none has been confirmed as yet (Carayon, 1971; Staddon, 1979).

The small bugs of the genus *Oxycarenus* are well known in Africa and elsewhere in the world as pests of cotton and other malvaceous crops (Adu Mensah and Kumar, 1977). The two dorsally situated abdominal scent glands of the larva are replaced in the adult by a ventrally situated metathoracic scent apparatus. The metathoracic scent gland itself shows features which appear to be peculiar to Oxycareninae within Heteroptera (Carayon, 1971). The presence of capacious lateral scent reservoirs in addition to a median scent reservoir offers one feature of great interest to the analyst. We wish to report here the results of a gas chromatographic–mass spectrometric investigation on the composition of the scent oils from the metathoracic scent gland of *Oxycarenus hyalinipennis* (Costa). The work has revealed the presence in the scent oils of an array of isoprenoids in addition to fatty aliphatic materials. It has also revealed something of the changes in composition in the secretion from the gland tubules which occur during the first day or so after adult emergence. An assessment of the biological significance of the chemical findings is attempted.

METHODS AND MATERIALS

The bugs were identified as *O. hyalinipennis* from the descriptions of African Oxycareninae supplied by Samy (1969). They were maintained in the laboratory in continuous culture at 26°C under a 14:10 light–dark photoperiod in a Gallenkamp IH-282 cooled incubator on a supply of cotton seeds and drinking water. Body weight statistics are given in Table 1. Adults were chilled before excising the metathoracic scent gland under 200 mM NaCl.

Gas chromatography–mass spectrometry (GC-MS) was performed with a Varian MAT CH5D mass spectrometer at 70 eV, ion source temperature 190°C separator 180° and 200 μA ionizing current. Separations were performed on a silicone OV column; 2 m × 2 mm 3% OV-225 on 60–80 mesh Gas Chrom Q, 10 ml helium/min, 70°C isothermal for 5 min, and then temperature programed to 200°C at 10°/min. During the initial stages of the work, GC-MS was carried out on acetone extracts of glandular material. In

TABLE 1. BODY WEIGHT OF *Oxycarenus hyalinipennis* ADULTS AT SEXUAL MATURITY

	Weight (mg)[a]
Males	1.33 ± 0.26(6)
Females	2.9 ± 0.5(6)

[a]Mean ± standard deviation of the mean (number of adults).

subsequent work, the solvent was dispensed with and the glandular material (entire gland, median reservoir, lateral reservoir, or secretory tubule) was injected by a simple open column procedure (Staddon et al., 1979).

The gas chromatographic analyses (GC) were carried out with a Varian series 1400 gas chromatograph equipped with flame ionization detector and 2 m × 2 mm ID silicone OV-225 column; basic routine, injector 170°C, 30 ml nitrogen/min, column 80° isothermal for 20 min, and then temperature programed at 10°/min to 200°C. For peak area calibration external standards were prepared from dodecane in acetone.

Standard materials were obtained from several sources: α-pinene, (*E*)-oct-2-enal, (*Z*)-oct-2-enyl acetate from Proprietary Perfumes Limited, Ashford, England; (*E*)-hex-2-enal from Aldrich Chemical Company, Milwaukee, U.S.A.; (*E*)-hex-2-enyl acetate from Dr. D.W. Knight, Nottingham, England; and limonene and 1,9-cineole from Sigma Chemical Company, St. Louis, U.S.A.

RESULTS

Diagrams indicating aspects of gland structure, filling, and emptying are given in Figure 1. Capacious lateral reservoirs (Figures 1A and B, lr) are present in both sexes. They occur in some other lygaeid bugs but only in the male adults (Carayon, 1948; Johansson, 1957). They fill up with secretion later than the median reservoir but are quite full by around the third day after adult emergence. They appear quite shrunken in the newly formed gland (Figure 1B, lr). The secretory tissue consists of a compact mass of secretory tubules (Figures 1A and B, gt) and opens by a single opening near the apex into the lateral reservoir. The tubules are unpigmented, unlike the epithelium of lateral and median reservoir tissue which is pigmented an orange color. Accessory glands (Figure 1A, ag) are present in the well of the median reservoir. Suggested functions for these structures in the metathoracic scent gland in other Heteroptera include enzymatic catalysis of the final step in the production of the scent aldehydes from precursors secreted by the gland secretory tubules (Gilby and Waterhouse, 1967; Games and Staddon, 1973;

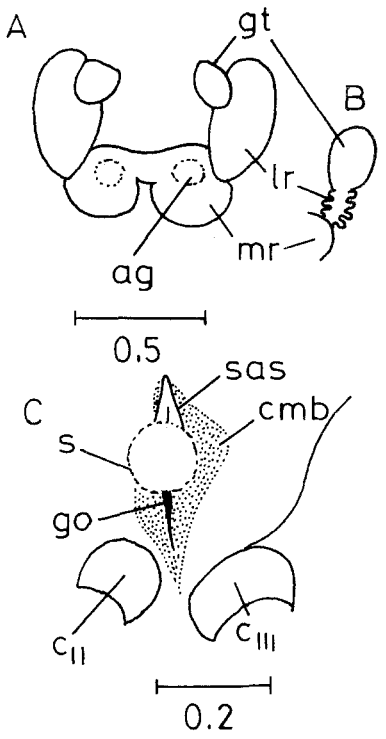

FIG. 1. Morphological aspects of scent oil accumulation and emission: (A) metathoracic scent gland fully distended with secretions; (B) secretory tubule-lateral reservoir complex prior to filling of lateral reservoir; (C) droplet of secretion adhering to the external scent accumulation surface. ag, accessory gland; C_{II} mesothoracic and C_{III} metathoracic coxa; cmb, cuticular microsculpture border; go, external opening of metathoracic scent apparatus; gt, metathoracic secretory tubules; lr, lateral reservoir; mr, median reservoir; s, droplet of secretion; sas, scent accumulation surface. Scale lines indicate length in mm.

Aldrich et al., 1978b; Everton et al., 1979). When adult bugs are handled or otherwise disturbed, the stored secretion is expelled to accumulate in a temporary droplet on a special raised area formed from metapleuron (Figure 1C, sas). Transfer of the extruded oil to alien surfaces can be effected on the mesotarsus by defensive movements of the middle leg.

The scent oil accounts for around 1% of the total body volume of a sexually mature adult. The greater part is contained in the two lateral reservoirs (80% of the total), the remainder occupying the lumen of the median reservoir (20% of total).

Figure 2 shows total ion current traces from acetone extracts of entire gland (Figure 2A) and lateral reservoir (Figure 2B) material. Table 2 supplies

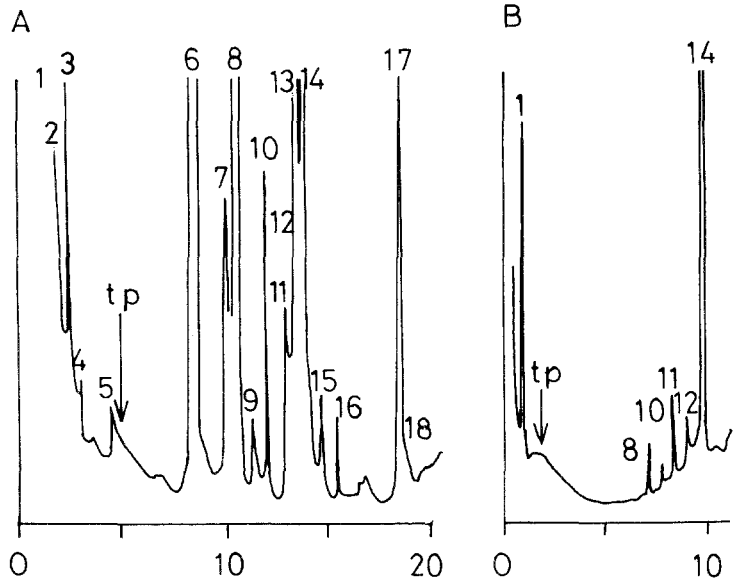

FIG. 2. Separation of scent volatiles by GC-MS. Total ion current monitor tracers of (A) sample of entire metathoracic scent gland material, and (B) a lateral reservoir sample, in acetone. One division of time scale = 5 min. OV-225, 10 ml helium/min, 70° isothermal for 5 min, and then temperature programmed (tp) at 10°/min to 200° C. For peak identification see Table 2.

mass spectral data for 18 different peaks and gives the identities of these peaks so far as they could be asscertained from the mass spectral data.

Peaks 1, 2, and 4 were found to be from α-pinene, limonene, and 1,8-cineole (eucalyptol), respectively. When authentic samples of similar materials were analyzed on two different GC columns (OV-225, Apiezon L) results obtained were identical with those from the natural products. It should be noted that formation of α-pinene by thermal rearrangement was not observed when β-pinene was analyzed.

Peaks 3 and 6 were found to be from hex-2-enal and oct-2-enal, respectively; peaks 5 and 8 from hex-2-enyl acetate and oct-2-enyl acetate; and peaks 7 and 13 from 4-oxohex-2-enal and 4-oxo-oct-2-enal. These fatty aliphatic materials are widely present in the secretions from the scent glands of Heteroptera (Staddon, 1979; Blum, 1981). The mass spectrum of 4-oxo-hex-2-enal from *Oxycarenus* matched that published for (*E*)-4-oxo-hex-2-enal from *Nezara viridula* (Gilby and Waterhouse, 1965). Quantitative differences in the mass spectra of authentic (*E*)- and (*Z*)-4-oxo oct-2-enal have been recorded (Macleod et al., 1977). Peak 13 from *Oxycarenus* was found to be from (*E*)-4-oxo-oct-2-enal. It is probable that the other fatty aliphatic

TABLE 2. MASS SPECTROMETRIC IDENTIFICATION OF VOLATILES FROM THE METATHORACIC SCENT GLAND OF *Oxycarenus hyalinipennis*

No.	Identity	Mass spectral data, m/z (intensity, %)
1.	α-Pinene	136(M^+;6), 121(8), 107(5), 105(9), 93(100), 92(37), 91(41), 80(9), 79(22), 77(28), 68(5), 67(10), 65(6), 55(6), 53(9), 51(6), 41(22)
2.	Limonene	136(M^+;5), 121(5), 107(24), 105(25), 94(35), 93(49), 92(56), 91(22), 79(33), 77(21), 68(100), 67(57), 53(10)
3.	Hex-2-enal	*98(M^+*;18), 97(13), 83(54), 69(48), 57(48), 55(41), 42(57), 41(100)
4.	1,8-Cineole	154(M^+;23), 139(28), 111(17), 108(40), 96(18), 95(14), 93(30), 84(44), 83(11), 81(57), 71(39), 69(31), 68(16), 67(16), 55(28), 43(100), 41(25)
5.	Hex-2-acetate	142(M^+;1), 100(16), 99(6), 82(28), 71(7), 67(34), 57(12), 43(100), 55(15), 41(18)
6.	Oct-2-enal	126(M^+;absent), 125(1), 111(2), 98(10), 97(13), 93(11), 83(56), 82(35), 70(66), 69(27), 67(26), 57(53), 55(79), 42(48), 41(100)
7.	4-Oxo-hex-2-enal	112(M^+;18), 84(13), 83(100), 57(22), 55(50)
8.	Oct-2-enyl acetate	170(M^+;1), 128(33), 110(46), 95(26), 82(57), 81(80), 71(27), 69(49), 68(74), 67(69), 57(38), 55(61), 54(9), 43(100), 41(74)
9.	Sesquiterpene hydrocarbon ($C_{15}H_{24}$)	204(M^+;45), 189(28), 161(100), 136(36), 133(61), 121(40), 119(48), 107(39), 105(89), 95(45), 93(67), 91(79), 81(43), 79(44), 77(26), 67(41), 41(42)
10.	Sesquiterpene hydrocarbon ($C_{15}H_{24}$)	204(M^+;4), 161(11), 119(100), 107(24), 105(23), 93(78), 91(32), 79(24), 77(17), 69(35), 55(23), 41(42)
11.	Sesquiterpene hydrocarbon ($C_{15}H_{24}$)	204(M^+;5), 161(13), 133(24), 127(17), 120(23), 119(26), 105(18), 93(82), 91(23), 81(22), 79(26), 69(100), 67(24), 55(23), 41(84)
12.	Sesquiterpene hydrocarbon ($C_{15}H_{24}$)	204(M^+;26), 161(39), 136(50), 133(37), 121(100), 119(39), 111(29), 107(27), 105(40), 98(43), 93(68), 91(42), 79(34), 69(27), 55(28), 41(59)
13.	4-Oxo-oct-2-enal	140(M^+;2), 125(19), 110(93), 98(97), 96(23), 93(12), 85(12), 84(36), 83(92), 70(58), 69(11), 67(51), 56(18), 55(100), 41(71)

TABLE 2. (*Continued*)

No.	Identity	Mass spectral data, m/z (intensity, %)
14.	Sesquiterpene hydrocarbon ($C_{15}H_{24}$)	204(M^+;14), 189(9), 161(14), 147(12), 135(17), 121(18), 119(69), 107(44), 105(28), 93(100), 91(33), 81(22), 79(33), 77(20), 69(27), 67(28), 55(28), 41(66)
15.	Sesquiterpene hydrocarbon ($C_{15}H_{24}$)	204(M^+;26), 161(37), 135(33), 133(48), 121(96), 119(91), 107(60), 105(89), 93(100), 91(67), 81(59), 79(58), 77(36), 67(53), 55(46), 41(86)
16.	Sesquiterpene oxide ($C_{15}H_{24}O$)	220(M^+;6), 205(19), 204(16), 135(58), 119(12), 107(100), 105(21), 93(42), 91(33), 79(17), 77(14), 57(19), 43(16) 41(35)
17.	Sesquiterpene oxide ($C_{15}H_{26}O$)	222(M^+;4), 137(33), 119(21), 109(46), 105(20), 95(34), 93(24), 84(100), 83(45), 81(41), 69(47), 67(39), 55(35), 41(53)
18.	Sesquiterpene oxide ($C_{15}H_{24}O$)	220(M^+;5), 205(21), 97(53), 83(76), 70(81), 69(100), 57(82), 56(91), 55(99), 43(44), 41(72).

materials from the metathoracic scent gland of *Oxycarenus* are also in the *E* configuration.

Peaks 9–12 and peaks 14–18 are from sequiterpenoids. This group of materials has not previously been recorded from the scent glands of Heteroptera. The major peak in this series (peak 14) showed a molecular ion at m/z 204 and high-resolution mass spectrometry agreed with the expected composition $C_{15}H_{24}$ (m/z found, 204.1905; m/z expected, 204.1878). Peaks 9–12 and peak 15 also appear to be from sequiterpene hydrocarbons of molecular composition $C_{15}H_{24}$. The remaining sesquiterpenoid peaks appear to derive from oxides. The molecular formula $C_{15}H_{24}O$ has been assigned tentatively to peaks 16 and 18. The weak mass spectra obtained from these components showed peaks at m/z 220 (M^+) and 205 (M − 15). A prominent but unexplained peak at m/z 204 was observed in the mass spectrum recorded from peak 16. Peak 17 has been assigned the molecular formula $C_{15}H_{26}O$. The mass spectrum of this component showed peaks at m/z 222 (M^+), 207 (M − 15 and 204 (M − 18).

The structures of the sesquiterpenoids have not been elucidated. A close

match between the mass spectrum of minor peak 11 and that published for (*E*)-β-farnesene is noted (Murray, 1969).

Figure 3 reveals the existence of a marked difference in composition of entire metathoracic scent gland secretion in a 1-day-old female adult (Figure 3A) and a 7-day-old female adult (Figure 3B). Peaks for oct-2-enal (6) and oct-2-enyl acetate (8) dominate the chromatogram from the 1-day-old gland, peaks for α-pinene (1) and the major sequiterpene (14), by contrast, dominate the 7-day-old gland. Figure 4 reveals the existence of similar differences in lateral reservoir samples. There is a preponderance of oct-2-enyl acetate (8) in the 1-day-old lateral reservoir; the older (7-day) sample is dominated by peaks for α-pinene and the major sequiterpene. In fact, peaks for oct-2-enal and oct-2-enyl acetate were not detected in the 7-day-old lateral reservoir. It was possible to calculate that fatty aliphatic materials, if at all present in the 7-day-old lateral reservoir, could not have accounted for more than 0.1% of the total sample. These data seem to indicate that gland filling is associated with a switch from the production of fatty aliphatic materials dominated by

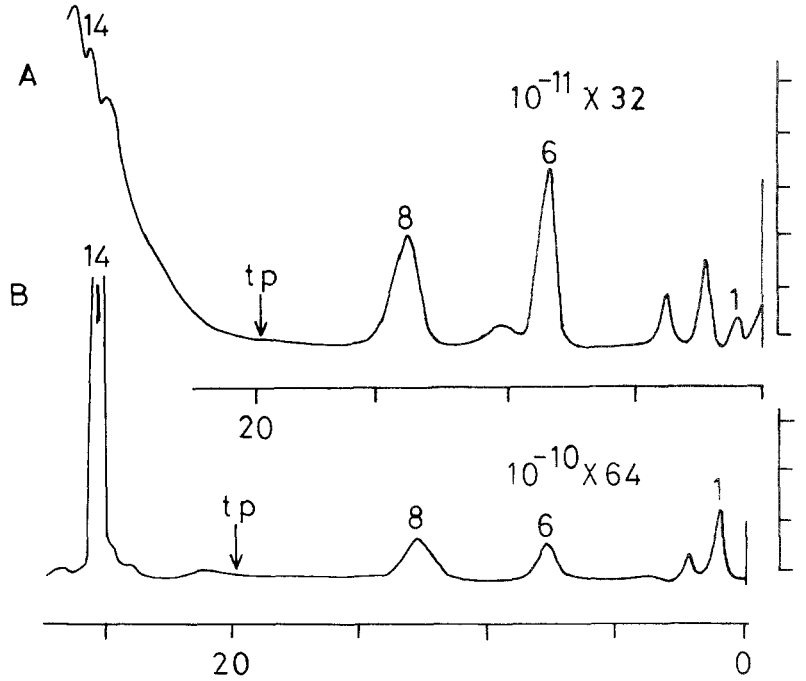

FIG. 3. Composition of volatile material from the entire metathoracic scent gland in (A) a 1-day old female and (B) a 7-day-old female adult. GC on 3% OV-225, 30 ml nitrogen/min, 80° for 20 min, and then temperature programed (tp) at 10°/min to 200° C. One division of time scale = 5 min.

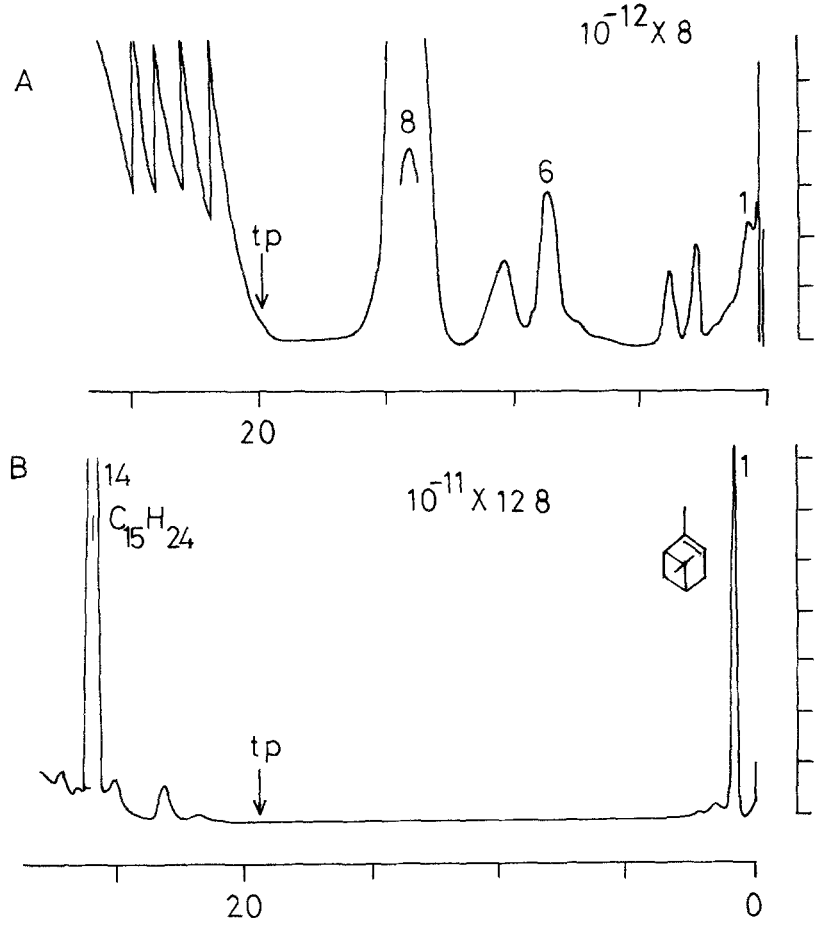

FIG. 4. Composition of volatile material from the secretory tubule-lateral reservoir complex in (A) a less than 1-day-old adult and (B) a 7-day-old female adult. GC on OV-225, 30 ml nitrogen/min, 80° for 20 min, and then temperature programed (tp) at 10°/min to 200° C. One division of time scale = 5 min.

oct-2-enal and oct-2-enyl acetate to the production of isoprenoids dominated by α-pinene and a sequiterpene hydrocarbon (peak 14). This change in emphasis occurs after adult emergence, during the period of sexual maturation.

An indication that the isoprenoid fraction of the secretion is undergoing quantitative changes in composition during gland filling was obtained. Table 3 supplies for comparison data arising from GC analysis of secretory tubule-lateral reservoir complex (Figure 3A) and median reservoir material (Figure 3B) from a single gland from a 4-day-old *Oxycarenus* adult. It may be seen

TABLE 3. SCENT OIL COMPOSITION IN MEDIAN RESERVOIR (MR) AND LATERAL RESERVOIR (LR) OF METATHORACIC SCENT GLAND FROM A 4-DAY-OLD *O. hyalinipennis* ADULT

Scent oil component	Composition (%)	
	MR	LR
α-Pinene	0.2	11
Oct-2-enal	23	1
Oct-2-enyl acetate	58	4
Major sesquiterpenoid (peak 14)	17	82
Total percentages	98.2	97
Sample volume (=μl dodecane)	0.0001	0.0036

that α-pinene is more abundant relative to the sequiterpene (peak 14) in the lateral reservoir sample. A possible explanation is that α-pinene is increasing allometrically relative to the sequiterpene during gland filling (allometry in connection with accumulation of fatty scent volatiles has been reported; Staddon and Daroogheh, 1981, 1982).

The data reported in Table 2 indicate that oct-2-enyl acetate persists as a major component relative to oct-2-enal in the median scent reservoir. Figure 5 shows from GC analyses of whole gland samples a plot of peak area response of oct-2-enal on that of oct-2-enyl acetate (24 analyses, adults ranging in age from less than 1 day to 7 days). These data indicate that the ratio of oct-2-enyl acetate to oct-2-enal changes little during gland filling. The isomolar line $y = 0.74x$ which could be fitted to the data points supplies a reasonable indication of the form of this relationship.

The occurrence of oct-2-enyl acetate as a major component in median reservoir scent oil is unexpected in veiw of previous work on Heteroptera. In *Oncopeltus fasciatus*, for example, where fatty scent esters dominate the secretion stored in the lateral reservoirs of the male adults, the fatty esters occur only as minor components in the scent oil stored in the median reservoir. The place of the scent esters in the median reservoir is taken by the aldehydes corresponding to the alcohols of the esters from which they are presumably derived by biosynthetic modification. It was also unexpected to find a peak for oct-2-enal in lateral reservoir sample materials in *Oxycarenus*. It was decided therefore to perform further GC-MS analyses specifically on lateral reservoir and secretory tubule material. The results obtained were entirely in accord with interpretations previously derived from the GC work.

Secretory tubule tissue alone from two 1-day-old adults showed, on analysis, peaks for oct-2-enyl acetate and the major sesquiterpenoid (peak 14); one of the samples also showed peaks for hex-2-enal, hex-2-enyl acetate,

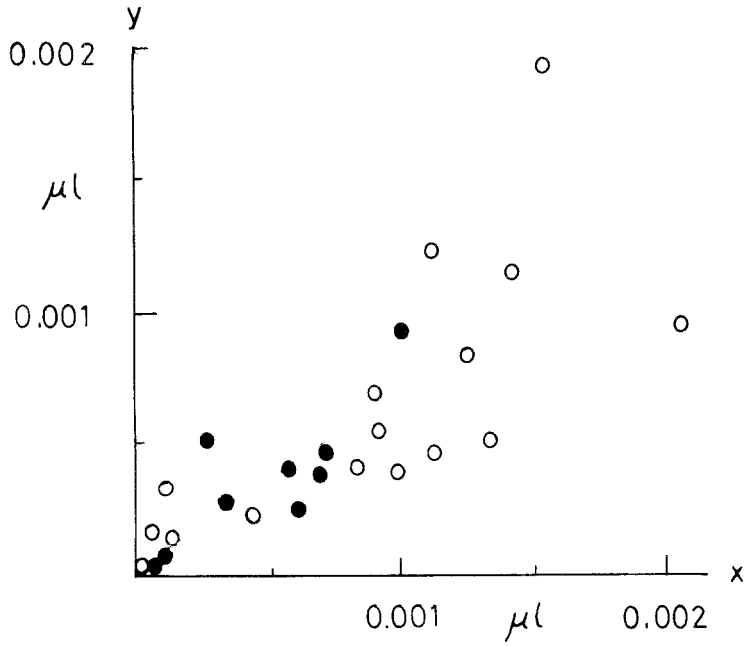

FIG. 5. Graph of accumulation of oct-2-enal (y) on oct-2-enyl acetate (x) for the entire metathoracic scent gland. From GC on 3% OV-225 using dodecane as external standard. Closed circles, male adults; open circles, female adults. Adult age range from less than one day to seven days. Number of data points, 25.

oct-2-enal, and oct-2-enyl acetate. It is an interesting difference that oct-2-enyl acetate dominated one of the two secretory tubule samples while the major sesquiterpene (peak 14) dominated the other. Thus there is an indication that the switch from fatty aliphatic material production to isoprenoids could be occuring within one day after adult emergence. However, the data fail to exclude the scent aldehydes from secretory tubule tissues. In a final pair of analyses, marked similarity in composition in lateral reservoir and median reservoir scent oil from a 16-hr-old adult was observed. α-Pinene, the major sequiterpene, hex-2-enal, hex-2-enyl acetate, oct-2-enal, and oct-2-enyl acetate were detected in both samples and in both the dominant peak was found to be from oct-2-enyl acetate.

An interesting feature of these further results is that they failed to reveal the presence of either 4-oxo-hex-2-enal or 4-oxo-oct-2-enal in samples of material from young adults. It is possible that the formation of the 4-oxo compounds takes place relatively slowly in the median reservoir.

Finally, analyses (GC) performed on two samples of ejected scent oil clearly indicated that the isoprenoids are ejected together with the aliphatic scent volatiles when the adults are disturbed by handling. Presumably,

Oxycarenus lacks a mechanism for the selective elimination of isoprenoid materials from the distended lateral reservoirs.

DISCUSSION

The work here reported indicates that the scent oils from the metathoracic scent gland of *Oxycarenus hyalinipennis* are composed of at least 18 different compounds comprising several fatty aliphatic materials as well as monoterpenoid and sequiterpenoid materials. It also indicates that the composition of the scent oil from the secretory tubules is not fixed once and for all at the moment of onset of secretory activity. While fatty aliphatic materials dominate the secretion from the newly activated gland tubules, isoprenoids dominate that formed from about the first day after adult emergence. The result of this apparent shift in biochemical emphasis from fatty aliphatic to isoprenoid production is a difference in composition of the secretions stored in the lateral and median reservoirs in the newly filled gland. The distended lateral reservoirs contain the isoprenoids and little else, while the median reservoir continues to contain a high proportion of fatty aliphatic materials. Features of further biochemical interest include the persistence of oct-2-enyl acetate together with oct-2-enal as major components of the scent oil in the median reservoir, the apparent production of hex-2-enal and oct-2-enal in secretory tubule material, the late appearance of the 4-oxo-alk-2-enals, and a possible allometric increase of α-pinene relative to the major sequiterpenoid (peak 14).

Monoterpenoids have been reported previously from the scent glands in five different species of Heteroptera distributed in three families: linalool, terpinen-4-ol, α-terpineol, and *cis* -piperitol from the abdominal scent gland of the male in the predaceous bug *Podisus maculiventris* (Pentatomidae; Asopinae) (Aldrich et al., 1978a); β-pinene, myrcene, limonene, α-terpinene, terpinolene, terpinene-4-ol, piperitone, and thymol from the metathoracic scent gland of the plant bug *Niesthrea louisianica* (Rhopalidae) (Aldrich *et al.*, 1979); perilla alcohol from the dorsal abdominal scent gland of *Niesthrea louisianica* (Aldrich et al., 1979); α-pinene, β-pinene, myrcene, and limonene from the abdominal scent glands of *Jadera haemalotoma* (Rhopalidae); and linalool from the metathoracic scent gland of *Dysdercus intermedius* and *D. superstitiousus* (Pyrrhocoridae) (Everton et al., 1979; Daroogheh and Olagbemiro, 1982). This series can now be extended to include α-pinene, limonene, and 1,8-cineole from the metathoracic scent gland of *Oxycarenus hyalinipennis* (Lygaeidae). The metathoracic scent gland of *Oxycarenus* is, so far as is now known, unique among the scent glands of Heteroptera in secreting sesquiterpenoid materials.

Previous workers find no apparent link between diet and the presence of monoterpenoids in the scent glands of Heteroptera. Thymol, for example, a major component of the metathoracic scent gland of *Niesthrea* was not detected in the food plant of *Niesthrea* (Aldrich et al., 1979). The composition of the scent oil from the metathoracic scent gland of *Oxycarenus* shows a parallel with that of cotton seed oil (Kumamoto et al., 1979) in that α-pinene and limonene are present in both. However, the major sesquiterpenoid of cotton leaf oil is different from that of *Oxycarenus* scent gland. We have not studied the matter specifically in *Oxycarenus*, but all data obtained to date indicate that scent oil composition in Heteroptera is specified by the bugs themselves and not by diet.

The only previous demonstration of age-related changes in scent oil composition comparable in magnitude to those described here for *Oxycarenus* is from work on the metathoracic scent gland of *Dysdercus intermedius* reported by Everton et al. (1979). In *Dysdercus* a gradual displacement of fatty scent esters by the monoterpene alcohol linalool was observed in the small lateral reservoir supplied by the gland secretory tubules. Quantitative changes of an allometric nature have been recorded from the scent glands of other Heteroptera in connection with the accumulation of fatty scent aldehydes and esters (Staddon and Daroogheh, 1981, 1982), and similar changes are perhaps occurring on *Oxycarenus* in connection with the accumulation of α-pinene and the major sesquiterpene (peak 14). However, the sharp discontinuities in composition observed in two analyses of secretory tubule material from 1-day-old *Oxycarenus*, the one sample showing octenyl acetate as dominant, the other showing the major sesquiterpene as dominant, is an indication perhaps that fatty aliphatic biosynthesis is abruptly rather than gradually replaced by isoprenoid biosynthesis during gland filling in *Oxycarenus*. This speculation goes beyond the available facts, but it is difficult to resist advancing the suggestion that humoral factors may be involved in the control of this biochemical transformation in secretory tubule tissue.

It is a further peculiarity of the metathoracic scent gland in *Oxycarenus* that oct-2-enyl acetate persists together with oct-2-enal in the median reservoir. Indeed, the two are present in approximately equimolar proportions. The situation in *Oxycarenus* can be contrasted with that in the milkweed bug *Oncopltus fasciatus* where the fatty aliphatic acetates of the secretory tubules are almost completely displaced in the median reservoir by the fatty aliphatic aldehydes corresponding to the alcohols of the tubule produced esters (Games and Staddon, 1973). Moreover, the scent aldehydes have not been detected in secretory tubule material in *Oncopeltus*. The explanation of the pattern of variation to be found in *Oncopeltus* and in at least some other Heteroptera seems to be that the scent aldehydes are formed

extracellularly in the median scent reservoir, under the action of enzymes secreted by the accessory gland, from the alcohols of the esters supplied by the gland secretory tubules (Gilby and Waterhouse, 1967; Games and Staddon, 1973; Aldrich et al., 1978; Everton et al., 1979; Staddon et al., 1979). In *Oxycarenus* not only do we find oct-2-enyl acetate persisting in the median reservoir, but we also find oct-2-enal in secretory tubule and lateral reservoir material. Although it cannot be excluded as possible that the oct-2-enal observed in lateral reservoir or secretory tubule tissue in *Oxycarenus* was a contaminant picked up during dissection from median reservoir scent oil, it is clearly necessary to propose that the scent aldehydes as well as the scent esters originate in *Oxycarenus* in the tissue of the gland secretory tubules.

Several isoprenoids have been documentated as repellents and alarm pheromones in a variety of insects from different orders. They include α-pinene and β-pinene in the defensive resinous discharges from a sawfly larva *Neodiprion sertifer* (Eisner et al., 1974) and termites of the genus *Nasutitermes* (Moore, 1964; Eisner et al., 1976). There is evidence that limone functions as an alarm pheromone in the termite *Drepanoterms rubriceps* (Moore, 1968). Sesquiterpenoids have been found in the osmeterial secretions of *Papilio* (Lepidoptera) larvae (Eisner et al., 1971; Burger et al., 1978; Honda, 1980a,b, 1981). Sesquiterpenoid alarm pheromones have been isolated from a variety of aphids (Homoptera) (Bower et al., 1972, 1977; Edwards et al., 1973; Wientjens et al., 1973; Nishino et al, 1977; Pickett and Griffiths, 1980).

The biological function of isoprenoids in the metathoracic scent gland of *O. hyalinipennis* remains to be elucidated. In *Dysdercus*, the vapor from linalool elicits feeding responses (Everton et al., 1979). Ghandi (1979), in contrast finds, that α-pinene is repellent to *Oxycarenus hyalinipennis*. Moreover, Adu-Mensah and Kumar (1977) were unable to find clues to the function of the metathoracic scent gland from observations on *Oxycarenus* adults in nature. No real support exists at present for the presumption that the scent volatiles are chiefly used in defense against predators. It is perhaps the case that the isoprenoids play some role in the sexual activities of the adults, since their late appearance in the secretion could supply a chemical distinction between immature and sexually mature adults. It would be possible to advance other ideas on possible roles for the scent isoprenoids in a variety of short- or long-range interactions or encounters. The main hope is that the problem of function will yield to studies using purified materials separately and in combination in suitably designed bioassays.

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TRIUNSATURATED HYDROCARBONS, SEX PHEROMONE COMPONENTS OF *Caenurgina erechtea*[1,2]

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Abstract—(*Z,Z,Z*)-3,6,9-Eicosatriene and (*Z,Z,Z*)-3,6,9-heneicosatriene have been identified as components of the sex pheromone of the noctuid, *Caenurgina erechtea* (Cramer), the forage looper. Structural assignments were made on the basis of spectroscopic and chromatographic data and were confirmed by comparison with synthetic material. Flight tunnel behavioral studies demonstrated that either component, when tested individually, would elicit wing fanning responses in males; however, mixtures of the two components increased this response and were essential for initiation of upwind flight and landing. In field experiments, traps baited with either component alone captured few or no adult forage looper males while those baited with both components captured several target males.

Key Words—Behavior, *Caenurgina erechtea* (Cramer), forage looper, Lepidoptera, Noctuidae, sex pheromone, (*Z,Z,Z*)-3,6,9-eicosatriene, (*Z,Z,Z*)-3,6,9-heneicosatriene, flight tunnel.

INTRODUCTION

Sex pheromones and attractants have been reported for some 150 species of temperate North American Noctuidae representing nearly 10% of the species of this family within the region (Roelofs, 1979; Steck et al., 1982). The chemistry of the components comprising these sex pheromones and attractants has been summarized (Steck et al., 1982) as even-numbered

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($C_{10,12,14,16}$), straight-chain compounds bearing a terminal alcohol, acetate, or aldehyde functional group and possessing a single double bond in the *cis* configuration at odd-numbered carbons in the chain; only a few are diunsaturated or saturated. Blends of these compounds attract males representing nine of the 13 subfamilies of Noctuidae (system of Rockburne and Lafontaine, 1976), although none have been reported as attractants of species within the other four subfamilies, namely, Sarrothripinae, Agaristinae, Euteliinae, or Catocalinae (Steck et al., 1982).

Adult *Caenurgina erechtea*, forage looper, of the sub family Catocalinae, have been regularly captured in a blacklight trap operated nearby but none have been caught in any of our survey traps chemically baited with noctuid sex attractants (Steck et al., 1979, 1980, 1982). Preliminary to the work reported here (unpublished), measurements were made of the antennal responses (EAG) of forage looper males to some 300 synthetic, saturated and monounsaturated, even-numbered, C_{10-18} alcohols, acetates, and aldehydes. All of the responses were near background, which suggested some fundamentally different class of chemical entity may constitute the sex pheromone of this species.

We report here the isolation, identification, and synthesis of two components of the sex pheromone of *C. erechtea*, and the results of flight tunnel and field trapping experiments using the two components.

METHODS AND MATERIALS

Identification and Synthesis. A laboratory culture of *C. erechtea*, started in 1979 from field-collected adults, was maintained at 22°C under a 16:8 light–dark photoperiod with larvae reared on a modified wheat germ diet (Shorey and Hale, 1965). Pheromone was obtained from 1 to 4-day-old virgin females by everting their ovipositors and washing the exposed surface with ca. 5 μl of hexane (Klun et al., 1979). After rinsing, the ovipositors were excised and extracted for several hours with methylene chloride to obtain greater recoveries of pheromone. A preliminary purification of the methylene chloride extract was necessary, and this was carried out by thin-layer chromatography (TLC) using Whatman Silica Gel K6F plates developed with hexane. Pheromone components were located and recovered by sequentially scraping 0.5-cm bands from the plates, extracting each with diethyl ether–methylene chloride (1:1), and assaying for biological activity by electroantennography.

Gland washes and TLC-purified extracts were fractionated by high-resolution capillary gas chromatography (GC) using SP-2100 and SP-2250 analytical columns (25 m × 0.22 mm ID) and an OV-101 preparative column (25 m × 0.32 mm ID) which were programed from 90°C to 220°C at

4°C/min. When required, components eluting from the columns were collected via an effluent splitter which diverted 15% of the effluent to the flame detector and the remainder to a heated collection port. Components were condensed in Dry-Ice cooled glass capillaries (125 mm × 0.6 mm ID). An electroantennograph was used to measure pheromone activity in crude extracts, thin-layer and gas chromatographic fractions, as well as the antennal stimulatory activity of synthetic compounds (Chisholm et al. 1975). Chemical ionization (CI) and electron impact (EI) mass spectra were obtained by gas chromatography–mass spectrometry (GC-MS) using a Finnigan 4000 mass spectrometer equipped with a SP-2100 glass capillary (25 m × 0.22 mm ID). Methane was used as both carrier and reagent gas for CI-MS. Proton nuclear magnetic resonance ([^{1}H]NMR) spectra of insect-derived pheromone components in $CDCl_3$ were obtained using a Bruker WH-400 spectrometer; [^{1}H]NMR spectra of synthetic compounds were recorded with either Varian HA-100 or XL-100 instruments.

Diunsaturated and triunsaturated hydrocarbons, C_{18}–C_{22}, were synthesized starting from C_{18} methyl esters (99% pure, NuChek Corp., Elysian, Minnesota). Reduction of methyl linolenate [methyl (Z,Z,Z)-9,12,15-octadecatrienoate] in ether with Red-Al® [sodium bis-(2-methoxyethoxy)aluminum hydride] afforded linolenyl alcohol from which the tosylate was prepared in pyridine and purified by silicic acid chromatography using hexane–ether (9:1). A portion of the tosylate was reduced with lithium aluminum hydride to (*Z,Z,Z*)-3,6,9-octadecatriene [(*Z,Z,Z*)-3,6,9–18:H]. This hydrocarbon and those to follow were purified by silicic acid chromatography using hexane. The remainder of the tosylate was used to form the series of (*Z,Z,Z*)-3,6,9-hydrocarbons with 19–22 carbon atoms using a Grignard coupling reaction mediated by dilithium tetrachlorocuprate (Fouquet and Schlosser, 1974). Grignards derived from methyl iodide, ethyl iodide, propyl bromide, and butyl bromide were used to synthesize C_{19}, C_{20}, C_{21}, and C_{22} hydrocarbons, respectively. A similar sequence of reactions starting from methyl (*Z,Z,Z*)-6,9,12-octadecatrienoate (methyl-γ-linolenate) afforded (*Z,Z,Z*)-6,9,12-hydrocarbons with chain lengths of 18, 20, 21, and 22 carbons. Starting from methyl (*Z,Z*)-9,12-octadecadienoate, the series (*Z,Z*)-6,9-hydrocarbons (C_{18}–C_{22}) were prepared. IR and [^{1}H]NMR spectra were recorded and each was consistent with the expected product; all hydrocarbons, when assayed by capillary GC, were >98% pure.

Flight Tunnel Tests. The flight tunnel (89 × 95 × 266 cm long), a modification of an earlier design (Miller and Roelofs, 1978) and made of Plexiglas, has been described elsewhere (Palaniswamy et al., 1983). Illumination within the tunnel was at an intensity of 0.1 lux and a laminar flow of air at 25.7 cm/sec and 21 ± 1°C was maintained. Chemicals were released from rubber septa placed on glass rods and positioned 22 cm above the tunnel floor, 206 cm upwind from the point of moth release.

Adult males, 2–5 days old, which had been maintained under a 16:8 light–dark photoperiod, were held in cylindrical wire screen release cages (7 cm diameter × 12 cm long) for at least 45 min before experimentation. All assays were carried out 0.5–2 hr into scotophase. At the start of the test the release cage was positioned on a disposable stand at the downwind end of the tunnel in the path of the chemical plume, the position of which had been previously established using titanium tetrachloride. After a 3-min baseline period, if the insect remained calm, test chemicals were introduced at the upwind end of the tunnel. Males were considered to be nonresponsive if they failed to show the wing fanning response within 3 min after sample introduction. Responding insects were released from their cages ca. 30 sec after commencement of wing fanning. Behavioral response experiments were carried out on several days, but each day all treatments (Table 1) were included, although not always with equal frequency. Moths were exposed only once to a treatment and in random order; all moths were discarded at the end of the day in which they were used.

The behavioral responses were videotaped using a Panasonic ER Newvicon 1850 camera, and this was supplemented by audio recording an observer's description. In this report the term plume-oriented flight initiation refers to the forward or upwind progress of those moths which, when released from their holding cages, orient in and follow the plume for at least 10 cm from the point of release. Moths which flew out of the cage but failed to orient in and follow the plume for at least 10 cm were considered not to have initiated plume-oriented flight. Plume-oriented flight refers to continued upwind flight along the plume or to hovering and looping flights in the plume area but without upwind progress. Plume-oriented flight was considered terminated when the moth left the plume area and did not return to reorient in the plume.

Field Trapping. Pherocon 1CP® traps (Zoecon Corp., Palo Alto, California) or white Conor 3CW (Conor Trap Co., Saskatoon, Canada), similar to the cone-orifice model 3 trap described by Steck and Bailey (1978), were used for field trapping. Traps contained either synthetic lures impregnated in rubber septa (A.H. Thomase #8753-D22) or 2–3 laboratory-reared virgin females held within a wire screen cage. Female-baited traps were inspected daily and new females were replaced as needed. In replicated tests, traps were set out in randomized block designs.

Statistical Analysis. The significance of treatment effects was determined by χ^2 analysis of transformed (arc sin $X^{\frac{1}{2}}$) percentage data, and the means were compared by Duncan's new multiple-range (DNMR) test using a pooled binomial error variance. Treatment effects on transformed latency data (ln X) were compared by DNMR test using the error mean square from one-way analysis of variance. Transformed trap capture data $(X + 0.5)^{\frac{1}{2}}$ were subjected to analysis of variance and the means were compared by DNMR test.

RESULTS AND DISCUSSION

GC analysis (SP-2100 column) of hexane washes of forage looper female ovipositors exhibited two well-resolved peaks. The first of these two peaks eluted at 27.0 min, between those of standard nonadecane, 19:H (25.4 min), and eicosane, 20:H (27.9 min) while the second component eluted at 29.4 min, between 20:H and 21:H (30.3 min). When the column effluent was split and timed fractions were collected and assayed by EAG, major antennal stimulation was associated with those fractions collected during the elution of these two peaks. The greatest EAG activity was elicited by the first component to elute and its peak area was 20% of the second. Using the more polar SP-2250 column, two peaks were observed with peak ratio of 1:4 and strong EAG activity was associated with their elution. Their elution times were greater from this column relative to hydrocarbon standards; here, the first minor component eluted at 25.1 min between 20:H (24.6 min) and 21:H (27.2 min) and the second eluted at 27.7 min between 21:H and 22:H (29.6 min).

Components having similar retention times were present in considerably greater quantities in methylene chloride extracts of female ovipositors; however, even greater amounts of EAG-inactive impurities were also present, necessitating a preliminary purification. Both EAG-active components migrated as a single band, R_f 0.4–0.6, on plates developed with hexane and were recovered free of the major contaminants which remained near the origin. It was apparent from their TLC migration that neither component was likely to be a long-chain alcohol, acetate, aldehyde, ketone, or epoxide since these compounds remain at or near the origin when hexane is used as the developing solvent. The mobility of the EAG-active components exhibited here is similar to the migration of unsaturated hydrocarbons. Repeated injections of concentrated tip washes and TLC-purified extracts on a wide-bore capillary column led to the separation and purification of the two components.

Both components exhibited similar fragmentations on CI-MS and in each spectrum ions corresponding to $(M - 1)^+$, $(M + 1)^+$, and $(M + 29)^+$ were present, giving molecular weights of 276 for the first component to elute and 290 for the second. Absent from both spectra were ions indicative of alcohol or aldehyde $[(M + 1) - 18]^+$, $[(M - 1) - 18]^+$, and acetate $[(M + 1) - 60]^+$, $[(M - 1) - 60]^+$, functional groups. The base peak m/z 109 and ions of the series $(C_nH_{2n-5})^+$ were observed in each spectrum indicating C_{20} and C_{21} triunsaturated hydrocarbon structures for the two components. Their EI mass spectra were also consistent with these structures, showing molecular ions at m/z 276 and 290 and ions characteristic of polyunsaturated olefins (Zeman and Scharmann, 1972) including the series, 107, 121, etc. $[CH_3(CH_2)_n(CH{=}CH)_3]^+$. Also present were ions at m/z 55, 108, and 220 (C_{20} component) and

at 55, 108, and 234 (C_{21} component). The presence of these ions permitted the tentative assignment of two of the three double-bond positions in each structure.

Several authors (Lee et al., 1970; Blumer et al., 1970; Youngblood et al., 1971; Karunen, 1974) have found that the positions of the outer double bonds in nonconjugated polyunsaturated olefins can be assigned by MS data alone and tentative assignments were similarly made here. One of the pair of outer double bonds was assigned at C_3 for both components based on the presence of ions at m/z 55, $(CH_3CH_2CH{=}CH)^+$, and m/z 108 $[CH_3CH_2(CH{=}CH)_3H]^+$. Ions at m/z 220 and 234, which correspond to $[CH_3(CH_2)_9(CH{=}CH)_3H]^+$ and $[CH_3(CH_2)_{10}(CH{=}CH)_3H]^+$, located the other outer double bond position at C_9 in both compounds (or, counting from the other end of the C_{20} and C_{21} structures, at C_{11} and C_{12}, respectively). The UV spectrum of each component, recorded in pentane, showed no absorption maxima above 210 nm and thereby precluded the possibility of a conjugated system of double bonds (Lee et al., 1970). Thus tentative structures of 3,6,9-eicosatriene and 3,6,9-heneicosatriene were assigned.

The mass spectra, thin-layer mobility, and retention times on SP-2100 and SP-2250 columns of the two insect-derived hydrocarbons coincided exactly with those of synthetic (*Z,Z,Z*)-3,6,9-eicosatriene and (*Z,Z,Z*)-3,6,9-heneicosatriene which were prepared by chain extension of methyl linolenate. From a [^{1}H]NMR spectrum of synthetic (*Z,Z,Z*)-3,6,9-heneicosatriene obtained at 400 MHz, the following chemical shifts and coupling constants were assigned: ($CDCl_3$) δ: 0.85 (t, J = 6.6 Hz, $\underline{CH_3}CH_2CH_2$, 3 H), 0.95 (t, J = 7.2 Hz, $C\underline{H_3}CH_2CH$, 3 H), 1.2–1.4 (m, CH_2, 18 H), 2.0–2.1 (m, apparent sextet, J_{app} 7.2 Hz, singly allylic H, 4H), 2.79 (m, apparent t, J_{app} 6.0 Hz, doubly allylic H, 4H), 5.36 (complex m, apparent septet, HC=C, 6H). These data are in agreement with the published data obtained at lower field strength (Conner et al., 1980).

In the [^{1}H]NMR spectrum obtained from the natural material (ca. 6 μg recovered) also at 400 MHz could be clearly distinguished the symmetrical multiplet of olefinic protons, the pseudotriplet of the doubly allylic protons, and five peaks of the apparent sextet of the singly allylic protons. The correspondence of the peaks of the allylic and vinyl protons in the natural and synthetic materials support the assignment of an all-*cis* configuration for the pheromone component.

The results obtained from EAG assays, flight tunnel observations of male behavioral responses, and field trapping experiments using synthetic (*Z,Z,Z*)3,6,9-20: H and (*Z,Z,Z*)3,6,9-21: H all supported the conclusion that both compounds are pheromone components. EAG responses were recorded using *n*-saturated (C_{12-22}), *Z* and *E* monounsaturated ($C_{18,20}$), (*Z,Z*)-6,9 diunsaturated (C_{18-22}), (*Z,Z,Z*)-3,6,9 triunsaturated (C_{18-22}), and (*Z,Z,Z*)-

6,9,12 triunsaturated ($C_{18,20-22}$) hydrocarbons. Only the 3,6,9 series of hydrocarbons and (*Z*,*Z*)6,9-20:H elicited responses appreciably above background. The greatest antennal stimulation was in response to (*Z*,*Z*,*Z*)3,6,9-20:H; the application of 0.1 μg to EAG filter paper disks resulted in a mean response of 3.8 mV ($N = 6$). The next most potent compound was (*Z*,*Z*,*Z*)3,6,9-21:H which gave an equivalent stimulation at an applied dose of 1.0 μg. This was followed, in decreasing order, by (*Z*,*Z*,*Z*)3,6,9-19:H, (*Z*,*Z*,*Z*)3,6,9-18:H, (*Z*,*Z*)6,9-20:H, and (*Z*,*Z*,*Z*)3,6,9-22:H.

In flight tunnel tests forage looper males exhibited wing fanning, plume-oriented flight initiation, and landing in response to mixtures of (*Z*,*Z*,*Z*)3,6,9-20:H and (*Z*,*Z*,*Z*)3,6,9-21:H. During several preliminary tests using 1:4 (C_{20}-C_{21}) mixtures, as were found present in washes and extracts of female ovipositors, males responded positively to rubber septa containing 250 μg of material. Lures containing either component alone elicited wing fanning; 21% of males tested responded to (*Z*,*Z*,*Z*)3,6,9-21:H while 72% responded to (*Z*,*Z*,*Z*)3,6,9-20:H (Table 1). Not only was there a greater response to the latter component, but the latency in this response (time in seconds between chemical introduction and commencement of fanning) was 31.9 ± 30.9 sec ($N = 22$), significantly less than observed in tests with the C_{21} component, 99 ± 64.3 sec ($N = 10$). Compared with the foregoing responses to single components, a greater fanning response was observed in males (96%) which were exposed to lures containing 1:4 mixtures of C_{20}-C_{21} (250 μg), and the latency in this instance was significantly shorter (14.2 ± 7.7, $N = 40$) than had been observed with (*Z*,*Z*,*Z*)3,6,9-20:H alone. No differences were observed in either the percentage of males responding or the latency of their response (range 12.1–14.5 sec) to other blends and doses which were tested.

When tested individually neither component initiated plume-oriented flight (Table 1). The presence of both components was obligatory for plume-oriented flight initiation, upwind flight, and landing on the baited septum, and there were no significant differences in male responses to the blends and doses tested. In addition to these responses to the synthetic attractant source, males also displayed clasper extension and copulatory attempts with high frequency, behavioral responses consistent with sex pheromone activity. Somewhat over half (50–64%) of the males which initiated plume-oriented flight landed on the source. Those that did not land were observed to exhibit sudden arrestment in their forward flight progress which was followed by looping flight patterns within the plume area and subsequent flight termination. Approximately 70% of flight terminations occurred between 160 and 200 cm upwind of the point of insect release. Such flight behavior may be associated with synthetic lures which have not been optimized with respect to component ratio, release rate, or complement (cf. Palaniswamy et al. 1983). In

TABLE 1. FLIGHT TUNNEL RESPONSES OF *C. erechtea* MALE MOTHS TO (*Z*,*Z*,*Z*)-3,6,9-EICOSATRIENE AND (*Z*,*Z*,*Z*)-3,6,9-HENEICOSATRIENE

Lure formulation (μg)					
3,6,9–20:H	3,6,9–21:H	*N*[a]	Wing fanning (%)[b]	Plume-oriented flight initiation (%)[c]	Landing on source (%)[d]
10	40	56	95a	49a	61a
50	50	35	91a	63a	50a
50	100	42	83ab	57a	55a
50	200	50	96a	52a	64a
50	0	25	72b	0b	
0	200	47	21c	0b	

[a] Total number of moths tested.
[b] Based on total number of moths tested (*N*).
[c] Based on total number of moths which fanned.
[d] Based on total number of moths initiating plume-oriented flight.

addition to the range of component ratios and doses listed here, several others were examined using fewer males per flight tunnel test, but none indicated a change in flight behavior or an increase in response.

Captures of forage looper males in field traps baited singly or with various mixtures of (*Z*,*Z*,*Z*)3,6,9–20:H and (*Z*,*Z*,*Z*)3,6,9–21:H (Table 2) corroborated the flight tunnel observations. Traps baited with either

TABLE 2. CAPTURE OF *C. erechtea* MALES BY TRAPS CONTAINING VARIOUS RATIOS OF (*Z*,*Z*,*Z*)-3,6,9-EICOSATRIENE AND (*Z*,*Z*,*Z*)-3,6,9-HENEICOSATRIENE

Lure formulation (%)		Total males captured	
3,6,9–20:H	3,6,9–21:H	Test A[a]	Test B[b]
100	0		6 e,f
95	5	0	3 e,f
90	10		14 b,c,d,e
80	20	8	21 a,b,c
50	50	10	25 a,b
20	80	6	38 a
10	90		22 a,b,c
5	95	0	2 e,f
0	100		0 f
0	0	0	0 f

[a] 3× replicated; total lure dose 100 μg; Zoecon ICP traps; May 15–21, 1981.
[b] 3× replicated; total lure dose 500 μg; Conor-3CW traps; July 9–August 4, 1981.

component alone captured few or no target males. Greater numbers of males were captured in traps containing mixtures of the two components which ranged from 4:1 to 1:9 (C_{20}-C_{21}). Trap captures in all cases were highly specific for forage looper males. The effect of different applied doses of the two components on male captures was studied in a separate 3× replicated field test (May 19–July 7). In this test traps baited with 60, 200, 600, 2000, and 4000 μg of a 1:1 mixture of the two components captured a total of 40, 59, 95, 68, and 77 males, respectively, none significantly different. In another field test (3 × replicated) additions of 100-μg amounts of third components to a 1:1 blend of (*Z,Z,Z*)3,6,9-20:H and (*Z,Z,Z*)3,6,9-21:H (1.1 mg total applied dose) yielded no significant differences in trap captures; compounds added included, (*Z,Z,Z*)3,6,9-18:H, (*Z,Z,Z*)3,6,9-19:H, (*Z,Z,Z*)3,6,9-22:H, (*Z,Z,Z*)6,9,12-20:H, (*Z,Z,Z*)6,9,12-21:H, (*Z,Z*)6,9-20:H, and (*Z,Z*)6,9-21:H.

The potency of a synthetic attractant [(*Z,Z,Z*)3,6,9-20:H (100 μg + (*Z,Z,Z*)3,6,9-21:H (400 μg)] was assessed by comparing forage looper male captures in female baited and synthetic baited traps (Table 3). In the first test the number of males captured in traps containing the synthetic lure greatly exceeded the number caught in female baited traps. However, in the subsequent test the relative attractancy of females and synthetic lure for target males was similar. At present, we can only suggest that the low number of males which were attracted by females in the first test may have occurred as a result of a low incidence of female calling.

Multiunsaturated hydrocarbons and certain related oxygenated derivatives have, within the past 2–3 years, been identified as sex pheromone components of species from three families of Lepidoptera. (Z,Z,Z)3,6,9-Heneicosatriene was first isolated (Conner et al., 1980) from the arctiid *Utethesia ornatrix* and its 9,10-epoxy derivate, (*Z,Z*)-3,6-*cis*-9,10-epoxyheneicosadiene, together with (*Z,Z*)-9,12-octadecadienal and (*Z,Z,Z*)-9,12,15-octadecatrienal have been established as components of the sex

TABLE 3. CAPTURES OF *C. erechtea* MALES BY TRAPS BAITED WITH VIRGIN FEMALES AND SYNTHETIC LURE

Attractant (μg)	Total males caught	
	Test 1[a]	Test 2[b]
(*Z,Z,Z*)3,6,9-20:H (100) + (*Z,Z,Z*)3,6,9-21:H (400)	53	43
C. erechtea females	9	54

[a] 3× replicated, May 21–June 21, 1981.
[b] 3× replicated, July 13–30, 1981.

pheromone of two additional arctiids, the saltmarsh caterpillar (Hill and Roelofs, 1981) and the fall webworm moth (Hill et al., 1982). The sex pheromone of the geometrid, *Operophtera brumata* (L.), has been found (Roelofs et al., 1982) to be comprised of a structurally related tetraene, (*Z*,*Z*,*Z*)-1,3,6,9-nonadecatetraene. Recently, (*Z*,*Z*,*Z*)-3,6,9-eicosatriene and (*Z*,*Z*,*Z*)-3,6,9-heneicosatriene have been identified as pheromone components of another noctuid species, *Anticarsia gemmatalis*, the velvetbean caterpillar (Heath et al., 1983), which is of the same subfamily as the forage looper.

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CHEMICAL BASIS FOR HOST SELECTION BY *Hemileuca oliviae*: Role of Tannins in Preference of C_4 Grasses

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Abstract—When provided a choice between grass species with C_3 or C_4 photosynthetic pathways, larvae of range caterpillar, *Hemileuca oliviae* Cockerell, selected C_4 grasses. The basis for host selection was examined by conducting analyses of moisture, crude protein, total available carbohydrate, sucrose, glucose, astringency, condensed tannin, silica, and pubescence of 14 grass species, and correlating host plant chemical characteristics with host preference. Most of the variation in host preference was explained by tannin characteristics (astringency and condensed tannin); C_3 grass species had significantly higher tannin levels than C_4 species.

Key Words—Range caterpillar, *Hemileuca oliviae*, Lepidoptera, Saturniidae, C_4 plants, C_3 plants, tannins, grasses.

INTRODUCTION

The selection of food by insect herbivores is a complex process (van Emden and Way, 1973) which is governed by a series of factors including availability, stage of development, physiological state, and morphology of the host plant, as well as behavior, physiology, and ecology of the phytophagous insect. Plant chemistry generally is the most important factor governing acceptance and suitability of foliage (Bernays and Chapman, 1978). Many diverse herbivore–plant interactions mediated by host chemistry are known, and a few general principles that help explain host selection and suitability and the coevolution of plants and phytophagous insects, are beginning to emerge.

Rhoades and Cates (1976) and Feeny (1976), for example, independently

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proposed a general theory of herbivory in which apparent (predictable) plants and plant tissue purportedly utilize digestibility-reducing substances to deter herbivory, while unapparent (ephemeral) plant species and tissue use toxins. The nearly universal importance of nitrogen (protein) and water has recently received renewed interest (Mattson, 1980; Scriber and Slansky, 1981). Also, Caswell et al. (1973) suggest that plant species with the C_4 photosynthetic pathway are less suitable and hence less preferred by insect herbivores because bundle sheath cells reduce access to nutrients. Useful compilations of papers on the basis for host plant selection and suitability appear in Jermy (1976), Wallace and Mansell (1976), Harborne (1978), and Rosenthal and Janzen (1979).

The range caterpillar, *Hemileuca oliviae* Cockerell, feeds on rangeland grasses in New Mexico and adjacent areas. Occasionally it becomes sufficiently abundant on rangeland dominated by blue grama, *Bouteloua gracilis* (H.B.K.) Lag ex Steud., to warrant concern, and extensive suppression programs are initiated. Little is known about other host plants. Capinera (1978) studied the preference of young range caterpillar larvae for various wild and cultivated grasses, and noted that C_4 species were preferred. While preference for C_4 grasses relative to C_3 species is contrary to prevailing ecological theory (Caswell et al., 1973), other species, particularly grasshoppers, are known to feed readily on C_4 grasses (Boutton et al., 1980). From an evolutionary perspective, it would be surprising if some insects were not able to feed on C_4 species or even to specialize on these plants, given the abundance of this resource. What is curious, however, is that no mechanism to explain how herbivores distinguish C_4 and C_3 plants has been proposed. The purpose of this study was to evaluate the basis for host preference in range caterpillar with emphasis on how *H. oliviae* distinguishes C_4 and C_3 grasses.

METHODS AND MATERIALS

Range caterpillar eggs were field collected from Branson, Colorado, and stored at 4° C. Egg masses were exposed to 25° C and 75% relative humidity to stimulate eclosion. For preference tests, hatching larvae were transferred to potted blue grama plants and reared until the 5th instar at 25–27° C. Preference studies determined the quantity of blue grama leaf blade consumption relative to alternate host foliage. Details of the testing procedure and data treatment are provided by Capinera (1978). There were 8–22 replicates of each blue grama–alternate host plant combination. Plant species used in this study were: sudan grass, *Sorghum sudanese* (Piper) Stapf.; corn, *Zea mays* L.; side-oats grama, *Bouteloua curtipendula* (Michx.) Torr.; sorghum, *Sorghum bicolor* (L); wheat, *Triticum aestivum* L.; blue grama, *Bouteloua gracilis* (H.B.K.) Lag. ex Steud.; foxtail millet, *Setaria italica* (L.)

Beauv.; oats, *Avena sativa* L.; Japanese millet, *Echinocholoa crusgalli* var. *frumentacea* (Roxb.) W.F. Wight; barley, *Hordeum vulgare* L.; western wheatgrass, *Agropyron smithii* Rydb.; buffalo grass, *Buchloe dactyloides* (Nutt.) Engelm.; Russian wildrye, *Elymus junceus* Fisch.; and Kentucky bluegrass, *Poa pratensis* L.

For evaluation of host plant preference, a plant preference index was developed (% consumption of blue grama/% consumption of alternate host) and used to assign a relative ranking. Thus, the most preferred host had the lowest index and rank of 1, and the least preferred host had the highest preference index and rank of 14. Blue grama was assigned a preference index value of 1 and a rank of 7.

Caterpillar preference for certain carbohydrates was tested using a simulated foliage assay (Bristow et al., 1979). The assay involved saturating millipore filters with a blue grama extract prepared by extracting 2 g grama foliage with 10 ml hexane for 30 min. After the filters had air dried, 0.1 ml of 2.3% carbohydrate solution was pipetted onto filters and air dried. Check filters received 0.1 ml distilled water. Fifth-instar caterpillar larvae were presented with a choice of carbohydrate-treated and check filters pinned to a cork. Each filter was positioned ca. 1 cm from the cork and filters were in close proximity to one another. A single larva was allowed access to the filters and the test was terminated when ca. 50% of either filter was consumed. This level of consumption usually required 12–24 hr. Area consumed was determined with a LI-COR 3000 area meter. Consumption of check and treated filters was tested for significant differences with Student's t test.

Host plant characteristics determined were photosynthetic pathway, pubescence, moisture, crude protein, total available carbohydrate, sucrose, glucose, astringency, condensed tannin, and silica. Photosynthetic pathways are given by Downton (1975) and Krenzer et al. (1975). Pubescence was described using the terminology of Hitchcock (1935). Moisture content was the difference between fresh weight and oven-dried weight. Crude protein (nitrogen $\times$ 6.25) was determined with a micro-Kjeldahl technique (McKenzie and Wallace, 1954). Total available carbohydrate levels were determined by the Shaffer-Somogyi copper idiometric method as outlined by Heinze and Murneek (1940) and Smith et al. (1964). Glucose was measured using a modification of the total available carbohydrate method in which plant material was extracted with 80% ethyl alcohol instead of sulfuric acid. We assumed that the phenolics would not interfere with the total carbohydrate and glucose assays. Sucrose was determined with the ferricyanide procedure of Ting (1956) and Lechtenberg et al. (1971). Astringency was determined with the protein precipitation method of Hagerman and Butler (1978) using bovine serum albumin as a protein source. An estimate of condensed tannin concentration was determined using Bate-Smith's (1973a) leucoanthocyanin technique. Silica content was determined

with a dry ash method (Piper, 1944). At least three samples of foliage were analyzed for host plant characteristic tests; stem material was not included in the samples.

Plant characteristics, except for photosynthetic pathway and pubescence, were treated as independent variables, with the preference index or preference rank the dependent variable, and subjected to all possible subsets multiple regression analysis. Correlation coefficients were derived for all dependent and independent variables, and Student's t test was used to contrast preferences and host plant characteristics for C_3 and C_4 species, except for pubescence.

RESULTS AND DISCUSSION

Only two test grasses, sudan grass and corn, were significantly more preferred than the dominant natural host, blue grama (Table 1). Several species were consumed about as readily as blue grama: side-oats grama, sorghum, Kentucky blue grass, foxtail millet, buffalo grass, and oats. The other grasses tested were significantly less preferred.

Comparison of fifth instar *H. oliviae* preference rank with that of second instars (Capinera, 1978) indicates some shifts in preference. Buffalo grass, sorghum, and foxtail millet were less preferred as larvae matured. Three cultivated grasses (corn, wheat, oats) increased in relative preference. Differences between second- and fifth-instar preferences may be due to different insect-rearing methods or other factors. Second-instar larvae used in the previous study were reared on artifical diet and, therefore, had not been exposed to grasses prior to evaluation of preference. However, due to survival problems associated with long-term culture of larvae on artificial diet, we elected to rear caterpillars on blue grama prior to testing fifth instars. Jermy et al. (1968) have demonstrated that once insects have fed on a particular plant species, they may choose that species over other potential hosts, a behavior known as induction. However, the shifts in host plant preference observed in this study cannot be attributed solely to induction and may reflect age-related changes in behavior and physiology. Insect host preferences sometimes change with maturity. For example, Bernays and Chapman (1970) determined that change in diet by the grasshopper *Chorthippus parallelus* (Zetterstedt) was due to changes in the grasshopper rather than changes in vegetation. Alternatively, the plants tested may have been qualitatively different due to slightly different culture or age conditions.

These observed shifts are somewhat alarming from the plant protection viewpoint, as wheat, sorghum, and corn are often grown adjacent to blue grama rangeland. Despite the shifts in preference, however, range caterpillar larvae again chose C_4 grasses over C_3 species (Tables 2 and 3). Statistically

TABLE 1. PREFERENCE OF RANGE CATERPILLAR LARVAE FOR BLUE GRAMA AND ALTERNATE GRASS HOSTS

Alternate host	No.	5th instar grass preference[a] (% consumption ± SD) Blue grama	Alternate host		Preference index	Preference rank Instar 5	Instar 2	Mean rank
Sudan grass	10	13.2 ± 8.8	38.7 ± 7.2	**	0.34	1	4	2.5
Corn	18	17.5 ± 18.4	41.8 ± 18.0	**	0.42	2	11	6.5
Side-oats grama	13	22.3 ± 4.1	32.5 ± 22.8	NS	0.68	3	6	4.5
Sorghum	17	25.3 ± 16.8	33.2 ± 21.0	NS	0.76	4	3	3.5
Kentucky bluegrass	15	23.7 ± 13.2	28.9 ± 13.8	NS	0.82	5	5	5.0
Wheat	18	29.5 ± 18.1	30.1 ± 18.5	NS	0.98	6	12	9.0
Blue grama		NT	NT		1.00	7	7	7.0
Foxtail millet	18	24.9 ± 20.1	22.8 ± 17.8	NS	1.09	8	2	5.0
Buffalo grass	21	23.5 ± 15.5	21.5 ± 15.4	NS	1.09	9	1	5.0
Oats	18	27.6 ± 20.4	17.2 ± 15.8	NS	1.72	10	14	12.0
Japanese millet	8	31.4 ± 8.1	14.9 ± 16.2	**	2.10	11	10	10.5
Barley	8	37.0 ± 18.7	15.8 ± 10.3	**	2.34	12	13	12.5
Western wheatgrass	22	39.8 ± 15.9	15.8 ± 18.9	**	2.52	13	9	11.0
Russian wildrye	21	39.7 ± 24.2	11.9 ± 17.7	**	3.33	14	8	11.0

[a] ** *Indicates* $P < 0.01$; NS indicates no significant difference; NT indicates not tested.

TABLE 2. CHEMICAL AND STRUCTURAL CHARACTERISTICS OF GRASSES

Alternate host	Photo-synthetic pathway	Pubescence	Moisture (% ± SD)	Crude Protein (% ± SD)	Silica (% ± SD)	Carbohydrate (% ± SD)	Sucrose (% ± SD)	Glucose (% ± SD)	Astringency[a] (% ± SD)	Condensed tannins[b] (A 554 ± SD)
Sudan grass	C_4	Ciliate margin	76.2 ± 1.2	17.2 ± 0.8	0.9 ± 0.1	7.0 ± 0.2	0	2.2 ± 0.5	2.70 ± 0.4	0.158 ± 0.01
Corn	C_4	Ciliate margin	85.7 ± 0.6	23.5 ± 0.6	4.1 ± 0.0	5.5 ± 0.2	0	3.2 ± 0.5	3.55 ± 0.3	0.132 ± 0.001
Side-oats grama	C_4	Scabrous	68.5 ± 2.2	26.6 ± 1.7	2.4 ± 0.6	4.9 ± 0.1	0.3 ± 0.2	1.7 ± 0.8	3.30 ± 0.5	0.082 ± 0.001
Sorghum	C_4	None	77.4 ± 2.0	15.1 ± 0.2	1.5 ± 0.1	11.1 ± 0.8	1.8 ± 0.1	4.3 ± 0.4	3.35 ± 0.1	0.135 ± 0.01
Kentucky bluegrass	C_3	None	75.6 ± 1.8	23.1 ± 1.8	2.6 ± 0.3	12.3 ± 0.5	5.2 ± 0.4	2.0 ± 0.6	3.60 ± 0.3	0.186 ± 0.01
Wheat	C_3	Ciliate margin	81.1 ± 0.5	17.5 ± 0.9	3.6 ± 0.3	7.5 ± 0.3	2.7 ± 0.5	4.3 ± 0.4	4.90 ± 0.2	0.306 ± 0.03
Blue grama	C_4	Scabrous	63.5 ± 2.5	19.9 ± 0.3	1.5 ± 0.3	7.0 ± 0.8	1.9 ± 0.4	0.9 ± 0.5	1.05 ± 0.2	0.105 ± 0.02
Foxtail millet	C_4	Scabrous	80.1 ± 1.6	15.9 ± 0.9	5.2 ± 0.4	8.5 ± 0.1	0	2.5 ± 0.6	2.25 ± 0.2	0.048 ± 0.01
Buffalo grass	C_4	Pilose	62.0 ± 1.3	16.6 ± 1.0	2.4 ± 0.2	9.1 ± 0.1	4.0 ± 0.6	1.3 ± 0.4	1.65 ± 0.3	0.086 ± 0.01
Oats	C_3	None	85.6 ± 0.9	24.2 ± 1.7	3.1 ± 0.4	12.1 ± 0.5	8.8 ± 1.1	3.3 ± 0.7	3.75 ± 0.3	0.104 ± 0.01
Japanese millet	C_4	None	86.7 ± 2.3	19.4 ± 0.5	6.8 ± 0.7	6.2 ± 0.5	0.4 ± 0.2	2.0 ± 0.5	3.55 ± 0.6	0.044 ± 0.01
Barley	C_3	None	86.4 ± 0.2	22.8 ± 0.2	2.2 ± 0.1	8.2 ± 0.4	1.9 ± 0.6	2.7 ± 0.7	5.20 ± 0.4	0.183 ± 0.01
Western wheatgrass	C_3	Scabrous, ciliate margin	68.8 ± 1.0	20.0 ± 0.8	1.5 ± 0.3	4.9 ± 0.4	0.4 ± 0.1	1.1 ± 04	3.80 ± 0.5	0.141 ± 0.01
Russian wildrye	C_3	None	76.4 ± 0.6	23.0 ± 0.9	1.4 ± 0.2	9.7 ± 0.1	3.7 ± 0.3	3.5 ± 0.6	4.40 ± 0.6	0.126 ± 0.01

[a] Astringency = % mg tannic acid equivalent per mg dry wt of plant material (Bate-Smith, 1973b).
[b] Absorbance at 554 nm.

TABLE 3. ANALYSIS OF RANGE CATERPILLAR RESPONSE TO, AND COMPONENTS OF, C_3 AND C_4 GRASSES

Variable	Photosynthetic pathway, variable mean		Probability
	C_3	C_4	
Preference index, instar 5	1.95	0.93	0.028
Preference rank, instar 5	10.00	5.62	0.048
Preference rank, instar 2	10.16	5.50	0.032
Preference rank, mean	9.25	5.56	0.030
Moisture	79.98	75.01	0.400
Protein, crude	21.76	19.27	0.207
Carbohydrate, nonstructural	9.01	7.41	0.241
Sucrose	3.78	1.05	0.040
Glucose	2.81	2.26	0.374
Astringency	0.85	0.53	0.004
Tannin, condensed	0.17	0.09	0.028
Silica	2.40	3.10	0.075
Sucrose + glucose/astringency	7.97	7.38	0.827

significant differences in preference index and rank (C_4 relative to C_3) were observed. Similar observations were found for preference rank of second instar larvae (Capinera, 1978) and the mean rank of second and fifth instars.

Chemical and structural characteristics of the grasses tested are given in Table 2. Larval host preference (C_4 relative to C_3) was not significantly correlated with total available carbohydrate, moisture, crude protein, or silica levels (Table 3). Pubsecence apparently was unrelated to larval preference.

Although a wide variety of phagostimulatory materials are known for insects (e.g., amino acids, proteins, carbohydrates, salts, vitamins, etc.), sugars are the most common phagostimulants (Singh, 1977). Evaluation of starch, fructosan (inulin), sucrose, fructose, and glucose with the millipore filter test indicated that sucrose was highly phagostimulatory and that glucose was a moderate stimulant. Starch and inulin were feeding deterrents at the concentrations tested. Inulin was chosen as a fructosan in the millipore filter tests because it was the only fructosan that we could obtain commercially; however, grasses characteristically contain levans as their fructosan.

C_4 plants possess the C_4-dicarboxylic acid pathway of carbon fixation in addition to the C_3-Calvin cycle pathway found in C_3 plants (Caswell et al., 1973). The reserve polysaccharides of C_4 and C_3 plants are significantly different. Grasses of tropical or subtropical origin (C_4 plants) store principally starch and sucrose, while grasses of temperate origin (C_3 plants) store principally fructosans (Ojima and Isawa, 1968; Smith, 1968). The difference in

starch and fructosan storage is well known; the differential sucrose storage is much less appreciated.

Smith (1968) provided data on 45 grass species found in North America. In C_4 species, 56% of the nonstructural carbohydrate was found to be starch, while there was less than 5% starch in C_3 plants. Similarly, in C_4 grasses, 39% of the nonstructural carbohydrate was nonreducing sugar (mostly sucrose) while there was 10% nonreducing sugar in C_3 species (difference significant at $P < 0.01$; t test; our analysis). These data suggested that range caterpillar larvae might choose C_4 plants based on the higher content of these species, although the analyses of Ojima and Isawa (1968) did not show significant differences in sucrose levels between C_4 and C_3 species.

Storage carbohydrate analyses generally are conducted on grass stem or root material, but since range caterpillar larvae feed primarily on leaf blade tissue, we tested leaf blade material only. The stem tissue content may not reflect leaf blade carbohydrate because of carbohydrate conversion in the plants. We did not find more sucrose in C_4 leaf blade tissue than in C_3 tissue; rather, we found significantly less. Glucose levels were similar in C_3 and C_4 species. Hence, carbohydrate levels alone do not explain range caterpillar preference for C_4 plants.

Tannin analyses indicated that C_3 species contained significantly higher levels of astringency and total tannin (Tables 2 and 3). The ecological importance of tannins in plants is due to their effectiveness as feeding deterrents and as digestibility-reducing substances. Acid hydrolysis of plant materials to determine an estimate of condensed tannin levels is useful but may be somewhat deceptive because much of the tannin may not be readily available to the herbivore. A more relevant property is astringency (Bate-Smith, 1973a), which was measured in this study from a methanol extract of foliage. Astringency probably is a more valuable measure of the deterrent effects of tannin concentration on host selection by herbivores.

The ratio of feeding stimulants to deterrents could influence host selection; thus, the amount of sucrose and glucose relative to astringency was determined. However, we found no difference in stimulant–deterrent ratios between C_3 and C_4 grasses.

Depending upon the dependent variable employed, a number of independent variables contributed significantly to explaining variance of preference in the multiple regression analysis; however, astringency always was most important. The regression equation which explained the greatest degree of variance (71%) utilized mean preference rank as the dependent variable, although considerable variance was explained even where only preference of fifth-instar larvae was considered (49%) (Table 4).

Tannins are a diverse group of polyphenols that chemically precipitate proteins. Their presence in plant tissue may reduce the availability of plant protein to digestive degradation in the herbivore either by binding directly to

TABLE 4. MULTIPLE-REGRESSION ANALYSIS OF RANGE CATERPILLAR PREFERENCE (DEPENDENT VARIABLE) AND GRASS CHEMICAL CONSTITUENTS (INDEPENDENT VARIABLES)

Dependent variable	Independent variable	Coefficient	*t* statistic	Equation R^2
Preference rank, mean	Astringency	17.6874	4.67	0.71
	Sucrose + glucose/ astringency	0.6688	3.11	
	Condensed tannin	−23.1800	−2.20	
	Nonstructural carbohydrate	−0.7243	−1.94	
	(intercept)	−0.9212		
Preference index, instar 5	Astringency	4.2836	3.03	0.49
	Condensed tannin	−8.4095	−2.26	
	Moisture	−0.0513	−1.55	
	(intercept)	3.5406		

plant proteins or by binding to degradative enzymes. Condensed tannins (e.g., proanthocyanidins) are widespread in vascular plants, while hydrolyzable tannins are restricted to dicotyledons of the angiosperms. Other tannins are of little importance or are poorly known (Swain, 1979). Tannins generally are believed to act as feeding deterrents, although Bernays (1981) points out that insect response to tannins is quite variable, with hydrolyzable tannins sometimes acting as feeding stimulants.

Almost nothing is known about tannins in grasses, and little information on the response of graminivores to tannin concentration is available. Rice and Pancholy (1973) reportedly found condensed tannins in grasses, but we know of no other reports of their occurrence. Bernays and Chapman (1978) suggest that most grasses are devoid of antibiotic chemicals and that graminivorous grasshoppers lack the ability to tolerate antibiotics. Graminivorous grasshoppers are more deterred from feeding on tannin-containing foliage than are polyphagous grasshoppers, although digestibility does not seem to be affected by hydrolyzable or condensed tannins in either graminivorous or polyphagous species (Bernays et al., 1980, 1981). Lepidopterous insects seem to be more affected by tannins than are acridids, but Bernays (1981) accurately points out that there has been little attempt to differentiate tannin-related effects from other naturally occurring processes that affect herbivore performance. Thus, the results of this study are significant in that a number of host-related factors have been considered, and tannin concentration emerged as the most important variable affecting caterpillar preference. Ingested tannin also inhibits range caterpillar development and survival (Roehrig and Capinera, unpublished).

Seasonal and phenological patterns of tannin concentration in grasses are unknown. In oak trees, *Quercus robur* L., tanning concentration increases from 0.5% of dry leaf weight in April to nearly 5.0% in September (Feeny and Bostock, 1968). High concentration of tannin in oak leaves is correlated with a decrease in insect herbivore numbers. In bracken fern, *Pteridium aquilinum* Kuhn, tannin concentration increases with frond maturity (at least in the case of early maturing fronds) while fronds emerging in midseason have significantly less tannin than early- or late-emerging fronds (Tempel, 1981). There is less herbivore damage to bracken with high tannin concentration. Thus, bracken-feeding herbivores also may be influenced by seasonal phenological changes in host plant tannin content. Our preliminary studies (Capinera and Roehrig, unpublished) indicate that there is seasonal variation in tannin concentration in some native grasses, but it is not yet known whether changes in tannin levels are significant enough to result in shifts in host plant preference.

Blue grama is an apparent resource and, according to prevailing theory (Cates and Rhoades, 1977; Feeny, 1976; Rhoades and Cates, 1976), would be expected to utilize digestibility-reducing substances to inhibit herbivory.

Thus, the low levels of tannin found in blue grama foliage are somewhat surprising. However, the anatomy of C_4 plants such as blue grama may compensate somewhat for the lack of chemical defenses, resulting in reduced herbivory. C_4 species supposedly are a poorer source of food for herbivores because most nutritional materials are stored in tough, thick-walled vascular bundle sheath cells which herbivores are not able to break down readily (Caswell and Reed, 1975, 1976). Also, suitable foliage from a predictable or apparent resource may escape in time (Cates, 1980), or there may be another unknown explanation for this anomaly.

It appears that C_3 plants, which are more digestible and which apparently are more preferred by many herbivores (relative to C_4 species), contain higher tannin concentrations, which reduces palatibility. However, the number of grass species examined here was small and includes a number of cultivated varieties which may not be exposed to natural selection. Thus, general consideration of C_4 and C_3 chemical plant defenses awaits further analyses, although this study suggests the presence of different antiherbivore chemical defense systems and the ability of herbivores to distinguish among the tannin concentrations found in grasses.

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MUSHROOM CHEMICAL DEFENSE: Pungent Sesquiterpenoid Dialdehyde Antifeedant to Opossum

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Abstract—A new bioassay employing a natural fungivore, the opossum *Didelphis virginiana*, is described. Using this bioasssay, eighteen species of fungi were tested for palatability. Five species of mushrooms, all of which taste pungent to humans, were found to be unpalatable to the opossum. From the least palatable of these, *Lentinellus ursinus*, the pungent principle was isolated and identified as isovelleral, a previously described fungal metabolite. The compound was shown to be a potent antifeedant to opossums. By means of difference NOE and relaxation time NMR studies, the relative configuration and solution conformation of isovelleral were deduced.

Key Words—Tricholomataceae, *Lentinellus ursinus*, sesquiterpene, antifeedant, bioassay, isovelleral, mushrooms, opossum, *Didelphis virginiana*, pungency, dialdehyde.

INTRODUCTION

Mushrooms are a well-known source of toxins, including emetics, cathartics, hallucinogens, and liver poisons (Lincoff and Mitchel, 1977). Despite the considerable work that has been undertaken to elucidate the chemistry and pharmacology of these substances, their actual function in nature has remained unknown. Work recently done on mushrooms of the family Russulaceae has led to the isolation of a group of sesquiterpenes, including two which are pungent-tasting to humans (List and Hackenberg, 1969; Magnusson et al., 1972; Magnusson et al., 1973). We present evidence

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that the pungent Russulaceae are distasteful to a natural fungivore, the opossum *Didelphis virginiana*. An additional species, *Lentinellus ursinus*, a pungent nonpoisonous mushroom of a different family (Tricholomataceae), is also rejected by the opossum. We report here the isolation and characterization of the compound responsible for this antifeedant activity.

METHODS AND MATERIALS

Opossum Bioassay. Preliminary tests with opossums in captivity has shown these animals to feed readily on commercial mushrooms (*Agaricus bisporus*). Presented with pieces of mushroom, they approached these, seized them in their mouths, and then, with occasional manipulative aid of the front paws, proceeded to eat them in their entirety. An opossum commonly consumed several dozen such items in quick succession.

A measure of the relative palatablity of various wild mushroom species was obtained by means of a feeding protocol used previously in a bioassay with unpalatable insects and thrushes (Eisner et al., 1978). Individual opossums, tested in daily feeding sessions, were offered fresh pieces (approximately $2 \times 2 \times 2$ cm) of a given test species of mushroom, and comparable pieces of *Agaricus bisporus* as the edible control. A total of 18 species of mushroom common to the northeastern United States was collected for testing. Each species was presented in a single feeding session to several (6–10) opossums. Mushroom pieces were presented one at a time in three-item sequences consisting of two pieces of *Agaricus* and one randomly interspersed piece of the test mushroom. The total number of items (test plus control) presented to each opossum per session ranged from 15 to 27.

Results were scored as fate of individual mushroom pieces. If an item was totally consumed, it was scored as eaten; if it was partly eaten, rejected on close inspection (tasted, sniffed, and/or manipulated), or ignored from a distance, it was scored as rejected. If an item at the end of a session was rejected, it was not tallied since the negative response might have been due to satiation of the animal. For each mushroom, the scores from all the opossums were combined to calculate a palatability rating defined as the percent eaten.

For *Agaricus bisporus*, the total number of control items from all tests was used for the calculation. For the screening of purified compounds, we utilized a bioassay similar to the preceding one except that only *Agaricus bisporus* mushrooms were used. These mushrooms were of two kinds: the treated items to which a topical dosage of the compound (dissolved in pentane or hexane) was added, and control items to which the solvent alone was added. In each case the solvent was allowed to evaporate before the item was presented for feeding.

Extraction and Fractionation Procedures. A sample of the fungus was

extracted in a Soxhlet apparatus with pentane, ether, and methanol in succession. At each stage of the procedure, the extracts were tasted by one of us. The pentane extract, which was the sole pungent fraction, was partially crystalline. Recrystallization from hexane yielded a number of nonpungent, saturated fatty acids, while concentrating the pungency in the mother liquors. Subsequent fractionation of the mother liquors on a column of silica gel in hexane with increasing proportions of ether yielded a single, intensely peppery, crystalline compound (ca. 0.15% of the fresh weight of the fungus). This compound could be detected in a dosage as low as 1 μg, dissolved in hexane, placed on a 1-cm disk of filter paper applied to the tongue.

RESULTS AND DISCUSSION

It is evident from the opossum bioassay that *Didelphis* groups the mushrooms we tested into two distinct categories (Figure 1). The five species in the first group were generally rejected. The mean percent palatability ($\bar{X} \pm$ SD) of these mushrooms was 7 ± 7 (range 2–18). The other group, the palatable mushrooms, comprised 13 species whose mean percent palatability was 85 ± 13 (range 56–100). Members of nine families, the Hygrophoraceae, Paxillaceae, Boletaceae, Agaricaceae, Entolomataceae, Lycoperdaceae, Amanitaceae, Strophariaceae, and Tricholomataceae, are generally acceptable while those of the Russulaceae and a single member of the Tricholomataceae are quite unpalatable by comparison.

The unpalatability of the Russulaceae, given their peppery taste and similar chemistry (Andina et al., 1980), was expected. The opossums handled *Lentinellus ursinus* in much the same manner. The fungi were often spat out within seconds, and the animals frequently salivated profusely, frothed at the mouth, shook their heads from side to side, and wiped their muzzles in their fur or on the bottom of the cage. When an unpalatable item was rejected, it was nearly always approached beforehand and apparently sniffed.

This behavior prompted an investigation of the chemistry of *Lentinellus ursinus*. A single, crystalline compound was isolated (see Methods and Materials) which accounted for all of this mushroom's pungency. The physical and spectral data (melting point, optical rotation, proton and carbon-13 nuclear magnetic resonance, infrared, ultraviolet, and mass spectra) of this compound led to its unambiguous identification as isovelleral (Figure 2). Although this compound had previously been isolated from two pungent members of the Russulaceae, *Lactarius vellereus* and *L. pergamenus* (Magnusson et al., 1972), its relative stereochemistry had not been rigorously established. While earlier workers had presented good evidence that the bridgehead protons H-4, and H-10 were *cis* to each other, the orientation of the cyclopropane ring with respect to these protons remained unsubstantiated.

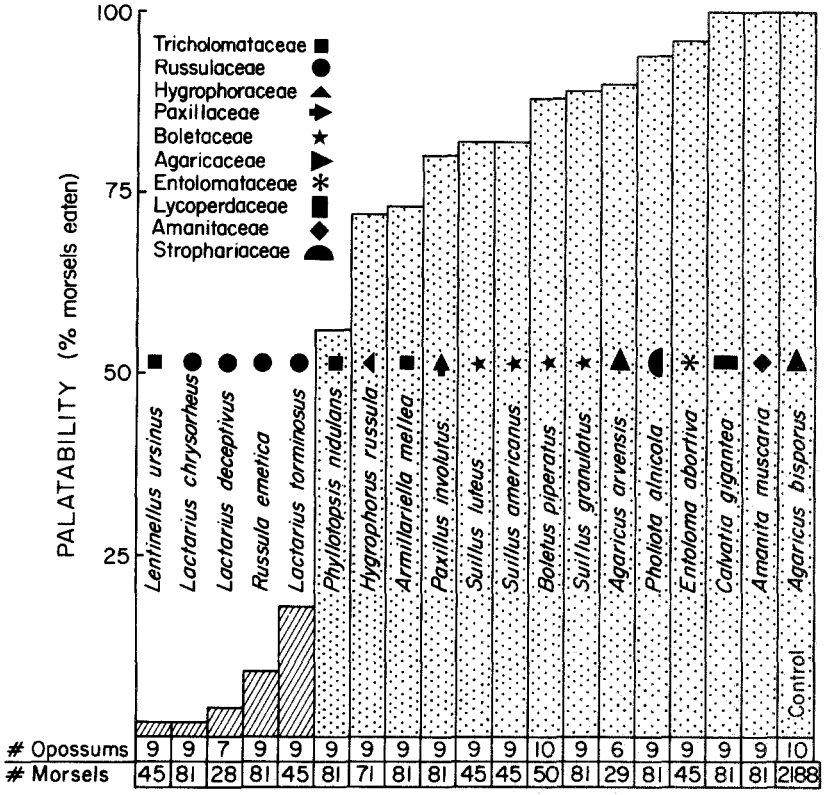

FIG. 1. Palatability of 18 wild mushrooms to the opossum *Didelphis virginiana*. The five least palatable species are shown cross-hatched; the more palatable species by stippling. Baseline figures give the number of opossums fed each species of fungus and the total number of test morsels offered (the controls of all tests are treated as a single category).

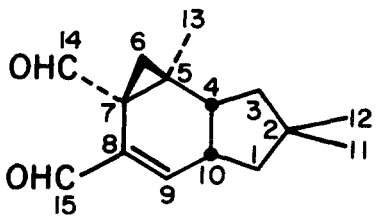

FIG. 2. Isovelleral.

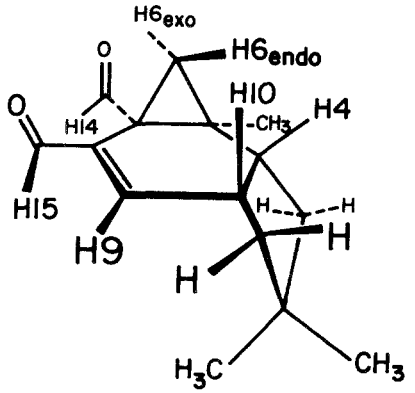

FIG. 3. Conformational formula for isovelleral.

Inspection of Dreiding models of isovelleral revealed that the cyclopropane H-6$_{endo}$ should be quite near both H-4 and H-10 in the proposed structure (Figure 3). As a test of this relationship, we chose to exploit the nuclear Overhauser effect (NOE) in proton NMR spectroscopy (Noggle and Schirmer, 1971). By means of the difference NOE technique (Hall and Sanders, 1980), we were able to show that both H-4 and H-10 contribute significantly to the relaxation of the H-6$_{endo}$ proton. Since the magnitude of this effect is inversely proportional to the sixth power of the internuclear distance, this result demonstrated that all three of these protons were on the same face of the molecule, thus confirming the proposed relative stereochemistry of the compound. (Although the proton NMR spectrum of isovelleral has been reported and assigned by earlier workers, the chemical shifts of H-4 and H-10 are unfortunately coincident in $CDCl_3$ solution. In 2:1 C_6D_6–$CDCl_3$, however, the coincidence is relieved and the chemical shifts of H-4, H-10, H-6$_{exo}$, and H-6 $_{endo}$ are δ 2.18, 2.09, 1.75, and 0.54 ppm, respectively. Irradiation at 0.54 ppm causes enhancements of approximately 28, 8.2, and 4.1% in the integrated intensities of the H-6$_{exo}$, H-10, and H-4 resonances.)

The fatty acid fraction of *Lentinellus* was found to comprise a surprising 16% of the dry weight. Methylation with diazomethane followed by gas chromatographic analysis (10% XF-1150 on Chromosorb W-AW-DMCS, 175° C) permitted the identification of five fatty acids. Relative amounts of these, and of four additional C_{20} acids, were as follows: stearic (10), oleic (3.1), linoleic (2.9), palmitic (1.5), linolenic (1), and higher acids (1.75).

Assays remained to be done with isovelleral itself. The compound was therefore tested with opossums at three dose levels. At the highest dosage (5.8 mg/g mushroom), all of the opossums rejected the treated *Agaricus* after a few

initial trials; only 33% ($N = 36$) of the treated pieces were eaten. At a dosage of 0.19 mg/g mushroom the compound was not deterrent; all of the treated pieces ($N = 90$) were eaten. At the intermediate dosage (1.7 mg/g mushroom) activity was borderline; 78% ($N = 27$) of the treated pieces were eaten.

The dosage at which isovelleral showed 100% deterrency to the opossums is actually higher by a factor of 3.9 than the level (1.5 mg/g) at which we isolated the compound from *Lentinellus*. We feel however, that actual levels of the sesquiterpene in the mushroom might be higher than the isolated yield, since the compound is labile and bound to be lost in part during isolation. Moreover, added to a highly palatable base such as *Agaricus*, isovelleral need not be as effectively deterrent as it is in the natural carrier. At any rate, there can be no question that the compound can account, in part at least, for the unacceptability of *Lentinellus* to opossums. The response of the animals to deterrent levels of isovelleral was indistinguishable from their response to *Lentinellus* itself: rapid rejection was accompanied by profuse salivation, frothing at the mouth, and cleaning of the muzzle.

The finding that *Lentinellus* is rich in fatty acids may itself be significant since long-chain fatty acids could air in the solubilzation of isovelleral in saliva. Indeed, in subjective tests by one of us (S.C.), the combination of isovelleral and stearic acid seemed to induce a stronger peppery sensation, over a more extended zone of the mouth, than isovelleral alone. Other data also suggest that the stimulative potency of isovelleral may be a function of the physical state of the compound. For example, pure crystals of isovelleral, when applied to the tongue, showed little if any pungency, whereas such crystals, when dissolved in hexane and placed on disks of filter paper, were potently and persistently peppery.

Our results demonstrate the mammalian antifeedant activity of a peppery-tasting compound isolated from an unpalatable mushroom. It is probable that all of the mushrooms in the unpalatable group (Figure 1) contain similar sesquiterpenoid dialdehydes which serve as the basis for their rejection by the opossum and the toxicity of some of these species to humans. [At least one other mushroom (*Lactarius deceptivus*) rejected by the opossums also contains isovelleral (A. Lupo and J. Meinwald, unpublished results).] However, not all animals reject peppery mushrooms. The red squirrel (*Tamiascurius hudsonicus*) is partial to *Russula emetica* and *Lactarius piperatus* (Hatt, 1929). Moreover, not even the opossum rejected all peppery muschrooms. In the palatable group, the pungent-tasting *Boletus piperatus* was found to be quite acceptable to the opossums. Studies are presently underway to isolate and characterize the pungent principle of this species.

Interestingly, a number of pungent-tasting isoprenoid dialdehydes has been isolated from a wide variety of natural sources (Kubo and Ganjian, 1981). The biological activity of these compounds is not limited to mammalian

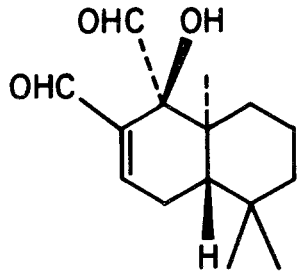

FIG. 4. Warburganal.

feeding deterrency. Warburganal (Figure 4), for example, is antimicrobial, molluscicidal, cytotoxic, antifungal, and antifeedant to various insects (Nakanishi, 1980).

All of the compounds contain neighboring aldehyde and enal functionality. In the case of isovelleral, we have shown these groups to adopt a rigid conformation in solution. The T_1 relaxation time of the H-14 proton (22.6 sec) is exceedingly long relative to that of other protons in the molecule. The corresponding T_1 relaxation time of the C-14 carbon nucleus, (2.06 sec), however, is similar to that observed for the four other methine carbons of isovelleral. These two observations indicate that the two aldehyde moieties of isovelleral must adopt a rigid conformation which keeps the H-14 proton remote from all other protons in the molecule (Martin, et al., 1980). This requirement may be satisfied only if the enal moiety of isovelleral adopts an *s-trans* conformation, an assignment which is supported by the observation of an unusually large NOE at H-9 (27%) on irradiation of the H-15 nucleus. Furthermore, the remaining aldehyde carbonyl at C-14 must adopt a conformation which roughly bisects the cyclopropane ring (Figure 5). This conformation is expected to be favored on theoretical grounds (Hoffmann, 1965), and the unusually low-field chemical shift of the H-6$_{exo}$ proton (1.88 ppm in $CDCl_3$) confirms this assignment. Whether other naturally occurring enal–aldehyde antifeedants adopt similar conformations in solution is not known.

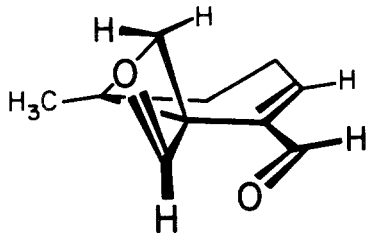

FIG. 5. Preferred conformation of aldehyde groups in isovelleral.

The insect antifeedant activity of such compounds as warburganal shows significant species selectivity (Kubo and Nakanishi, 1979). It is therefore not surprising that *Lentinellus ursinus* is acceptable to some fungivores. The slugs *Limax maximus* and *Arion subfuscus* eat these mushrooms in the laboratory and a number of insects (e.g., mycetophilid flies) can be reared from the fungi. These fungivores have evidently "broken through" the defensive chemical barrier of the mushroom and thereby have succeeded in utilizing a food source rejected by other animals. Such metabolic and behavioral adaptations could set the stage for the evolution of a means of appropriating fungal toxins for the animal's own protection.

Other mushroom compounds that act as antifeedants are likely to be found. The heterocyclic mushroom toxin, muscimol, found in *Amanita muscaria*, causes a delayed illness in opossums resulting in a long-lasting aversion to the mushroom (Camazine, 1983). Many of the classes of secondary metabolites shown to be antifeedant in plants are also present in fungi (Turner, 1971). A number of bitter or pungent mushrooms contain unidentified toxins which cause gastrointestinal upset in humans (Lincoff and Mitchel, 1977). We suspect that these compounds may also protect the mushrooms against natural fungivores.

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REGENERATION AND BIOSYNTHESIS OF DYTISCID DEFENSIVE AGENTS (COLEOPTERA: DYTISCIDAE)

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Abstract—The defensive secretions of the dytiscid species, *Agabus seriatus* (Say) and *Agabus obtusatus* (Say), were qualitatively and quantitatively analyzed by high-pressure liquid chromatography. The intrinsic ability of *A. seriatus* and *A. obtusatus* to regenerate their prothoracic gland defensive secretions under laboratory conditions was determined by analyzing the secretions every seventh day for five weeks. Both beetles regenerated ~80% of their prothoracic gland components within two weeks. *A. seriatus* was injected with [4-^{14}C]cholesterol and after a three-week regeneration period 7.5% of the ^{14}C label was found in the steroidal defensive secretion from the prothoracic glands.

Key Words—Coleoptera, Dytiscidae, *Agabus seriatus*, *Agabus obtusatus*, defensive secretions, steroids, regeneration, pygidial glands, prothoracic glands, cholesterol.

INTRODUCTION

Aquatic beetles of the family Dytiscidae possess paired dermal prothoracic and pygidial defensive glands that secrete agents toxic to other organisms. The prothoracic secretions have been found to contain various C_{18}, C_{19}, and C_{21} steroids and related derivatives, including steroid hormones of the type normally found in higher vertebrates, such as estrogens, androgens and corticosteroids (Schildknecht, 1970; Miller and Mumma, 1976). Using artifical bioassay conditions, the toxicity and anesthetic activity of some of these steroids have been demonstrated with potential predators, such as fish and amphibia (Blunck, 1917; Schildknecht, 1970; Miller and Mumma, 1976). Although steroids are the primary component of the prothoracic secretion,

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other substances have been reported (Schildknecht, 1971, 1976). Other aspects of the biological activity warrant further investigation (Duffey, 1977). The pygidial glands secrete a variety of compounds that possess antimicrobial activity, lower the surface tension of water, act as cell poisons, and possess auxin activity (Schildknecht, 1970; Dettner and Schwinger, 1980).

A vast amount of information has been accumulated on the subject of arthropod defensive secretions (see Duffey, 1977, and Blum, 1978, for recent reviews), but little quantitative information is available on the ability of the arthropods to regenerate their defensive secretions. Averting the momentary attacks of large predators and decreasing the severity of microorganism infestation appear to be the functions of arthropod defensive agents. The composition has been found to fluctuate seasonally (Miller and Mumma, 1974; Newhart and Mumma, 1979; Dettner, 1979), change in captivity (Wheeler et al., 1970) and vary intraspecifically (Eisner et al., 1967; Tschinkel, 1975; Dettner, 1979). Diet, life cycle, temperature, predation pressure, and biased population sampling have been implicated in the observed variation in defensive secretions. Unfortunately, little is known about how or if these factors affect defensive potential. Information on regenerative ability would facilitate evaluation of the intrinsic potential for secretory production.

All insects that have been examined require a dietary or exogenous source of sterol to achieve normal growth, development, and reproduction (Clayton, 1964; Thompson et al., 1973; Svoboda et al, 1975; Downer, 1978). The only exceptions are those species in which a sterol source may be attributed to associated symbiotic microorganisms (Clayton, 1964; Thompson et al., 1973; Svoboda et al., 1975). Cholesterol fulfills the dietary sterol requirement of nearly all insects studied thus far and is the major sterol in body tissues. In vertebrates, steroid hormones are biosynthesized from cholesterol through the intermediary formation of pregnenolone (Sandor and Mehdi, 1979; Lehninger, 1975; Dorfman and Sharma, 1965). It is not surprising that the steroid secretions of dytiscids appear to be derived from cholesterol. Using *Acilius sulcatus* Schildknecht (1970) and Chapman et al. (1977) demonstrated that injected [4-^{14}C]cholesterol was incorporated into the components of the defensive steroid secretion. These studies have only begun to explain how the defensive steroids are biosynthesized.

In this study, the defensive agents of *Agabus seriatus* and *Agabus obtusatus* were analyzed qualitatively and quantitatively over a five-week period to determine their regeneration time. The information obtained was compared with the literature to assess intrinsic regeneration ability and its importance in maintaining peak defensive potential. [4-^{14}C]Cholesterol was injected into *A. seriatus* to determine the percentage incorporation into the defensive steroids and to help identify the presence of minor steroidal components.

METHODS AND MATERIALS

Beetle Collection and Handling. Water beetles were collected, one or two days before beginning an experiment, at night from a spring-fed and watercress (*Nasturtium officinale*) -choked tributary of "Buffalo Run" located three miles north of State College, Pennsylvania. *A. seriatus* was collected from the lotic erosional part of the spring on March 4, 1981, for the regeneration experiment and on February 15, 1981, for the biosynthesis experiment. *A. obtusatus* was collected from the lotic depositional part of the spring on November 13 and 14, 1980.

Collection of Defensive Agent. The prothoracic and pygidial secretions were collected simultaneously by electrical shocking with five 20-mA, 90-V DC, 1-sec pulses, with 5 min between each pulse. Groups of five to six beetles were shocked in 40-ml centrifuge tubes containing 5 ml of tap water. When electrically shocked, the beetles lose normal limb coordination and cannot swim. To allow the beetles to recover, they were removed from the centrifuge tubes after shocking and placed in a Petri dish in the incubator. The number of beetles recovering from shocking was recorded. The secretion process was readily observed because the prothoracic secretion of *A. seriatus* is colored blue and the pygidial secretion is white.

Regeneration Experiment. Ninety-five *A. seriatus* and 115 *A. obtusatus* beetles were randomly arranged five per centrifuge tube and electrically shocked. These aqueous solutions were used to determine the amount of secreted defensive agent in previously unalarmed beetles. To determine the amount of defensive agent remaining in the glands after shocking, 15 *A. seriatus and 25 A. obtusatus* beetles were randomly selected for whole-body extractions of beetles in groups of five.

A ten-gallon aquarium was divided vertically into six parts (10 × 25 cm) with perforated Plexiglas to allow water circulation produced by a bubble filter in the third slot. No beetles were placed in this slot. Rocks and corks were provided as shelter, and four inches of spring water was placed in the aquarium. As the beetles recovered from electrical shocking, they were randomly placed into five divisions of the aquarium (15–17 beetles per division). Every four days the beetles in each division were fed with four cut-up *Tenebrio molitor* larvae.

Sampling was performed every seventh day for five weeks. Beetles were removed from the designated aquarium division and placed in centrifuge tubes for shocking as previously described. Beetle mortality and water pH were recorded throughout the experiment.

Biosynthesis Experiments. Seventy *A. seriatus* beetles were shocked with electricity as previously described. After two days of recovery, the beetles were injected with radiolabeled cholesterol.

Using the method from Chapman et al. (1977), 50 μl of injection solution was prepared by emulsifying 10 μCi of [4-^{14}C]cholesterol (57.9 mCi/mmol, obtained from Amersham Corporation) in distilled water with Tween 20. Suction was used to hold the beetles by the elytra during the injection process. A 100-μl syringe fitted with a 27-gauge needle was used to inject 1 μl (approximately 0.2 μCi) of the injection solution through the intersegmental membrane between the 5th and 6th abdominal sternites. Constant injection volumes were obtained using a microapplicator (ISCO, Inc.) which was calibrated to 1.0 μl. Six sample injection volumes were taken periodically to determine the amount of [4-^{14}C]cholesterol being injected. Using this technique 42 beetles were injected.

After injection, the beetles were placed into a Petri dish in the incubator to allow the hemolymph to seal the injection hole and allow the beetles to recover from the stress of injection. After 30 min, the beetles were transferred to a plastic tray (32 × 16.5 × 9 cm) containing spring water (1500 ml). The beetles were kept in a dark, 10° C incubator and fed 10 cut-up *Tenebrio moliter* larvae every four days. The tray was checked daily for dead beetles. After three weeks, the surviving beetles (30) were removed from the plastic tray and electrically shocked. Whole-body extractions were obtained after the beetles were shocked as previously described.

Extraction of Defensive Agents and Radioactivity. The water in which the beetles were shocked was acidified to pH 2 and extracted three times with chloroform. The chloroform washes were combined, and the solvent was removed by flash evaporation under reduced pressure. Traces of water remaining in the extract were removed by azeotropic distillation with toluene. The extract was transferred to a vial with 3 ml of chloroform. The chloroform was removed under nitrogen, and the extract was redissolved in 200 μl of dioxane prior to high-pressure liquid chromatographic analysis. Radioactivity of each fraction was determined with a liquid scintillation counter. The efficiency of the extraction procedure was followed by determining the recovery of spiked steroid standards.

Pooled samples of whole-body extracts of shocked beetles were obtained using the Folch procedure (Folch et al., 1957). The chloroform layer was evaporated to 1 ml and placed on a Florisil column (750 mg Supelcosil-ATF-120, Supelco Inc., in a Pasteur pipet). The defensive agents were eluted with 2 ml of chloroform and 3 ml of chloroform–methanol (9:1). The column eluant was evaporated under nitrogen to dryness and the residue redissolved in 200 μl of chloroform prior to HPLC analysis. Radioactivity in the insoluble beetle residue was determined by combustion and subsequent liquid scintillation counting of the trapped carbon dioxide.

The water, in which the radiolabeled-beetles lived during the experiment, was filtered through Whatman No. 1 filter paper. Residue (uneaten diet and

frass) remaining on the filter paper was analyzed for radioactivity by combustion. The filtrate was divided into 500-ml aliquots and each aliquot was extracted three times with 250 ml of chloroform. All chloroform and water layers were combined in respective flasks and rotary evaporated under reduced pressure to near-dryness. Aliquots were removed for quantification of radioactivity following dilution of the chloroform and water extract to known volumes.

Quantification of Defensive Agents and Radioactivity. The components of the defensive secretions were fractionated using a model ALC/GPC 244 high-pressure liquid chromatograph equipped with a model 6000A pump, WISP-710B automatic injector and model 440 UV absorbance detector (Waters Associates). The UV detector monitored 254 and 280 nm simultaneously. A 4 mm ID × 30 cm μ Porasil column (Waters Associates) was used with the mobile phase of 87% hexane–12% dioxane–1% methanol at a flow rate of 2.0 ml/min for normal phase separation. A refractive index detector (Waters Associates model R401) was used to monitor cholesterol.

UV absorption was converted into peak area by a Data Module (Waters Associates). Known quantities of pure standards were used to calculate regression equations which were used to translate peak areas into micrograms.

The HPLC eluant of radioactive samples was collected every 15 sec for 25 min. Individual UV absorbing peaks were also collected. The radioactivity in these collected solutions was determined. The purity of [4-^{14}C]cholesterol was determined by injection of 0.08 μCi into the high-pressure liquid chromatograph and analyzing the eluant as described above.

GLC was performed with a MicroTek 220 instrument using flame ionization detection. The chloroform defensive extracts of the intial samples were analyzed using a 1% OV-1 phase on a 100/120 Supelcoport support in a 186 × 4 mm glass column, a nitrogen flow of 60 ml/min, and a temperature of 210° C.

Radioactivity was quantified using a Beckman liquid scintillation counter (model LS8000). ACS (Amersham Corporation) and Scinti Verse (Fisher Scientific Company) liquid scintillation cocktails were used to dissolve the respective aqueous and organic samples. Solid samples were prepared for liquid scintillation assay by combustion in a Packard Tri-Carb oxidizer (model 306). Combustion efficiency was determined by combusting samples that were spiked with 5000 dpm of [^{14}C]benzoic acid.

Characterization of Defensive Agents. Since shocking induced the secretion of the prothoracic and pygidial agents simultaneously, it was necessary to determine the respective origin of each defensive compound by collecting material from individual glands using a capillary pipet and subsequent analysis using HPLC as previously described.

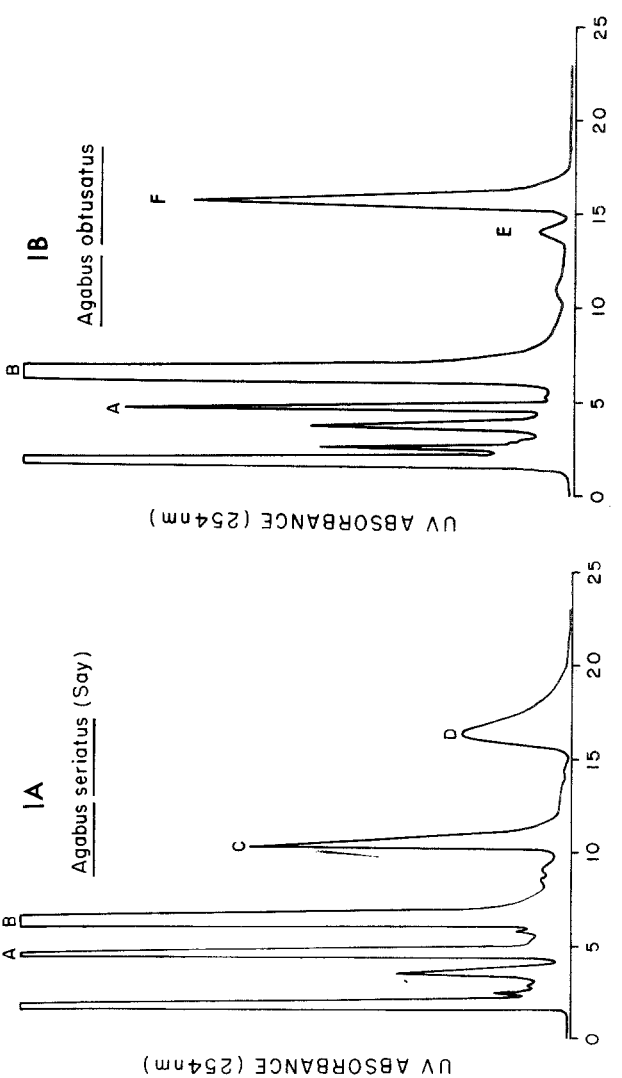

FIG 1. HPLC separation of the defensive compounds from *Agabus seriatus* (1A) and *Agabus obtusatus* (1B) employing a μPorasil column and mobile phase of hexane–dioxane–methanol (87:12:1) at a flow rate of 2.0 ml/min. Peak A, methyl *p*-hydroxybenzoate; B, *p*-hydroxybenzaldehyde; C, deoxycorticosterone; D, unknown III, E, Unknown I; and F, Unknown II.

RESULTS

Quantification of Pygidial and Prothoracic Gland Defensive Agents. High-pressure liquid chromatography of the defensive secretion of *A. seriatus* resulted in the identification of *p*-hydroxybenzaldehyde (PHB) and methyl *p*-hydroxybenzoate (MPHB) from the pygidial glands and deoxycorticosterone (DOC) and unknown III from the prothoracic glands (Figure 1A). Miller and Mumma (1973) reported isolating PHB and DOC from *A. seriatus* following thin-layer and gas-liquid chromatography of the secretions. MPHB and PHB were also isolated from the pygidial glands of *A. obtusatus*, and unknowns I and II were isolated from the pygidial glands (Figure 1B). Unknowns I, II, and III are presumably steroids. The electrical shocking conditions resulted in sufficient beetle survival (6% mortality for regeneration experiments) but only isolated 1.6–20% of the total pygidial gland secretions. Therefore, the pygidial components were quantified following total extraction of the beetles. The composition of the pygidial secretions from beetles collected on February 2, 1981, are presented in Table 1. The smaller *A. obtusatus* (0.5–0.7 cm long) has a much larger titer than *A. seriatus* (1.0–1.2 cm long).

Electrical shocking of the beetles resulted in nearly quantitative recovery of the prothoracic gland components as determined by whole-body extracts of electrically shocked beetles (DOC = 98%, unknown I = 97%, and unknown II = 92%). (Table 2). Considerable variation of titer occurred, especially with *A. obtusatus*. The coefficient of variation for the extraction technique was only 5%, indicating the variation of titer was not due to the extraction technique. Variations of defensive titer of dytiscid beetles have been observed by other investigators (Dettner, 1979; Miller and Mumma, 1973, 1974; Newhart and Mumma, 1979).

Regeneration of Prothoracic Defensive Agents. The ability of *A. seriatus* and *A. obtusatus* to regenerate their respective prothoracic defensive agents, DOC and unknowns I and II, was determined over a five-week period (Table 2

TABLE 1. QUANTIFICATION OF PYGIDIAL GLAND DEFENSIVE AGENTS

Species	Date collected	Samples[a] analyzed	Micrograms/Beetle[b] MPHB[c]	PHB[c]
A. seriatus	2/2/81	3	1.0 ± 0.3	21.7 ± 10.3
A. obtusatus	2/2/81	5	13.6 ± 2.9	64.8 ± 10.9

[a] Five beetles per sample.
[b] μPorasil column, UV absorbance at 254 nm; average ± standard error.
[c] MPHB = methyl *p*-hydroxybenzoate and PHB = *p*-hydroxybenzaldehyde.

TABLE 2. QUANTIFICATION OF PROTHORACIC GLAND DEFENSIVE AGENTS AFTER VARYING TIMES OF REGENERATION

Sampling time[a]	Replicates[b]	DOC (μg)/beetles[c]	Area/beetle[c]	
			Unknown I	Unknown II
Agabus seriatus				
Initial	19	23.8 ± 2.0		
Initial whole body extracts	3	0.6 ± 0.1		
Week 1	3	0.0 ± 0.0		
Week 2	3	11.6 ± 0.5		
Week 3	3	9.9 ± 1.3		
Week 4	2	14.9 ± 1.9		
Week 5	2	14.1 ± 2.3		
Agabus obtusatus				
Initial	15		52.0 ± 8.6	562 ± 91
Initial whole body extracts	5		1.6 ± 0.2	52 ± 10
Week 1	2		29.6 ± 17.6	196 ± 62
Week 2	2		45.0 ± 7.7	485 ± 69
Week 3	2		51.6 ± 21.2	633 ± 60
Week 4	2		57.6 ± 0.2	499 ± 22
Week 5	2		52.1 ± 11.7	427 ± 69

[a] Extracts were obtained after electrically shocking the beetles.
[b] Five or six beetles per replicate.
[c] μPorasil column, UV absorbance at 254 nm; average ± standard error.

and Figures 2 and 3). Eighty percent of the mortality, 12 of 15 beetles with *A. seriatus* and 22 of 29 beetles with *A. obtusatus*, occurred during the first week. No DOC and only 54% and 31% of unknowns I and II, respectively, were regenerated during this first week. By the second week, most of the DOC (80%) and unknowns I (82%) and II (77%) were regenerated, indicating recovery from shocking and adaption to environmental conditions had occurred. DOC and unknown I continued to be regenerated through the third week and leveled off between the fourth and fifth weeks at 15 μg/beetle for DOC and 56 area/beetle for unknown I. Unknown II, however, peaked at the third week at 633 area/beetle and then decreased through the fourth and fifth weeks where it began to level off at approximately 430 area/beetle.

Complete recovery from electrical shocking and adaption to the laboratory conditions did not occur because the beetles did not consistently regenerate their initial prothoracic defensive titer. *A seriatus* regenerated only 63% of the initial DOC. *A. obtusatus* regenerated 100% of unknown I and 113% of unknown II by the third week, but unknown II titer decreased to 77% by the fifth week, indicating that conditions were not optimum. *A. obtusatus* seemed to overcome the stresses of handling and electrical shocking and adapted to the laboratory environment more easily than *A. seriatus*.

The beetles were observed mating in the field and in the laboratory prior to their use in the regeneration experiments. They laid eggs on the underside of corks during the five-week experiments indicating the insects were sexually mature.

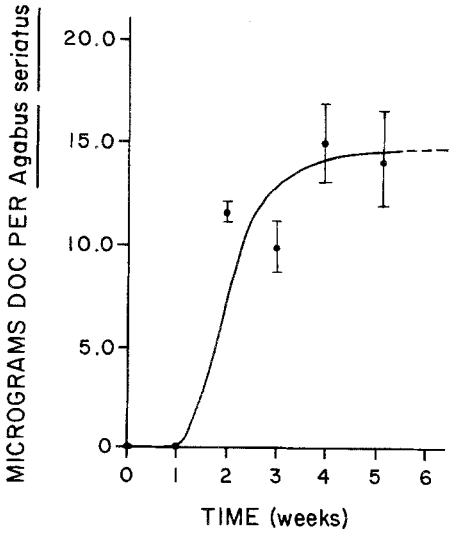

FIG. 2. Regeneration of deoxycorticosterone by *Agabus seriatus*. The bars represent standard error.

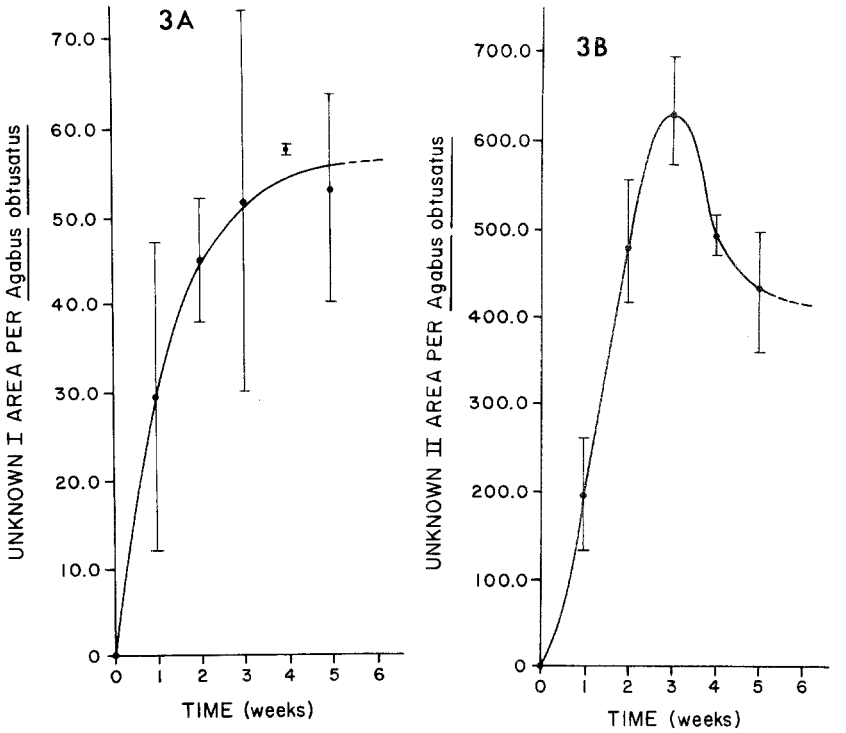

FIG. 3. Regeneration of unknown I (3A) and unknown II (3B) by *Agabus obtusatus*. The bars represent standard error.

The food (*Tenebrio molitor* larvae) was not their natural food but provided an adequate source of cholesterol. The *Tenebrio* larvae were readily eaten and were provided in excess. The beetles were found to eat almost anything they could catch. Dytiscids have been reported to be predators and/or scavengers (Johnson and Jakinovich, 1970).

The pH of the spring water in the aquarium (7.9–8.1) did not vary from the pH at the spring and the temperature used in the experiment (10° C) was the average yearly temperature of the spring. There was no predator pressure, but intraspecific aggression could have occurred and may have been enhanced by crowding in the aquarium.

Incorporation of [^{14}C]*Cholesterol into Prothoracic Defensive Agents.* Based on the results of the five-week regeneration experiment, three weeks after injection of radiolabeled cholesterol into *A. seriatus* was judged to be a satisfactory time to analyze the defensive secretions. This gave the beetles an extra week to recover from the added stress of injection and regenerate their defensive secretion.

TABLE 3. QUANTIFICATION OF DEFENSIVE AGENTS FROM *Agabus seriatus* FOR THREE-WEEK CHOLESTEROL INCORPORATION STUDY

Sampling time	Replicates	DOC (μg/beetle)[a]	Unknown III (area/beetle)[a]
Initial shocking	14[c]	33.3 ± 3.8	675 ± 33
Initial whole-body extracts[b]	3[c]	0.0 ± 0.0	60 ± 6
Final shocking	1[d]	2.5	106
Final whole-body extracts[b]	3[e]	0.0 ± 0.0	3 ± 1

[a] μPorasil column, UV absorbance at 254 nm; average ± standard error.
[b] Extracts were obtained after electrically shocking the beetles.
[c] Five beetles per sample.
[d] Thirty beetles per replicate.
[e] Ten beetles per sample.

To determine the amount of regeneration occurring during the experiment, the prothoracic defensive secretion was analyzed at the beginning (initial) and at the end (final) of the three-week experiment (Table 3). Only 7.4% and 15.7% of the initial DOC and unknown III were present at the end of the experiment. Very little regeneration had occurred relative to the results of the regeneration experiments. Analysis of the whole-body extracts showed all of the DOC and nearly all of unknown III (initial = 91.1% and final = 97.2%) were isolated from the beetles. Apparently the beetles had not recovered or were just beginning to recover from the added stress of injection.

The relative distribution of injected [4-^{14}C]cholesterol in the beetles and substrates coming in contact with the radiolabeled beetles is shown in Table 4. The majority of the injected radioactivity (64.7%) remained in the beetles. The Petri dish used to hold the beetles until they recovered from injection had a relatively large portion of the injected radioactivity (14.8%), indicating that some bleeding did occur. The rest of the radioactivity (11.7%) was found in the cage water (4.3%) and in the uneaten diet and frass (7.4%). Nearly all of the radioactivity in the cage water (91.2%) was found in the chloroform extract, indicating the lipid nature of the radioactivity. Sulfate and glucoside conjugates are the major known steroid excretory products in insects (Thompson et al., 1973).

Of the 42 beetles initially injected with [4-^{14}C]cholesterol, 30 survived to the end of the three-week experiment. The relative distribution of injected [4-^{14}C]cholesterol within the 30 surviving beetles is shown in Table 5. Most of the radioactivity was found in cholesterol (72.6%). The rest was found in the chloroform extract of the aqueous defensive solution (7.4%). Although the quantity of DOC regenerated was small (2.5 μg/beetle or 7.5% of the initial amount), the relative amount of radioactivity incorporated into the defensive

TABLE 4. DISTRIBUTION OF INJECTED [4-^{14}C]CHOLESTEROL IN *Agabus seriatus* AND SUBSTRATES COMING IN CONTACT WITH RADIOLABELLED BEETLES

Component	Radioactivity (μCi)	% of radioactivity injected
Total injected[a]	7.98	
Within insects	5.16	64.7
Surviving beetles[b]	3.99	50.0
Dead beetles[c]	1.17	14.7
External to insects	2.11	26.5
Injection recovery		
Petri dish	1.18	14.8
Cage water	0.34	4.3
Chloroform-extracted water	0.03	0.4
Chloroform extract	0.31	3.9
Diet plus frass	0.59	7.4
Total recovery	7.28	91.2

[a] Based on the average injection of 0.19 ± 0.02 μCi/beetle (42 beetles were injected).
[b] Beetles injected with [4-^{14}C]cholesterol that survived the three week experiment (30).
[c] Beetles injected with [4-^{14}C]cholesterol that died during the three week experiment (12).

TABLE 5. DISTRIBUTION OF RADIOACTIVITY WITHIN 30 SURVIVING *Agabus seriatus* BEETLES THREE WEEKS AFTER INJECTION OF [4-^{14}C]CHOLESTEROL

Component	Radioactivity (μCi)	% of radioactivity injected	% in defensive solution
Total injected[a]	5.70		
Chloroform extract of aqueous defensive solution[b]	0.42	7.4	100
DOC	0.33	5.8	78.6
Unknown III	0.04	0.7	9.5
Cholesterol	0.02	0.4	4.8
Unknowns (6–8 min)	0.03	0.5	7.1
Whole-body extract[c]	4.15	72.8	
DOC	0.01	0.2	
Unknown III	0.00	0.0	
Cholesterol	4.14	72.6	
Unknowns (6–8 min)	0.00	0.0	
Total recovery	4.57	80.2	

[a] Based on the average injection of 0.19 ± 0.02 μCi/beetle.
[b] Obtained by electrically shocking the beetles; pooled sample of 30 beetles.
[c] Extracts were obtained after electrically shocking the beetles; pooled sample of 30 beetles.

solution (7.4%) was larger than any of the previous studies (2.65–4.42%, Schildknecht, 1970 and Chapman et al., 1977). If *A. seriatus* had fully regenerated DOC (33.3 μg) under these conditions, the DOC would have contained nearly 100% of the applied [^{14}C]cholesterol. The distribution of the radioactivity of the HPLC fractionated chloroform extract of the defensive secretion was compared with the UV absorbance (Figure 4). The majority of the radioactivity in the extract (78.6%) was found in DOC. Unknown III contained 9.5% of the radioactivity, confirming its steroidal structure. Radioactivity (4.8%) was also observed in a substance eluting between 6 and 8 min and probably is also steroidal but was unresolved or undetected by HPLC. PHB also eluted during this time period. Decomposition of the steroid nucleus has not been observed in insects (Thompson et al., 1974; Svoboda et al., 1975), and aromatic substances, such as PHB, have been reported to be biosynthesized from acetate or the amino acids tyrosine or phenylalanine (Duffey, 1977; Blum, 1978). When the refractive index response was

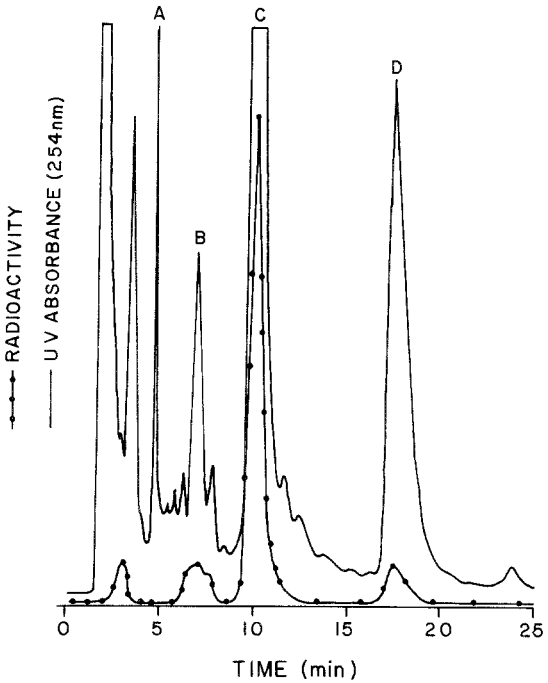

FIG. 4. Distribution of radioactivity in the chloroform extract of the aqueous defensive solution of *A. seriatus* when fractionated with HPLC and compared with the UV absorbance. Peak A, methyl *p*-hydroxybenzoate; B, *p*-hydroxybenzaldehyde; C, deoxycorticosterone; D, unknown III.

compared with the distribution of radioactivity in the HPLC fractionated chloroform extract, 4.8% of the radioactivity was found to chromatograph with standard cholesterol. Gas chromatographic analysis of the chloroform extracts of the initial aqueous defensive solutions showed cholesterol was present in amounts ranging from 1.08 to 4.44 μg/beetle.

DISCUSSION

A. seriatus and *A. obtusatus* have the intrinsic ability to regenerate their prothoracic defensive agents even under laboratory conditions: 80% of the defensive titer was regenerated by the second week. Although *A. seriatus*, *A. obtusatus*, and the majority of arthropods are capable of regenerating their defensive secretions, they do not appear to regenerate them very rapidly (requiring 1–2 weeks) (Blum, 1978). A small number of arthropods regenerate their defensive secretions rapidly (1–2 days), even when the biosynthetic pathway is the same as the "slower regenerators" (Blum, 1978). Regeneration in the wild would be expected to be at least as fast or even faster since the beetles would not have to adapt to a new environment following electrical shocking. This, of course, would be dependent upon sufficient reserves of cholesterol or an adequate dietary source. Frugal emission of defensive secretions is common in arthorpods. Fish and frogs often regurgitate dytiscids alive (Schildknecht, 1970), and frugal emission serves to ensure that adequate quantities of secretion are available if aggressive encounters occur soon thereafter. Frugal emission together with the ability to regenerate their defensive secretion probably enables *A. seriatus* and *A. obtusatus* to maintain peak defensive potential. The observed seasonal variation of defensive titer of *A. seriatus* (Miller and Mumma, 1974) may not be the result of predation pressures but may represent true biochemical capabilities at the time of collection.

A. seriatus incorporated 7.4% of the injected [^{14}C]cholesterol into the prothoracic defensive agents within three weeks. Unfortunately by this time, only 7.5% (2.5 μg/beetle) of the expected regeneration (33 μg/beetle) had occurred. Evidently the beetles require more time to adjust to the stresses of injection. This 7.4% incorporation of ^{14}C label is higher than that reported by Schildknecht (1970) and Chapman et al. (1977) and would have been much higher if the beetles had fully regenerated their biochemical capabilities. An expected benefit of this experiment was the detection of small but measurable amounts of ^{14}C-labeled minor components of the prothoracic glandular secretions and the incorporation of ^{14}C label into DOC and unknown III. Unknown III and the ^{14}C labeled minor components may be precursors or metabolities of DOC. These ^{14}C studies also confirmed the presence of cholesterol in the defensive secretion. Cholesterol was observed to be the

major component in the prothoracic secretion of *Acilius sulcatus* (Chapman et al., 1977), but in *A. seriatus* cholesterol is a minor component. When these beetles are electrically shocked, they often vomit and defecate and this could be the source of the cholesterol. A small amount of cholesterol (0.45 μg) was detected, using gas chromatography, in a capillary collection of the prothoracic gland secretion of five beetles.

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MANDIBULAR GLANDS OF STINGLESS BEES (HYMENOPTERA: APIDAE): Chemical Analysis of Their Contents and Biological Function in Two Species of *Melipona*

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Abstract—Workers of *Melipona fasciata* and *M. interrupta triplaridis* respond to their respective mandibular gland extracts with alarm recruitment and defensive behavior. Workers rapidly exit from the nest entrance, land on an intruding object, and bite with the mandibles while vibrating the flight muscles. These behaviors are accompanied by the release of the contents of the mandibular glands. Colonies of both species exhibited greater response to their own mandibular gland extracts than to those of other stingless bee species. Chemical analysis identified 2-heptanol as the major component in hexane extracts of each species. Undecane was a constituent of both species; skatole and nerol were identified only in extracts of *M. i. triplaridis*.

Key Words—*Melipona*, Hymenoptera, Apidae, mandibular glands, 2-heptanol, skatole, nerol, undecane, alarm response, stingless bees.

INTRODUCTION

A major function of alarm recruitment pheromones of social insects is the release of defensive behavior in the proximity of the nest (Wilson, 1971; Blum and Brand, 1972; Parry and Morgan, 1979). Volatile compounds of biting and stinging Hymenoptera are released from glands associated with the sting apparatus or mandibles of workers (Baroni Urbani, 1979). Groups of workers, many times those specialized for nest defense, respond to the release of alarm pheromones near the colony by leaving the nest, after which additional stimuli may cause them to attack an intruding object (Gary, 1974;

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Blum, 1979); in some species, however, workers are less inclined toward leaving the nest (Johnson and Weimer, 1982). Among the highly social bees, i.e., the honeybees (Apinae) and the stingless honeybees (Meliponinae), alarm pheromones have been identified for all species of *Apis* and for several meliponine species of the tribe Trigonini (Blum, 1966, 1979; Keeping et al., 1982). Here we present the first description of possible alarm substances in the genus *Melipona* and of the inter- and intraspecific alarm recruitment responses to natural extracts. We also consider evolutionary reasons for species-specific responses to substances used in nest defense.

METHODS AND MATERIALS

Studies with natural colonies of *Melipona fasciata* and *M. interrupta triplaridis* (Cockerell) near Gamboa, Panama, indicated pronounced response of workers to colony disturbance (Roubik, in preparation). One colony of each species was used as a source of mandibular glands and in testing colony response to the resultant extracts. For bioassay, eight workers of each *Melipona* species were taken from the nests; the glands were extracted by rotating the mandible 180°, then gently pulling the mandible from the cranium with fine forceps. The resultant two sets of eight glands and mandibles of each species were placed in vials containing 1 ml dichloromethane and refrigerated at 5–10° C. Similar extracts of *Lestrimelitta limao* and *Trigona* (*Scaptotrigona*) *pectoralis* were made from workers collected at their nests. The mandibular gland contents of *L. limao* consists of the two isomers of citral, geranial and neral, and is used during foraging raids on nests of other highly social bees (Blum et al., 1970; Sakagami and Laroca, 1963; Roubik, 1981).

Bioassay of colony response to mandibular gland extracts was made by counting the number of bees leaving the nest and/or biting the control stimulus, followed directly by another count after the presentation of one of the test extracts. The stimulus in both cases was a 7-mm filter paper disk held in place with an insect pin 2.5 cm over a dark blue nylon cloth ball, approximately 8 cm in diameter. Counts of bees leaving the nest were made for 1 min when the stimulus was held 2–3 cm below the nest entrance; the second 1 min count was made after the filter paper was impregnated with 6 μl (approx. 0.1 female equivalents, FE) of a test extract. Bioassays using randomly chosen test substances were made at 15-min intervals on each of 8 days. Tests on *M. i. triplaridis* were made between 0715 and 1000 hr local time, and those on *M. fasciata* between 1330 and 1645 hr. All tests were made in early April 1980.

A second series of bioassays testing the ability of a single worker to elicit a defensive response from nestmates was made by holding a live worker 2 cm

below the nest entrance, not within visual range of the guard bees. Counts of exiting workers were made as in the above bioassay. The control treatment preceding the presentation of the live worker was presentation of the fingers in which the bee was later held.

Chemical analysis was performed with mandibular glands extracted in 1.5 ml of hexane and stored in glass vials with Teflon-lined caps. After two weeks during shipping to the Uppsala University Ecological Station in Sweden, the samples were stored under nitrogen gas at 5° C. Prior to analysis the samples were concentrated to 100 μl by evaporation. Isolation and identification were performed with an LKB 2091 gas chromatograph-mass spectrometer fitted with a 25-m glass capillary column coated with WG-11 stationary phase. The temperature program was set from 50 to 200° C/min after an initial isothermal time of 4 min.

RESULTS

Bioassay. Colony response of *M. fasciata* and *M. i. triplaridis* to test substances is shown in Figure 1. Workers of both species responded maximally to conspecific mandibular gland extracts ($P < 0.01$, Wilcoxon signed ranks test). In addition, these species exhibited a biting response in the presence of their respective gland extracts; the latter was never or rarely observed with other extracts or controls. One to five *M. i. triplaridis* closed their mandibles on the nylon ball in 12 of 16 tests with their mandibular gland extract; the same numbers of *M. fasciata* showed this response to their glandular extract in all of eight tests.

The response of *M. fasciata* to glandular extracts of *L. limao*, *T. pectoralis*, and *M. i. triplaridis* was similar. This response, while significantly less than that to its own glandular extracts, was in all cases greater than the three controls; however, only the response to extracts of *M. i. triplaridis* was statistically higher ($P < 0.05$). Biting behavior was elicited in one or two of eight trials with extracts of each of the other species tested. Thus *M. fasciata* displayed alarm and biting response to mandibular gland extracts of other species, while *M. i. triplaridis* did not.

A single, live biting bee elicited alarm and biting responses from its nestmates for both *Melipona* species. Biting by exiting workers occurred only when a live bee was held under the nest entrance. The biting response was observed in seven of 13 trials with *M. fasciata* and in five of 10 trials with *M. i. triplaridis*. Furthermore, a significant alarm response was recorded in the number of bees leaving the nest after presentation of a live bee for both *M. fasciata* ($\bar{X}$ control = 4.0 bees/min; $\bar{X}$ with live bee = 10.1 bees/min: $P < 0.01$, Wilcoxon test, $N = 13$) and *M. i. triplaridis* ($\bar{X}$ control = 2.9 bees/min; $\bar{X}$ with live bee = 9.9 bees/min: $P < 0.01$, Wilcoxon test, $N = 10$).

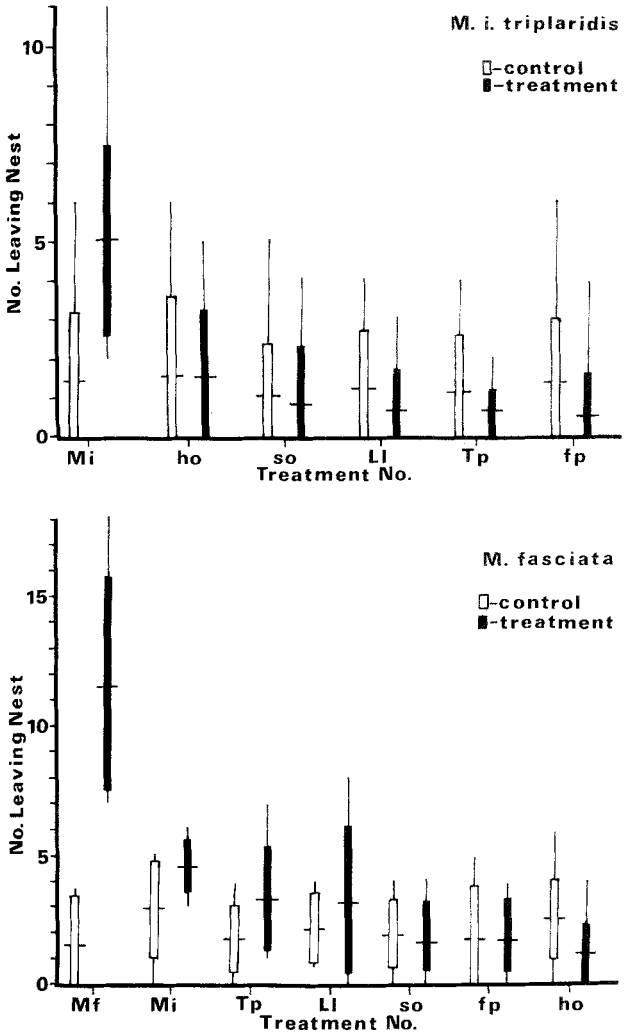

FIG. 1. Alarm response of *Melipona* species to the presentation of test substances at the nest entrance. Horizontal lines represent means, vertical lines the ranges, and boxes the standard deviations of the numbers of bees leaving the nest entrance in 1-min periods after the presentations. Open boxes represent controls; closed boxes represent the response to the test substance. In *M. i. triplaridis* the only test substance which elicited a greater response than the control was its own mandibular gland extract ($P < 0.01$*), which also elicited a greater response than any other test substance ($P < 0.01$**). In *M. fasciata* only its own mandibular gland extract and that of *M. i. triplaridis* elicited a higher response than controls ($P < 0.01$* and $P < 0.05$*, respectively); the former elicited a higher response than any other test extract, and the latter elicited a higher response than the fp, so, and ho tests ($P < 0.01$** and $P < 0.05$**, respectively). Mi, mandibular gland extract of *M. i. triplaridis*; Mf, mandibular gland extract of *M. fasciata*; Ll, mandibular gland extract of *Lestrimelitta limao*; Tp, mandibular gland extract of *Trigona pectoralis*; ho, honey from a *Melipona* nest; so, solvent + filter paper; fp, filter paper; *Wilcoxon signed ranks test; **Kruskal-Wallis test.

Chemical Analysis. Several major components were identified from the mandibular gland extracts. The component corresponding to peak 3 (Figure 2) has a mass spectrum, molecular ion, and retention time identical to that of 2-heptanol. This and other alcohols are common as constituents of the mandibular gland secretions of other species of stingless bees (Blum, 1979).

Only *M. i. triplaridis* exhibited skatole (peak 13, Figure 2). The mass spectrum, molecular ion, and retention time of this component are identical to synthetic skatole. It is conceivable that skatole is present in *M. fasciata*, but in amounts which were undetectable. However, since three separate extracts of each species were analyzed, and the same amount was used in each case (approximately 2 FE), it is reasonable to conclude that skatole is present in much larger quantities in *M. i. triplaridis*. Qualitatively, the odor produced by a biting *M. i. triplaridis* corresponds to skatole, and that of *M. fasciata* is not distinguishable from 2-heptanol.

Several hydrocarbons were present, most notable of which is undecane, which is present in substantial amounts in both species. This hydrocarbon has been reported as an alarm pheromone in several ant species (Parry and Morgan, 1979; Bergstrom and Lovquist, 1971). Nerol is present only in *M. i. triplaridis* and has previously been reported as a mandibular gland component of stingless bees (Johnson and Weimer, 1982).

DISCUSSION

We have presented evidence that two species of *Melipona* respond to mandibular gland extracts with defensive behavior including alarm recruitment from the colony. Skatole, reported here for one species, has not been previously identified in any bee species. Kerr and Lello (1962), however, reported a foul-smelling odor released by workers of some stingless bee species. It is known to repel predators of a trichopteran (Duffield et al., 1977), and the mandibular gland secretion of meliponine bees may function in this context as well.

It is notable, in light of the species-specific response of *M. fasciata*, that the major volatile component, 2-heptanol, is the same for both species. This substance is the major component used in the odor trails used by some *Trigona* species in the recruitment of foragers to food resources (Blum, 1979). In the honeybee, *Apis mellifera*, a freshly excised sting apparatus will elicit a more pronounced response than isopentyl acetate alone, the major component of a multicomponent alarm pheromone (Boch and Shearer, 1966). Grandperrin and Mauchamp (1982) show that a maximum alarm response in this species is elicited by mixtures containing both the major and trace constituents identified in sting extracts. The discrimination may then lie in the presence of quantitative variation in both the major and minor volatile

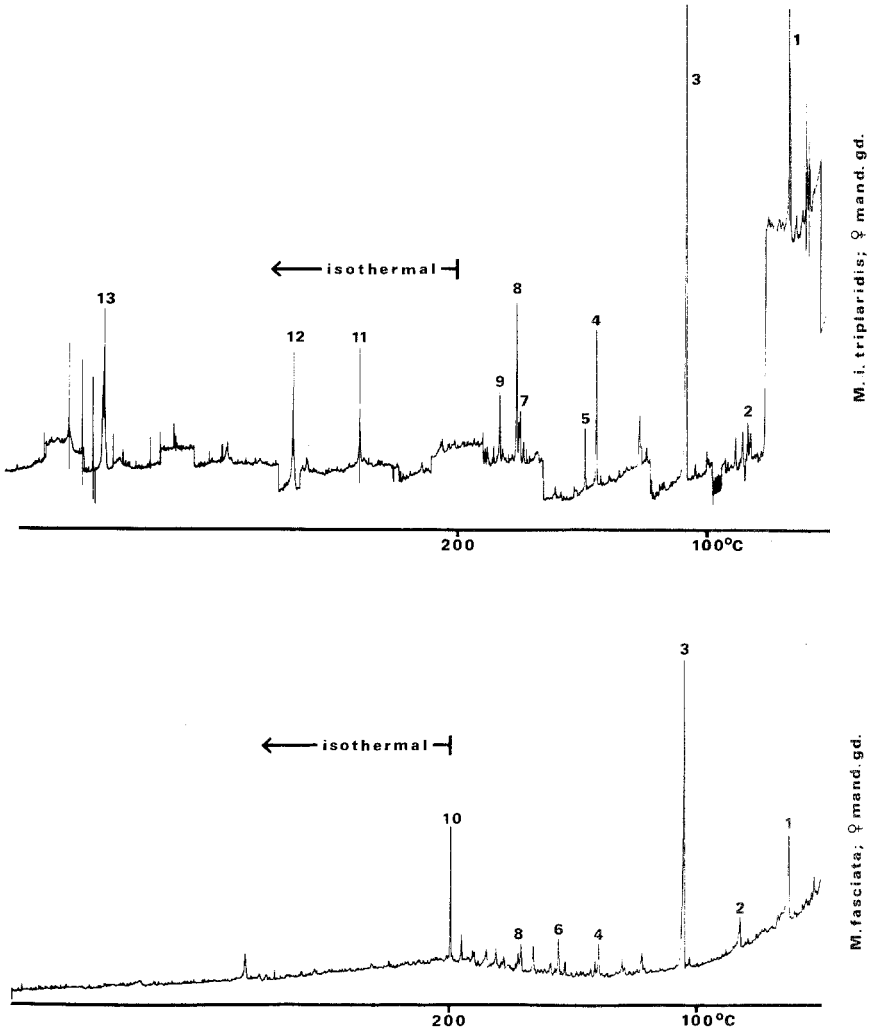

FIG. 2. Capillary gas chromatograms of mandibular gland extracts of *M. i. triplaridis* (top) and *M. fasciata* (bottom). Identification of secondary alcohols was based on the molecular ion, m-18 ion, *m/e* 45 ion, and comparisons with the mass spectra and retention times of synthetic alcohols. Skatole and nerol were identified by comparison of mass spectra and retention times with those of synthetic skatole, nerol, geraniol, and linolool. Peak identification: 1, undecane; 2, undecene; 3, 2-heptanol; 4, 2-nonanol; 5, nerol; 6, hexadecane; 7, 2-undecanol; 8, heptadecene; 9, unidentified aromatic ($m = 234$); 10, contaminant; 11, unidentified aromatic ($m = 234$); 12, ethyl hexadecanoate; 13, skatole.

constituents (Weaver et al., 1975). However, Johnson and Weimer (1982) show that in certain instances workers of *T. fulviventris* are not inclined toward leaving the nest upon presentation of the synthetic analog of the alarm pheromone. In the present investigation then, the observed decrease in response to the nonconspecific animal extracts may be explained either by the lack of trace constituents or by the alteration of the aggressive response by additional compounds. Work in progress with the synthetic compounds should indicate the relative importance of the various constituents, and how they vary within and between stingless bee species.

For four species of *Apis* there are species-specific blends of acetates in the sting-associated glands (Koeniger et al., 1979); the most pronounced defensive response to an extract of these glands is elicited from the species from which the extract is made. The response to these volatiles greatly enhances nest defense (Boch et al., 1962). Explanation of the species-specific response in *Melipona* must take into consideration the behavior of the robber bee *Lestrimelitta limao*. This species raids the nests of some species of stingless bees (Sakagami and Laroca, 1963; Roubik, 1981). During such raids the robber bees release the secretion from the mandibular gland, and this eventually disrupts the ability of the raided bees to muster a defense. It has been reported that some stingless bee species are not normally susceptible to this form of attack (Michener, 1974). It may be that this lowered susceptibility to raids of robber bees is due to the evoluation of lowered or altered responses to citral, the major component of the mandibular gland secretion of *L. limao*, and further development of a specific pheromone blend. Such a mechanism would facilitate the discrimination between nestmates and non-nestmates, and the maintenance of clear communication channels, which are the preliminary requirements in the defense of the nest against this form of attack.

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MUSHROOM CHEMICAL DEFENSE: Food Aversion Learning Induced by Hallucinogenic Toxin, Muscimol

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Abstract—Wild animals eat fungi, yet mushroom poisonings in nature are unknown. The opossum *Didelphis virginiana* readily consumed the toxic mushroom *Amanita muscaria*, became ill, and then developed an aversion to the fungus. Both the illness and the aversion were due, in part at least, to the toxin muscimol. This appears to be the first demonstration of a mushroom chemical defense against fungivores and the first reported role in nature for an hallucinogen.

Key Words—Mushroom, antifeedant, food aversion, muscimol, hallucinogen, opossum, *Didelphis virginiana*, *Amanita muscaria*, plant-herbivore coevolution.

INTRODUCTION

Approximately 350 cases of mushroom poisoning occur annually in the United States, and it is estimated that since 1900, 1500 people worldwide have died from eating toxic mushrooms (Lincoff and Mitchel, 1977). Surprisingly, nothing is known about mushroom poisonings of animals in the wild. Red squirrels (*Tamiasciurus hudsonicus*) eat some species of toxic fungi with impunity (Metcalf, 1925; Klugh, 1927; Hatt, 1929; Fogel and Trappe, 1978). Numerous other mammals eat fungi (Fogel and Trappe, 1978) and undoubtedly encounter potentially toxic mushrooms. Do these animals discriminate between toxic and edible fungi and learn to avoid those species that are poisonous or unpalatable?

In this report I show that the opossum *Didelphis virginiana,* a nocturnal marsupial that eats fungi in the wild (Fogel and Trappe, 1978), eats the toxic

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mushroom *Amanita muscaria* and becomes ill. The animal then develops an aversion to the fungus due, in part at least, to the mushroom's content of muscimol. A γ-aminobutyric acid agonist in the central nervous system of several vertebrates (Chilton, 1978), muscimol is an hallucinogen to humans (Lincoff and Mitchel, 1977; Chilton, 1978). This work appears to be the first demonstration of a chemical defense against fungivores, the first reported role in nature for muscimol, and the first demonstration of a role in nature for a compound hallucinogenic to humans.

METHODS AND MATERIALS

Ten opossums that readily accepted the commercial mushroom *Agaricus bisporus* were used in these experiments. There were four male and six female animals (ages 9 months to 2 years) either born in captivity or obtained as young from the pouch of their mother. They were maintained on a diet of dog food and water. The animals were tested once daily just prior to their regular feeding time.

Experiment 1: Dose-Response Tests with Muscimol. Tests were performed with the toxin muscimol to establish whether this compound could account for the toxicity of *Amanita muscaria.* This mushroom contains two related isoxazoles, muscimol and ibotenic acid. In man both induced symptoms of hallucination, delirium, muscular spasm, stomach upset, and vomiting. Ibotenic acid, however, is labile, readily decarboxylating to form muscimol, and it has been suggested that a significant portion of the activity of administered ibotenic acid may actually be due to muscimol formed secondarily by the decarboxylation reaction (Chilton, 1978). Ibotenic acid was therefore omitted from the tests.

Nine opossums were fed muscimol added to their standard diet of dog food pellets. Six dosages varying between 0.6 and 3.0 mg/kg body weight (1–7 mg/animal) were administered by topically applying the muscimol to a moistened dog food pellet so that the toxin would adhere. There were a total of 34 trials. A dosage of no more than 7 mg was employed in a test for fear of harming the animal. This dosage was chosen as the upper limit as it represents the amount of muscimol potentially available in a single *A. muscaria* mushroom (Chilton, 1978). The oral LD_{50} of muscimol for rats is 45 mg/kg body weight, far lower than that employed in these tests.

Experiment 2: Palatability of Different Mushroom Species. A measure of the relative palatability of various wild mushroom species was obtained in a feeding protocol previously described (Camazine et al., 1983). The assay was performed as follows. Individual opossums, tested in daily feeding sessons, were offered fresh pieces (approximately $2 \times 2 \times 2$ cm) of a given test species of mushroom and comparable pieces of *Agaricus bisporus* as the edible

control. A total of 18 species of mushroom common to the northeastern United States were collected for testing. Each species was presented in a single feeding session to several (6–10) opossums. Mushroom pieces were presented one at a time in three-item sequences consisting of two pieces of *Agaricus* and one randomly interspersed piece of test mushroom. Each item was left with the animal for a maximum of 30 sec. The total number of items (test plus control) presented to each opossum per session ranged from 15 to 27. Results were scored as fate of individual mushroom pieces. If an item was totally consumed, it was scored as eaten; if it was partially eaten, rejected on close inspection (tasted, sniffed, and/or manipulated), or ignored from a distance, it was scored as rejected. If an item at the end of a session was rejected, it was not tallied since the failure to eat might have been due to satiation of the animal. For each mushroom, the scores from all the opossums were combined to calculate a palatability rating defined as the percent of the total number of test items eaten. On a given day each opossum was presented with a single species of test mushroom. There were a total of 35 tests over a period of 5 months.

Experiment 3: Tests with Calvatia gigantea *and Muscimol.* Two additional experiments were performed using muscimol, both utilizing a bioassay similar to the mushroom assay described above except that test items consisted of a mushroom to which a topical dosage of the toxin was added. The mushroom was moistened with water so that the crystalline toxin would adhere to the surface. The control mushrooms to which no toxin was added were similarly moistened.

In the first of these experiments, I attempted to create a food aversion by adding toxic doses of muscimol to the mushroom, *Calvatia gigantea*. In experiment 2, three months earlier, nine opossums had been tested with *Calvatia gigantea* to determine whether it was palatable. Experiment 3 consisted of six trials spaced over 20 days using eight of the same nine opossums. On day 1, seven pieces of the *Calvatia gigantea* each poisoned with 1 mg of muscimol and 14 pieces of the control (*Agaricus*) were presented to the same opossums using the identical protocol as in the previous mushroom tests. The same test was repeated on day 2 except that only the first four of the seven pieces were poisoned as this amount was adequate to make the animals ill. In the remaining trials (days 3, 6, 13, and 20), no muscimol was added to the *Calvatia*.

Experiment 4: Tests with Panellus serotinus *and Muscimol.* The second experiment with muscimol assessed the significance of food novelty. In this case muscimol was applied to the familiar *Agaricus* mushroom on days 1 and 2, rather than to the test mushroom, and presented to the same eight opossums in conjunction with the nonpoisonous mushroom, *Panellus serotinus*, that the animals had never previously eaten. The protocol was identical to that in the *Calvatia*-muscimol experiment.

RESULTS AND DISCUSSION

Experiment 1: Dose-Response Tests with Muscimol. Dose-response tests with muscimol at dosages of 1–3 mg/kg body weight applied to the dog food pellet resulted in vomiting in 79% of the trials. Smaller dosages did not appear to induce any illness.

Experiment 2: Palatability of Different Mushroom Species. Of the 18 mushrooms tested for their palatability, only *Amanita muscaria* and *Calvatia gigantea* were completely eaten by all the opossums. The other fungi ranged in palatability from 2 to 96% (Camazine et al., 1983). At first this result was surprising due to the known toxicity of muscimol. However, within half an hour after ingestion of the first piece of *Amanita muscaria*, six of the nine opossums vomited. The initial acceptance of the *Amanita* mushrooms and the delayed illness induced by the toxin suggested the possibility that the opossums might learn to avoid the poisonous fungus in subsequent tests. The identical test was repeated one day later. The results (Figure 1) show that the acceptability of the *Amanita* decreased markedly from 100% to 17%. Four of the nine opossums refused the *Amanita* completely. The palatability of the

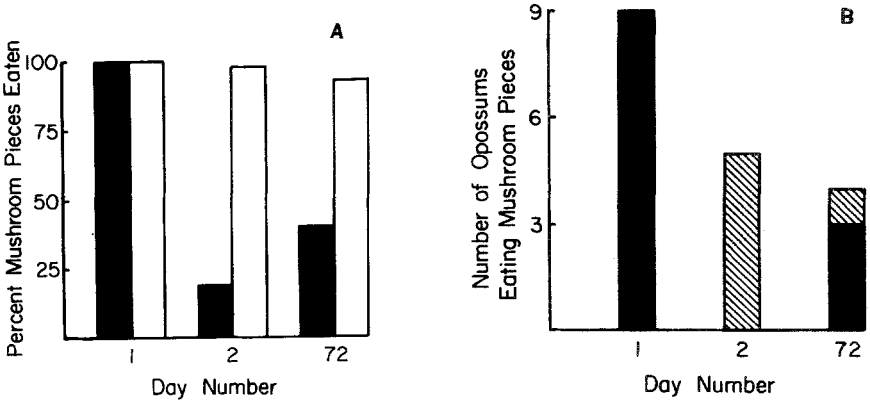

FIG. 1. (A) Palatability of the test mushroom, *Amanita muscaria* (solid bars), and the control mushroom, *Agaricus bisporus* (open bars) during three trials spanning 72 days. Data are expressed as percent total mushroom pieces eaten by all animals. Nine opossums were tested each day and each was fed nine test and 18 control pieces of mushroom on days 1 and 2. On day 72, five test and 10 control pieces were offered. There is significant difference (paired t test statistics) between the number of test pieces eaten on day 1 and day 2 ($P < 0.01$). (B) The same data for the palatability of the test mushroom, *A. muscaria*, expressed as the response of the individual opossums. Solid bars show the number of animals eating all the pieces of the test mushroom, and the cross-hatched bars show the number of animals eating some, but not all, of the pieces presented in the feeding session.

Agaricus control was essentially unchanged (98% consumed). The opossums had evidently learned to avoid the toxic *Amanita* mushroom yet continued to eat the nontoxic control mushrooms.

The animals were retested 70 days later to determine whether the food aversion still prevailed (Figure 1). Only 40% of the mushroom pieces were eaten. Five of the nine opossums again refused the *Amanita* mushrooms entirely (Figure 1B). Other animals also retain lasting food aversions, which may span weeks in the case of the slug *Limax maximus* or even decades for man (Gelperin, 1975; Garb and Stunkard, 1974).

Experiment 3: Tests with Calvatia gigantea *and Muscimol.* In experiment 2, three months earlier, each animal consumed all the pieces of *Calvatia gigantea*, confirming that the mushroom was palatable. On day 1 of experiment 3, all the mushrooms pieces were eaten, and within 75 min of eating the first mushroom piece, seven of the eight opossums vomited. On day 2, in which the identical protocol was repeated except that only the first four of the seven pieces of *Calvatia* were poisoned, 75% of the *Calvatia* pieces were consumed including all the poisoned pieces (Figure 2A). Four of the six animals that ate the *Calvatia* vomited with this dosage. The palatablity of the control mushroom was essentially unchanged (98% consumed). Only 16% of the *Calvatia* pieces were eaten on day 3; five of the animals refused any test mushroom and the remaining three ate some but not all of the pieces (Figure 2B). The palatability of the control mushroom was 75%. In further testing, there was a gradual extinction of the learned aversion; the palatability of the *Calvatia* increased nearly to baseline levels by day 20. This experiment demonstrates that, after two trials, a food aversion to a mushroom can be established using the toxin, muscimol.

In the *Amanita muscaria* and the *Calvatia*–muscimol tests, the mushrooms were initially palatable but were rejected after they had caused an illness in the animals. The opossums may not have associated the illness with the muscimol itself (which may lack a distinctive taste or odor) but instead may have learned to avoid the carrier mushroom which is remembered as the novel food item consumed just before the onset of the illness. The significance of food novelty was confirmed in experiment 4 in which muscimol was applied to the familiar *Agaricus* mushroom, rather than to the test mushroom, and presented to the opossums in conjunction with the novel mushroom, *Panellus serotinus*.

Experiment 4: Tests with Panellus serotinus *and Muscimol.* The results of this experiment (Figure 3) and the *Calvatia*–muscimol experiment are similar. This result would be expected if food novelty is an important factor in the evaluation of potential foods. Both the *Panellus* and the *Agaricus* were initially palatable (*Panellus*, 88%; *Agaricus*, 96%), but after two sessions in which the toxin was applied to the *Agaricus*, the novel *Panellus* mushroom was almost totally rejected (palatability = 2%), while the palatability of the

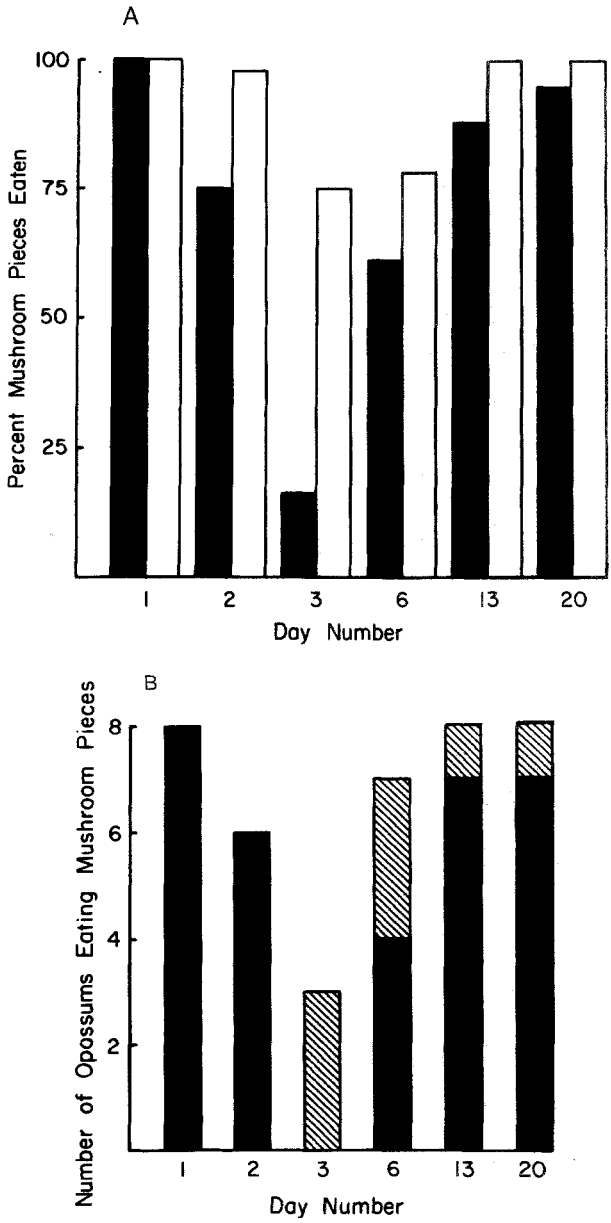

FIG. 2. (A) The palatability of the test mushroom, *Calvatia gigantea* (solid bars), and the control mushroom, *Agaricus bisporus* (open bars) over a period of 20 days. The test mushroom was poisoned on days 1 and 2 by topically applying muscimol. Data are expressed as percent total mushroom pieces eaten by all animals. During each trial eight opossums were offered seven pieces of test mushroom and 14 pieces of control mushroom. There is a significant difference between the number of test pieces eaten on day 1 and day 3 ($P < 0.01$). (B) The same data of the palatability of *C. gigantea* expressed as the response of the individual opossums, drawn as in Figure 1 (B).

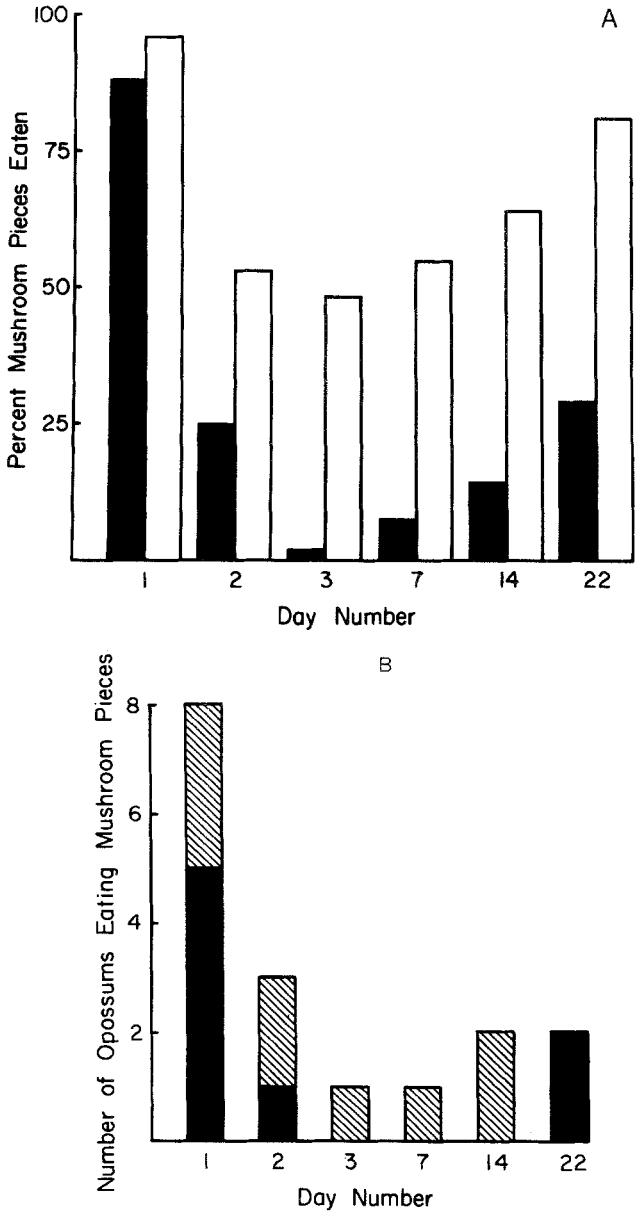

FIG. 3. (A) The palatability of the novel test mushroom, *Panellus serotinus* (solid bars) and the familiar control mushroom, *Agaricus bisporus* (open bars) over a period of 22 days. The *Agaricus* mushroom was poisoned on days 1 and 2 by topically applying muscimol. Data are expressed as percent total mushroom pieces eaten by all animals. The animals used in this experiment and the numbers of mushroom pieces presented in each session are the same as in the *Calvatia*–muscimol experiment except that one animal was not available for retesting on days 14 and 22. There is a significant difference between the number of test pieces eaten on day 1 and day 3 ($P < 0.01$). (B) The same data for the palatability of *P. serotinus* expressed as the response of the individual opossums, drawn as in Figure 1 (B).

familiar *Agaricus* mushroom decreased to 48%. This decrease in the acceptibility of the *Agaricus* control was somewhat unexpected but may be explained by a generalized association of the muscimol-induced illness with all foods presented to the opossums by the experimenters. The animals may begin to associate the entire experimental situation with their previous illnesses and become reluctant to eat under these conditions. Such behavior has been demonstrated in pigeons (Garcia and Hankins, 1977).

Learned aversions to a novel food item associated with a postingestional illness have been demonstrated in a variety of vertebrates including rats, dogs, guinea pigs, turtles, fish, and birds (Domjan, 1977). Polyphagous animals in particular tend to sample novel foods judiciously as if to assess the food for delayed ill effects before eating larger amounts that could be lethal. Deer cautiously taste new plants in small amounts (Nichol, 1938), and wild rats reluctantly sample novel substances (Wallace, 1976).

What sensory cues enable opossums to distinguish among mushroom species? Opossums are nocturnal and possess a keen sense of smell (Moulton, 1973). The distinctive odor of a particular mushroom may be the major sensory stimulus that the animal learns to associate with the toxic effect of a fungus and may serve as an olfactory warning signal (Edmunds, 1974; Eisner and Grant, 1981). Except in a few instances, pieces of mushroom are rejected after being closely approached and sniffed. Occasionally the mushroom is tasted, either being licked or placed briefly in the mouth, chewed, and then spit out. Working with rats, Garcia and his collaborators have shown that when taste and odor are combined to form a conditioning stimulus for a delayed poison, the odor alone may exert a depressive effect upon consumption even after the aversion to taste is completely extinguished (Garcia and Rusiniak, 1980). The adaptive value of this form of learning is apparent; on subsequent encounters, an animal may reject a toxic food on the basis of odor alone and thus avoid repeated, potentially lethal, taste "trials."

These experiments demonstrate that the palatable mushroom toxin, muscimol, causes a delayed illness that effectively conditions a learned food aversion in a vertebrate fungivore. This aversion is striking in that it occurs after a few trials, may be retained for many days, and can be established when the delay between food ingestion and illness is as long as an hour or more. In these respects, food-aversion learning is well suited to meet the demands placed upon a polyphagous animal, which may continually encounter in its environment a variety of novel foods with toxic effects. As an adaptation to poisonous compounds present in a variety of potential foods, the oppossum is able to learn and to retain for long periods the knowledge that a particular fungus is toxic. Some mushrooms, in turn, may have adapted to fungivory by evolving noxious compounds along with distinctive cues (such as tastes, odors, or colors) that facilitate learned food aversions.

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DENSITOMETRIC THIN-LAYER CHROMATOGRAPHIC ANALYSES OF CHOLESTEROL IN *Schistosoma mansoni* (TREMATODA) ADULTS AND THEIR EXCRETORY-SECRETORY PRODUCTS

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Abstract—Densitometric thin-layer chromatographic analysis was used to quantitate cholesterol in 6-week-old male, female, and worm-pairs of *Schistosoma mansoni*, and their excretory-secretory (E-S) products. Males extracted immediately after removal from mice had 1.2–1.5 μg cholesterol/worm, whereas those incubated for 0.5 hr in Earle's balanced solution at 37° C contained 0.8–1.5 μg cholesterol/worm. Females extracted immediately after removal from hosts contained 130 ng cholesterol/worm. Females accumulated considerable cholesterol during incubation and had 420 ng cholesterol/worm at 0.5 hr. Worm-pairs extracted at 0 hr had 1.9–2.8 μg cholesterol/pair and 1.2–1.5 μg cholesterol/pair when extracted at 0.5 hr postincubation. Following incubation for 0.5 hr, males released 12–28 ng cholesterol/worm (average 21), females released 8–13 ng cholesterol/worm (average 11), and worm-pairs released 3–13 ng cholesterol/worm-pair (average 8).

Key Words—*Schistosoma mansoni*, Platyhelminthes, Trematoda, blood flukes, thin-layer chromatography, densitometry, lipids, cholesterol, excretory-secretory products, in vitro maintenance.

INTRODUCTION

Acetone and ether extracts of male adult *Schistosoma mansoni* enhance growth and vitellogenesis of females in vitro, suggesting that male lipophilic

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factors influence the development of females (Shaw et al., 1977). Males of *S. mansoni* maintained in Earle's balanced salt solution released free sterols into the medium within 1 hr postincubation (Fried et al., 1981). Analyses by gas–liquid cromatography (GLC) and thin-layer chromatography (TLC) showed that cholesterol is the only free sterol in *S. mansoni* adults (Meyer et al., 1970; Smith and Brooks, 1969).

Quantitative TLC analyses of cholesterol in *S. mansoni* adults and their excretory–secretory (E-S) products are not available. The purpose of this study was to quantitate cholesterol in extracts of adult *S. mansoni* and in the E-S products released by adults maintained in vitro.

METHODS AND MATERIALS

Pairs of *S. mansoni*, 6 weeks old, were perfused from experimentally infected mice (Imperia et al., 1980) and placed in complete Earle's balanced salt solution (Clegg, 1965). To obtain separate males and females, pairs were separated by gentle prodding (Imperia et al., 1980). Worms were washed rapidly in several changes of Earle's and used within 10 min of necropsy.

To obtain sterol E-S products, males, females, and pairs were incubated 10/tube in 0.2 ml Earle's medium for 0.5 hr at 37° C, and the worm-free incubate was removed for subsequent TLC analyses. The worm-free incubate was extracted in 2 ml chloroform–methanol (2:1) (Folch et al., 1957); the lipophilic layer was used for analysis, and the hydrophilic layer was discarded.

To examine worm sterols, males, females, and pairs were either extracted immediately in chloroform–methanol (2:1) or incubated for 0.5 hr as described above and then extracted. Extraction studies used either 1 or 10 males or worm-pairs per trial. Extraction studies on females used only 10 worms/trial since preliminary studies on single females were spurious.

Prior to analysis, the lipophilic fraction was dried under nitrogen and reconstituted with chloroform–methanol (2:1). The neutral lipid standard 18-4A (Nu-Chek-Prep., Inc., Elysian, Minnesota) and a cholesterol standard (Supelco Inc., Bellefonte, Pennsylvania) were prepared in chloroform and applied to separate lanes of the preadsorbent zone of a LK6DF 20 × 20-cm silica gel plate (Whatman Inc., Clifton, New Jersey) (Berger and Fried, 1982; Perkins and Fried, 1982). Worm samples were applied to separate lanes of the plate. Chromatograms were developed 10 cm from the top of the preadsorbent zone in a glass tank containing 100 ml of petroleum ether–diethyl ether–acetic acid (70:30:1) (Mangold, 1969; Smith and Brooks, 1969). After development, plates were dried and lipids were stained by impregnation with 5% ethanolic phosphomolybdic acid prior to development (Berger and Fried, 1982). Lipid bands were scanned by a Kontes fiberoptic densitometer (K-495000) with baseline corrector and strip-chart recorder (Sonenshine et al., 1981; Perkins and Fried, 1982).

To determine the presence of free sterols other than cholesterol, argentation TLC (Morris, 1966) was done on the sterol fraction isolated preparatively from E-S lipids from males, females, and worm-pairs (Zibulewsky et al., 1982). Plates were developed in the dual solvent system of Skipski et al., (1965).

RESULTS

All worms were live and active following incubation in Earle's medium. The Earle's medium alone was always lipid negative as determined by TLC analysis described in this paper.

A representative chromatogram of worm extracts is shown in Figure 1.

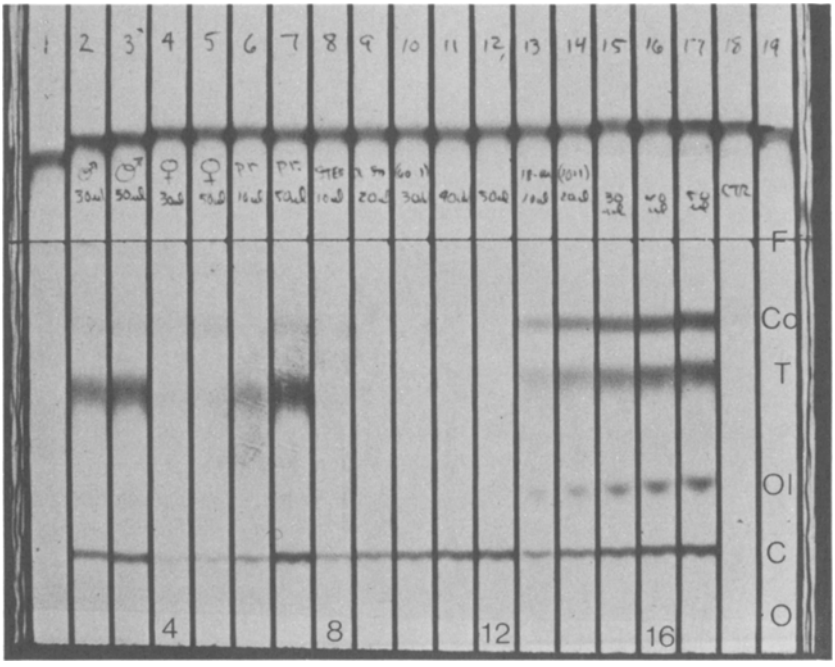

FIG. 1. A photograph of a representative chromatogram showing neutral lipids extracted from 0-hr, male, female, and worm-pairs of *Schistosoma mansoni*. This analysis was based on spotting various aliquots of samples from 10 males, females, and worm-pairs. Lanes 1 and 19 were not used; lane 18 is an Earle's BSS control; lanes 2 and 3 contain male samples; lanes 4 and 5 contain female samples; lanes 6 and 7 contain worm-pair samples; lanes 8–12 contain the cholesterol standard; lanes 13–17 contain the 18-4A standard. Abbreviations: C, cholesterol; CO, cholesterol oleate; F, solvent front; O, origin; Ol, oleic acid; T, triacylglycerols.

TABLE 1. ANALYSIS OF CHOLESTEROL (μg/worm) (MEAN ± SD) OF MALE, FEMALE AND PAIRS[a] OF EXTRACTED *Schistosoma mansoni*

Exp.	No. of trials	No. of worms/trial	Time	
			0 hr	0.5 hr[b]
Male	2	10	1.50 ± 0.707	1.53 ± 0.420
Male	4	1	1.23 ± 0.208	0.79 ± 0.465
Female	2	10	0.13 ± 0.140	0.42 ± 0.007
Pairs	2	10[c]	2.83 ± 1.910	1.16 ± 0.071
Pairs	4	1[c]	1.88 ± 0.457	1.45 ± 0.208

[a]For pairs, cholesterol in μg/worm-pair.
[b]Worms incubated for 0.5 hr in Earle's medium at 37°C prior to extraction.
[c]Worm-pairs/trial.

As reported previously (Smith and Brooks, 1969; Fried et al., 1981), the major neutral lipid fractions in adult *S. mansoni* are cholesterol and triacylglycerols. Results of densitometric TLC analyses of worm extracts are presented in Table 1. Males extracted at 0 hr contained 1.2–1.5 μg/worm of cholesterol, whereas those extracted at 0.5 hr postincubation contained 0.8–1.5 μg/worm. Females accumulated cholesterol during incubation and at 0.5 hr contained more than 3 times the weight of cholesterol of fresh worms. Pairs contained 1.9–2.8 μg of cholesterol/pair at 0 hr and 1.2–1.5 μg of cholesterol/pair at 0.5 hr, suggesting that worm-pairs release or utilize cholesterol during incubation.

The major lipophilic E-S product of adult worms was cholesterol. A representative linear calibration curve of cholesterol E-S products from 10 worm-pairs is shown in Figure 2. Results of cholesterol release at 0.5 hr in two trials using 10 males, females, or worm-pairs per trial were as follows: Males released 12–28 ng/worm (average 21); females released 8–13 ng/worm (average 11); and worm-pairs released 3–13 ng/worm (average 8).

Argentation TLC showed that cholesterol was the only free sterol present in E-S products of males, females, and worm-pairs. The R_f values were ergosterol, 0.21; stigmasterol, 0.26; cholesterol, 0.28; and coprostanol, 0.34.

DISCUSSION

Previous studies have reported cholesterol as the only free sterol in *Schistosoma mansoni* adults (Smith and Brooks, 1969; Meyer et al., 1970). GLC analysis has identified cholestane in *S. mansoni* adults (Smith and

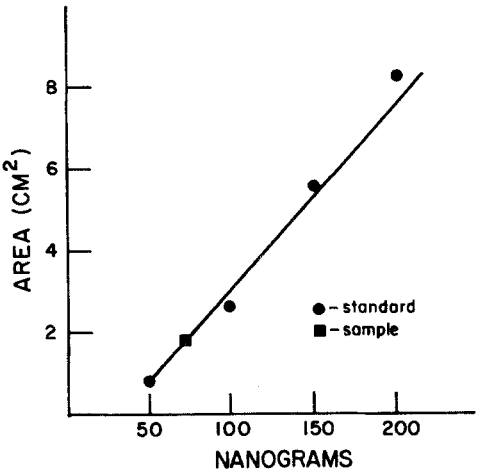

FIG. 2. A representative calibration curve showing the interpolation of *Schistosoma mansoni* cholesterol E-S products from cholesterol standards. In this trial 75 ng of cholesterol were released from 10 worm-pairs (7.5 ng/worm-pair) following 0.5 hr of incubation.

Brooks, 1969). In TLC studies with the Mangold (1969) solvent system, this steroid would migrate with or near the solvent front (Fried et al., 1980). In our study, argentation TLC of *S. mansoni* E-S sterols showed only cholesterol.

A recent study has reported the presence of ecdysteroids in *S. mansoni* adults (Torpier et al., 1982). As mentioned previously (Fried et al., 1980), ecdysones would remain at the origin in the Mangold (1969) solvent system.

Our studies report for the first time weights of cholesterol in adult *S. mansoni* and show that males contain about 10 times the amount of cholesterol found in females. Extracts of males and worm-pairs contained less cholesterol after 0.5 hr of incubation, suggesting, that males and worm-pairs release or utilize this steroid within 0.5 hr in vitro. During 0.5 hr of maintenance, females accumlate considerable amounts of cholesterol, and the significance of this finding is unclear at present. Previous studies indicate that de novo synthesis of cholesterol does not occur in *S. mansoni* adults (Meyer et al., 1970).

Worms incubated in vitro released 3–28 ng/worm in 0.5 hr with males releasing considerably more cholesterol than either females or pairs. These findings suggest that low nanogram amounts of cholesterol probably influence growth, development, and behavior of *S. mansoni* adults. The significance of lipophilic factors in growth and development (Shaw et al., 1977) and in chemoattraction (Imperia et al., 1980) of *S. mansoni* has been discussed.

In previous trematode studies, accumulation and release of neutral lipids has been associated with either the excretory or digestive system. In *Fasciola hepatica* and in *Echinostoma revolutum*, neutral lipids accumulate in the excretory system and are released via the excretory pore (Burren et al., 1967; Fried et al., 1980). In the brachylaimids, *Leucochloridimorpha constantiae* and *Amblosoma suwaense*, neutral lipids accumulate in the intestine and are released from the mouth (Fried and Shapiro, 1975; Fried and Robinson, 1981). We have no evidence that either the excretory or digestive system of *S. mansoni* is involved in neutral lipid accumulation and release. Studies are in progress to associate particular structures involved with the accumulation and release of neutral lipids in *S. mansoni*.

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INSECT SEX PHEROMONES: Determination of Half-Lives from Formulations by Collection of Emitted Vapor

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Abstract—A new method for determining half-lives for the evaporation of sex pheromones from formulations has been developed. A previously unexploited mathematical relationship makes possible the determination of half-lives of over 1000 days by collecting evaporated pheromone for 1–4 hr and measuring the amount collected and the amount left in the formulation. Purified nitrogen flows over the test material and the evaporated pheromone is qualitatively collected on an adsorbent of 80–100 mesh silica gel silanized with octadecyltrichlorosilane. Quantitation is by gas chromatography. Quantitative mass balance was achieved and half-lives determined by this new method are in agreement with half-lives previously determined by measuring the amount of pheromone left in septa after a sequence of time intervals. Previously undetermined half-lives for acetates and alcohols are reported.

Key Words—Insect attractants, pheromone formulations, pheromone evaporation rate, sex pheromones, Lepidoptera pheromones, measuring pheromone evaporation, silanized silica gel.

INTRODUCTION

In the last several years the structures of sex attractants for a large number of economically important lepidopteran pests have been reported (Klassen et al., 1982). The primary uses for sex attractants are in survey and monitoring traps and for population suppression by mating disruption. For either one of these applications, the formulation is critical to success. Conrel® hollow fibers (Ashare et al., 1975) and Hercon® plastic laminates (Kydonieus et al., 1976) are examples of commercially available formulations which have proved

useful for mating disruption. Polyvinyl chloride (PVC) pellets have also proved useful for this purpose (Daterman, 1974, 1982; Fitzgerald et al., 1973). In addition, these three formulations have been used to control pheromone release rates in survey and monitoring traps. Also, rubber septa are widely used for this purpose.

To obtain maximum efficacy from formulations, one needs quantitative knowledge of all the factors which affect evaporation rates of pheromones from given formulations. Besides aiding in the development of efficacious formulations, such information would be useful for studies of insect behavioral response to pheromones. Such extensive information will require a large number of determinations of evaporation rates.

Several methods for collecting and analyzing pheromone vapors have been reported. (Byrne et al., 1975; Cross et la., 1980; Baker et al., 1981; Wiesner and Silk, 1982; Weatherston et al., 1982; Leonhardt and Moreno, 1982; Meighen et al., 1982; Szittner et al., 1982). These methods utilized solvents, glass, or polymeric powders to adsorb pheromone vapors, and gas chromatography, scintillation counting, or bioluminescence for quantitation.

In previous work (Butler and McDonough, 1979, 1981), we determined evaporation rates from rubber septa for acetate and alcohol pheromones to provide information on the relationship between chemical structure and evaporation rates. Our previous method was too slow to be practical for the determination of effects of temperature and air movement. Consequently, we sought a faster method.

The evaporation of pheromnes from PVC pellets, Hercon laminate dispensers, and natural rubber septa is first order (Daterman, 1974; Leonhardt and Moreno, 1982; Butler and McDonough, 1979). For a first-order evaporation, a mathematical relationship exists which makes possible a considerably more rapid determination of half-life by measuring the amount evaporated during a discrete time period and the amount left in the formulation (McDonough, 1978). We have developed a method based on this principal which utilizes a novel adsorbent which is not hygroscopic and collects pheromone vapors quantitatively. Collected pheromone is then determined by gas chromatography.

METHODS AND MATERIALS

Preparation of Adsorbent

The method of Kingston and Gerhart (1976) was used to synthesize the octadecyl silane surface of the silica gel.

The silica gel (Baker's analyzed reagent; catalog No. 5-3405, 60–200 mesh) was screened. The 60–80 mesh fraction was retained and dried for 12 hr

at 100°C. Then 150 g of it was transferred to a 2-liter round-bottom flask equipped with a magnetic stirrer. After 500 ml of toluene and 45 g of octadecyltrichlorosilane (Aldrich Chem. Co., Milwaukee, Wisconsin) were added, the mixture was refluxed and gently stirred for 14 hr. If the mixture is not stirred continuously, the silica gel surface may not become adequately silanized. After the mixture was cool, the solvent was decanted and 150 ml of toluene was added, swirled, and again decanted. The silanized silica gel and remaining solvent were filtered by vacuum through a sintered-glass filter. The silica gel was washed three times with toluene. After most of the toluene was removed, the silica gel was added to 300 ml of acetonitrile–water (1:1) and stirred for 2 hr at room temperature.

The solvent was decanted, and the silica gel was transferred into a fritted glass funnel and washed with water. The silica gel was dried for 12 hr at 110°C and transferred to a 2-liter flask. Then 500 ml 10% trimethylchlorosilane in toluene was added, and the mixture was stirred and refluxed for 2 hr. The solvent was decanted after the mixture was cooled, and the silica gel was again washed with toluene and then with methyl alcohol in the same manner as above. The silanized silica gel was dried in air for 3 hr at 110°C.

Preparation of Adsorbent Column

The glass tube for the adsorbent and test rubber septum was 27 cm long (including an inner and outer standard taper 24/40 connector) and 20 mm in diameter. It was packed with 6 cm of the adsorbent (8 g) held in place with two plugs of silanized glass wool. Materials in the adsorbent packing that would give gas chromatographic peaks were removed by washing with 300 ml of dichloromethane (Mallinckrodt, Omni HPLC grade). The last 100 ml was concentrated to 1 ml on a rotary evaporator and analyzed by gas chromatography. If peaks were present, the column was washed with another 100 ml of dichloromethane which was also concentrated and analyzed. This procedure was continued until no GC peaks were obtained. Usually 300 ml of dichloromethane was sufficient. This procedure was repeated with methanol; usually 200 ml was enough. Finally the column was washed with 200 ml of dichloromethane.

Then a column of similarly conditioned adsorbent was connected to the inlet of the adsorbent column and the remaining solvent was removed by vacuum until the supports were dry, about 1.5–2.0 hr. The first column prevents contamination of the adsorbent column by ambient vapors. If the adsorbent is not adequately dried, it will not quantitatively adsorb evaporated pheromone. When the column is not in use, the precolumn is left on and the ends are covered with aluminum foil to avoid adsorption of ambient vapors.

Determination of $t_{1/2}$

The following items, in order, were connected in series to a tank of high-purity nitrogen: tube of molecular sieve (6 Å), rubber tubing, a flowmeter (Gilmont Instruments, distributed by Van Waters and Rogers, Seattle, Washington), rubber tubing, a precolumn 20 mm in diameter packed with 6 cm of the adsorbent (8 g) (to assure that the incoming nitrogen is adequately pure), and the adsorbent column. The test substrate, usually a rubber septum impregnated with a pheromone or pheromone analog was placed in the unfilled section of the adsorbent column. The nitrogen flow rate was set at 2.5–3.0 liters/min (linear velocity of N_2 = 13–16 cm/sec). The collection period was 1–4 hr. The longer period was used for the less volatile compounds. After the collection, the apparatus was disassembled and the septum was extracted with 50 ml of dichoromethane by shaking for 1 hr in a 125-ml Erlenmeyer flask. Then the extract was decanted into a 200-ml volumetric flask; the extraction flask was washed several times with dichoromethane, and the washes were added to the volumetric flask and made up to volume. The adsorbent column was extracted twice with 100 ml of dichloromethane. The second 100 ml was used as a check to ensure complete removal of the test compound. Each 100 ml was concentrated to less than 1 ml with a rotary evaporator and made up to volume in a 1-ml volumetric flask. The adsorbent column was dried as before and was then ready to use again. The solutions were analyzed with a Hewlett Packard model 5710A gas chromatograph equipped with two flame ionization detectors. The analytical columns were 1.8 m × 6 mm OD silanized glass; one was packed with 5% SE-30 on 80/100 mesh Gas Chrom Q® and the other with 5% Carbowax 20 M on 80/100 mesh Gas Chrom Q.

Preparation of Rubber Septa

Test compounds were impregnated in natural rubber septa (West Co.) in 100 μl or more of dichloromethane. Sufficient solvent (100 μl or more) is needed to ensure nearly quantitative penetration of the test compound. Even then, a small percent of test compound might be left on the surface of the septum. Because this surface material would evaporate faster than the impregnated material, the septa were allowed to age for a few days to ensure that the loss rates did not include surface-deposited material. Aging periods were 1 or 3 days or more for 10- and 12-carbon alcohols and acetates, and 6 days or more for 14- and 16-carbon alcohols and acetates.

Mass Balance Experiments

For these experiments separate glass tubes were used for the adsorbent and the test substrate. This allowed the walls of the sample tube to be

extracted and analyzed separately from the adsorbent. The test compounds were made up in a dichloromethane solution so that the desired amount would be contained in 0.10 ml. The solution of test compound was added dropwise from a 0.10-ml pipet to a microscope slide. The solvent from each drop was allowed to evaporate before the next drop was added. The microscope slide was immediately placed in the chamber, and the experiment was conducted as previously described.

Theoretical Factors

Derivation of Equation for Calculating Evaporation Rates. The analytical method is based on the assumption that the pheromone will be released from the formulation by a first-order evaporative loss. For rubber septa, this is well established by our previous studies (Butler and McDonough, 1979, 1981).

For a first-order loss, the rate of loss is proportional to the amount present in the septum. The differential equation for this relationship is:

$$-\frac{dP}{dt} = kP \tag{1}$$

where P is the amount of pheromone present at time t in a septum, and k is a proportionality constant. The following substitutions may be made (McDonough 1978):

$$R = -\frac{dP}{dt} \tag{2}$$

$$k = \frac{\ln 2}{t_{1/2}} \tag{3}$$

Here R is the instantaneous rate of loss of pheromone, ln is the natural logarithm, and $t_{1/2}$ is the half-life. Then:

$$R = \frac{P \ln 2}{t_{1/2}} \quad \text{or} \quad t_{1/2} = \frac{P \ln 2}{R} \tag{4}$$

Therefore, equation (4) can be used to determine $t_{1/2}$ by measuring P and R. The instantaneous rate of loss (R) cannot actually be measured, but it can be approximated by determining the amount lost, $-\Delta P$, during a discrete time period, Δt, i.e.:

$$\mathrm{R} \approx -\frac{\Delta P}{\Delta t} \tag{5}$$

The percentage error introduced by this approximation will be greater the larger the $\Delta P / P$ ratio is, and this ratio will be larger the shorter the half-life. Since $t_{1/2} = 2$ days is the minimum $t_{1/2}$ that we expect to determine ($t_{1/2} = 2.2$

days for (*E*)-5-decen-1-ol; Butler and McDonough 1981), this value may be used to estimate the maximum error introduced by the assumption of equation (5).

The integral form of equation (1) is

$$P = P_o \exp(-kt) \tag{6a}$$

or by substitution for k from equation (3)

$$P = P_o \exp(-t t_{1/2}^{-1} \ln 2) \tag{6b}$$

Here P_o is the amount initially present in the septum (when t = o) and P is the amount present at the time t. For a half-life of 48 hr and an initial dosage of P_o = 5000 μg/septum, we would have left after t = 2 hr of evaporation:

$$P = 5000 \exp[-(2)(48)^{-1} \ln 2] = 4857.66 \ \mu g$$

The difference between P_o and P is the amount that would evaporate and be collected during 2 hr, which from equation (5) gives R = 71.17 μg/hr. Then using this value of R, and P = 4857.66 μg, and equation (4), one would calculate $t_{1/2}$ = 47.3 hr instead of 48.0 which is an error of 1.5%.

This error could be reduced further by collecting for a shorter time period, e.g., 0.5 hr, and this would be practical because of the relatively high evaporation rate. Since a 1.5% error is well within experimental error, equation (4) can be used to determine $t_{1/2}$.

Effect of Apparatus on Evaporation Rates. In an enclosed tube, evaporative and condensation processes may occur that do not occur in an open environment. The rate of loss in an enclosed space may be different from that in an open environment because of these additional processes.

In an open environment three processes must be considered. They can be summarized as follows:

$$P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} P_v \xrightarrow{k_3}$$

where P and P_v are, respectively, the concentration of the pheromone in the rubber septum and in the vapor near the septum; k_1, k_2, and k_3 are, respectively, the rate constants for (1) loss from the septum, (2) recondensation into the septum, and (3) movement to a position where recondensation does not occur. Here the rate of evaporation will be:

$$-\frac{dP}{dt} = k_1 P - k_2 P_v + k_3 P_v$$

Thus, the processes $k_1 P$ and $k_3 P_v$ contribute to the loss of pheromone from a septum, while $k_2 P_v$ decreases the rate of loss. In an enclosed glass tube, two additional processes may occur:

$$P_v \underset{k_5}{\overset{k_4}{\rightleftharpoons}} P_g$$

where P_g is the amount of the pheromone on the wall of the glass tube, and k_4 and k_5 are, respectively, the rate constants for condensation onto and evaporation off the glass wall. Here the rate of evaporation will be:

$$-\frac{dP}{dt} = k_1 P - k_2 P_v + k_3 P_v + k_4 P_v - k_5 P_g$$

If the measured rates of evaporation are to correspond to actual use situations, the process $k_5 P_g$ must not occur to a significant degree because it decreases the rate of evaporation (therefore it increases $t_{1/2}$). k_3, which is determined by the diffusion and convection of air, must be large enough to suppress k_4 because $k_4 P_v$ makes $k_5 P_g$ possible. A flow rate of 2.5 liters/min appears to fulfill this requirement.

As the flow decreases, a point will be reached where $k_5 P_g$ will influence the evaporation rate. If there were no flow (i.e., a static system), the then closed system would continuously approach and eventually reach an equilibrium state in which the values P, P_v, and P_g would be constant. If one were to measure evaporation rates with such a system, the rates would vary as the collection period varied (i.e., a 0 to 2-hr collection period would give a higher rate than a 0 to 4-hr collection period). If conditions of measurement were standardized, a closed system might be adequate for determining relative rates of test materials, but would not be adequate for absolute rates.

RESULTS AND DISCUSSION

Test for Mass Balance. Table 1 shows the mass balance for compounds of varying volatility after evaporation from a glass plate and collection by the adsorbent in the collection tube. The sample size was 10.0 μg for each compound. In addition 55 μg was used for 10:OH because all of the 10-μg sample evaporated during the test period. The uniformly high recoveries establish the high efficiency of collection. For the alcohols Z7–12:OH and Z11–14:OH, the amount found on the sample chamber walls was 7–8% of that found in the collection tube. Consequently, the $k_4 P_v$ and $k_5 P_g$ processes were not completely suppressed. Our next experiments indicated that this 7–8% level is adequately low.

Determination of $t_{1/2}$ of Compounds in Septa. The validity of our new method was further tested by determining and comparing $t_{1/2}$ values with values determined by our previous method (Butler and McDonough, 1979, 1981) as shown by the top four entries in Table 2. Over the range of $t_{1/2}$ = 2.09–662 days, the agreement between the two methods is very satisfactory. The confidence limits of the two methods overlap for three of the compounds (E5–10:OH, Z9–14:Ac, and Z11–16:Ac) and are close for the other one (393 vs. 410 for Z11–16:OH). Also, the agreement between the two methods shows

Table 1. Determination of Mass Balance of Compounds Volatilized from Glass Plate in Sample Chamber (Air Speed = 2 Liters/Min; 1 Hr/Determination)

Compound	Amount collected (μg) By column	Wall of sample chamber	From plate	Recovery (%)
10:OH[a]	9.91	0	0	99
	32.24	0.59	24.26	103
Z7-12:OH	5.33	0.44	3.77	95
Z11-14:OH	0.73	0.05	9.30	101
Z11-16:OH	0.24	0.0	9.43	97

[a]The letters after the colon indicate functional group: OH, alcohol; Ac, acetate. The number between the dash and colon indicates the number of carbon atoms in the longest continuous chain. The letters and numbers before the dash indicate the configuration and position of the double bonds.

that k_4P_v and k_5P_g are not affecting the $t_{1/2}$ values appreciably. If they were, the new method would give substantially longer $t_{1/2}$ values than the previous method.

Our previous method of determining evaporation rates from septa, based on measuring the amount of pheromone left in septa after time intervals, assumed that pheromone was lost by evaporation alone, and chemical decomposition did not occur to a significant extent. This conclusion was based on the following facts. The only apparent, potential decomposition reaction of the saturated acetates would be hydrolysis to the alcohol. If this

Table 2. Half-Lives ($t_{1/2}$) of Compounds of Varying Volatility Determined by Both Previous and New Methods at Room Temperature (22-23° C)

Compound	New method average $t_{1/2} \pm$ SE	Previous method average $t_{1/2}$ and 95% confidence limit
E5-10:OH	2.09 ± 0.06	2.2, 2.2-2.3
Z9-14:Ac	189 ± 6	202, 189-216
Z11-16:OH	379 ± 14	432, 410-456
Z11-16:Ac	662 ± 14	635, 525-801
E8,E10-12:Ac	46.5 ± 1	47.1, 40.4-56.5
Z9,E12-14:Ac	305 ± 11	319, 285-361
Z7,Z11-16:Ac	169 ± 9	
Z7,E11-16:Ac	159 ± 14	
Gossyplure[a]		159, 137-187

[a]Gossyplure is a 1:1 mixture of Z7,Z11-16:Ac and Z7,E11-16:Ac.

reaction occurred, the alcohol would be detected during GC analysis, but none was ever found. The monoene acetates had the additional potential of decomposing via air oxidation of the allylic C–H bonds. If this reaction occurred to a significant degree, the monoenes would be uniformly lost from septa at a higher rate than the saturated analogs. Instead, most of the monoenes had $t_{1/2}$ values about the same as the saturated analogs. The most sensitive of the unsaturated compounds to oxidative degradation are the conjugated and methylene interrupted dienes. We measured three of these (*E*8, *E*10–12:OH, *E*8,*E*10–12:Ac, and *Z*9,*E*12–14:Ac), and all three had half-lives equal to or larger than the saturated analogs. Gossyplure with ethylene-interrupted double bonds would be more stable than these compounds, and we therefore attributed its short half-life (relative to the saturated analog) to its effective molecular size as controlled by its most common conformations rather than to chemical decomposition. With the development of our new method for determining $t_{1/2}$, it became possible to test these conclusions directly. The half-lives of *E*8,*E*10–12:Ac, *Z*9,*E*12–14:Ac and gossyplure (*Z*7,*E*11–and *Z*7,*Z*11–16:Ac) determined by both methods are given in Table 2. In all three cases, the agreement between the two methods is completely satisfactory. This result validates our previous determinations by our former method.

New Determination of $t_{1/2}$ of Compounds in Septa. The new determinations of $t_{1/2}$ are summarized in Table 3. The five dodecen-1-ols have

TABLE 3. NEWLY DETERMINED $t_{1/2}$ VALUES BY VAPOR COLLECTION METHOD

Compound	$t_{1/2} \pm$ SE (days)
*E*7–12:OH	12.6 ± 0.3
*E*8–12:OH	13.3 ± 0.3
*Z*8–12:OH	13.5 ± 0.3
*E*9–12:OH	16.6 ± 0.1
*Z*9–12:OH	16.8 ± 0.3
*E*8–12:Ac	36.1 ± 0.2
*Z*8–12:Ac	36.8 ± 0.6
*Z*9–14:OH	117 ± 8
*E*11–14:OH	98 ± 3
*E*9–14:Ac	191 ± 2
*E*7–16:OH	918 ± 20
*E*9–16:OH	647 ± 68
*Z*9–16:OH	524 ± 66
E11–16:OH	398 ± 17
*E*7–16:Ac	1,168 ± 65
*Z*7–16:Ac	794 ± 24
*Z*9–16:Ac	738 ± 42
*E*11–16:Ac	617 ± 14

half-lives similar to half-lives reported earlier for 12:OH and *Z*7–12:OH (Butler and McDonough 1981). The values of these seven compounds range from 12.6 days for *E*7–12:OH to 16.8 days for *Z*9–12:OH. Also, *E*- and *Z*8–12:Ac are similar to other 12:Ac. Except for *Z*9–12:Ac, five of these were in the range 35.4–38.4 days; *Z*9–12:Ac was 44.8 days. Baker et al. (1980) found the average ratio of the emission rates from rubber septa of *Z*8–12:OH to *Z*8–12:AC from three determinations to be 2.70. Our $t_{1/2}$ values give 2.73 for this ratio, which is in excellent agreement with theirs. The two tetradecen-1-ols in Table 3 and the two previously determined by us ranged from $t_{1/2}$ of 98–130 days.

*E*9–14:Ac was previously reported to have $t_{1/2} = 331$ days. This value was estimated from data for *Z*9–14:Ac which, based on gas chromatographic analysis, underwent an apparent change in isomer ratio from 3.3% to 5.1% *E*9- over a period of 325 days (Butler and McDonough, 1981). The value by our present method of 191 ± 2 days is within experimental error the same as that of *Z*9–14:Ac. We consider our newly determined value to be the correct one.

The 16-carbon alcohols and acetates both show an increase in $t_{1/2}$ as the double bond becomes more centrally located in the molecule. This is in contrast to the 14 carbon acetates where the opposite effect is found.

Practical Factors. Determination of evaporative release rates by collection of emitted vapor followed by gas chromatographic analysis has two potential problems: (1) lack of quantitative collection of emitted vapor and (2) interference in the GC analysis by extraneous vapors. The octadecyl silane coating of the silica gel provides a highly adsorptive, nonhygroscopic surface. Since it is not hygroscopic, its adsorptivity will not be affected by humidity. When hygroscopic materials such as silica gel and alumina were tried, we could obtain neither consistent nor quantitative adsorption of emitted vapor.

After sufficient use (75–100 determinations), enough octadecyl will be removed from the adsorbent to expose a significant degree of silica gel surface. At this point, quantitative collection of vapors will not be possible. This event is generally signaled by slower elution of the pheromone from the adsorbent column with dichloromethane and nonrepeatability of results; instead of 100 ml, 200–500 ml of dichloromethane may be required to elute the test compound. In this regard, the original preparation of the adsorbent must be carefully done to ensure complete formation of the silanized surface. To guard against erroneous results due to inadequate collection of pheromone vapor, a compound with a known half-life is frequently determined.

The adsorbent in the precolumn serves to ensure purity of nitrogen. Otherwise interfering peaks were sometimes found during GC analysis. To further ensure validity of the analysis, dual GC column determinations (polar and nonpolar) are made. Quantitative agreement between the determinations on each GC column is required before a value is accepted.

In addition to the newly reported $t_{1/2}$ values (Table 3), this method is also being used to determine $t_{1/2}$ values of acetates and alcohols at different temperatures and to determine $t_{1/2}$ values for evaporative loss of aldehydes. These studies, still in progress, will be reported later.

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CANID SEX ATTRACTANT STUDIES

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Abstract—Behavioral studies using anestrous female beagles, and olfactory tests with dogs in the absence of females, indicate that methyl *p*-hydroxybenzoate cannot be considered a key sexual attractant for male beagles, even though this compound has been found in estrous vaginal secretions.

Key Words—Methyl *p*-hydroxybenzoate, sex pheromone, dog, *Canis familiaris*, Carcidae, estrous, chemical communication, sex stimulant, sex attraction.

INTRODUCTION

Olfactory communication in the family Canidae has been shown to serve in individual and specific recognition, sexual attraction, excitation, and other forms of communication (Dunbar, 1978; Anikso, 1977; Hart, 1974; Graf and Meyer-Holzapfel, 1974; Doty and Dunbar, 1974; Beach and Gilmore, 1949; von Uexküll and Sarris, 1931), but there are limited data on the specific attractive chemical compounds involved (Goodwin et al., 1979; Jorgenson et al., 1978). Goodwin et al. (1979) identified methyl *p*-hydroxybenzoate by gas chromatography–mass spectrometry as a component of vaginal odors of females beagles at the time of full estrous. They concluded that this compound was a male sex pheromone because vigorous mounting attempts by male dogs occurred after a saline-soaked cotton applicator, with methyl *p*-hydroxybenzoate absorbed on the tip, was inserted into the vagina of anestrous female beagles. Our studies with beagles, even though not definitive, suggest methyl *p*-hydroxybenzoate is no more attractive to males than saline alone, when these compounds are applied to the vaginas of anestrous females or presented in our standard odor-testing device. Two of the three behavioral experiments reported here closely followed Goodwin et

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al. (1979), except that saline "control" tests without methyl *p*-hydroxybenzoate were also conducted.

METHODS AND MATERIALS

Experiment 1. Experiment 1 was conducted with five sexually experienced male beagles (mean age 3 years) and five sexually experienced female beagles (mean age 4.5 years). All females had previously been exposed to a male during an estrous period and showed typical receptive behavior patterns as described by Goodwin et al. (1979). All males had previously been exposed to each female when she was in estrous and exhibited normal sexual behavior. They mounted within 10 sec and then were separated by observer.

The experiment consisted of three treatments: First, one of the untreated anestrous females was allowed to interact with one of the males for 10 min. A concealed experimenter recorded the behavior with a pen-event recorder activated by a keyboard of switches (see McKenna, 1981). Male-to-female directed activity and female-to-male directed activity was classified and recorded as: anogenital sniffing and licking, head investigation, body investigation, stand-overs, mounting attempts, urination, and defecation (McKenna, 1981). Anogenital investigation (combined sniffing and licking time) and mounting attempts were chosen as behaviors indicative of male sexual interest in the females.

At the end of the "control" period, the female was removed from the testing room for 5 min, and a saline-soaked cotton applicator was inserted into her vagina and rubbed over the vulvar area. She was then reintroduced into the testing room with the same male, and behaviors were recorded. Following this "saline" period, the female was again removed for 5 min, and her vagina and vulva were swabbed with a saline-soaked cotton applicator that had been rolled lightly over finely powered methyl *p*-hydroxybenzoate (shown to be pure by nuclear magnetic resonance). The female was reintroduced to the male, and allowed to interact for the third "methyl *p*-hydroxybenzoate" observation period.

Responses of the males were so prompt for all three treatments that all tests were later shortened from 10 to 5 min to avoid fatigue. Treatments were performed in the above order so that the same female could be used for one test; the odor contamination of the vagina following treatment with methyl *p*-hydroxybenzoate would have rendered a subsequent saline treatment invalid. For the same reason, tests were conducted at least four days apart to ensure that normal anestrous vaginal odors would stabilize between tests.

In experiment 1, eleven sets of tests with three treatments each were conducted. In addition, seven sets of tests were performed with only two

treatments each: four tests with a control followed by a methyl *p*-hydroxybenzoate treatment, and three sets with a control followed by a saline treatment.

Due to the small number of animals used and the variable estrous schedules, males and females were not paired randomly. Rather they were paired in an attempt to maximize interaction, based on prior observations. However, each female was paired with at least two males, and each male with at least two females to provide some degree of independence.

During the period of the 10-min tests, beagles were housed separately in small kennels. With the advent of the 5-min tests, the beagles were housed together in an outdoor 5-acre pen. The dominance hierarchy was as follows: Males: Endless (alpha), Renee, McClaren, Douglas, Chevy; Females: Val (alpha), Hula, Molly, MT, Katie. Katie demonstrated a greater interest and tolerance for Chevy than the other females and therefore was paired often with him, and Hula was paired with Renee for the same reason.

Experiment 2. Indications of a slightly stronger mounting response to the saline and the methyl *p*-hydroxybenzoate treatments over the control treatments prompted eight additional series of three 5-min observation tests with four males and four anestrous females (one male and female were not available for testing after experiment 1).

Cotton swabs were treated as in experiment 1, but rubbed on the outside of the vulva and on the backs of the flanks and not inserted into the vagina as in experiment 1 (in an attempt to remove the factor of mechanical stimulation of the vaginal walls).

Experiment 3. Tests were conducted to determine the olfactory attractiveness of compounds presented in special odor test devices without the added stimulation of behavioral cues from females. The odor test device consisted of disposable perforated plastic tissue capsules (39 × 10 mm) containing tips of treated cotton swabs, or a piece of absorbant cotton, held between two aluminum disks fastened together by screws. The disks were attached to a square stainless-steel plate (18 × 18 cm). The entire device was placed on the floor of the testing room (Timm, 1977). An individual male was presented with two odor devices, 2 m apart, containing two different odors for a 5-min observation period. Tests were conducted with swabs treated with saline or saline + methyl *p*-hydroxybenzoate and swabs treated as above with additional insertion into the vagina of an anestrous female. Tests with fresh estrous urine and vaginal secretions, compounds known to act as sexual releasers, were used for comparative purposes. The following pairs of treatments were presented individually to four male dogs twice: saline (S) versus saline with vaginal insertion (S-VA), saline + methyl *p*-hydroxybenzoate (SM) versus SM with vaginal insertion (SM-VA), estrous vaginal swab versus cotton control, fresh estrous urine versus cotton control.

The testing area was hosed down between individual tests, to minimize odors contributed by the previous test animal, and was deodorized with an ammonia solution following each testing day.

RESULTS

In experiment 1, anestrous females reacted differently to males; Katie was immediately submissive, often rolling on her back, whereas Val would jump around to face the male following anogenital investigation, rarely standing still for mounting attempts or stand-overs. The other females were intermediate in these behaviors; they generally were playful and accepted stand-overs and mounts.

Mounting attempts (with no erections or intromission) were observed in response to all three treatments by two males, whereas three males exhibited no mounting response to any treatment (Table 1). The number of tests where mounting attempts occurred is about equal for the saline and methyl *p*-hydroxybenzoate treatments, and in no case did mounting attempts occur in response to the methyl *p*-hydroxybenzoate treatment where they had not previously occurred in response to the saline treatment (Table 1). In four of the five sets of tests where mounting attempts did occur, the time spent mounting in response to the saline and methyl *p*-hydroxybenzoate treatments was greater than the time spent mounting during the control period.

The anogenital investigation data suggest no increase in interest in the methyl *p*-hydroxybenzoate treatment over the saline treatment (Table 1).

Due to the small number of dogs used, and the nonrandom pairing, a formal statistical evaluation could not be performed on the data. However, we believe the data cast sufficient doubt on the attractant qualities of methyl *p*-hydroxybenzoate.

In experiment 2, mounting attempts (with no erections or intromission) were observed in three tests: One male attempted to mount in response to all three treatments, and two other males attempted mounting in response to both the saline and the methyl *p*-hydroxybenzoate treatments (Table 2). As in experiment 1, no formal statistical analysis can be made; however, the number of tests where mounting attempts occurred is equal for the saline and the methyl *p*-hydroxybenzoate treatments, and in every case where mounting occurred in response to methyl *p*-hydroxybenzoate, it had previously occurred in response to saline. The overall mounting time was greater in response to saline and to methyl *p*-hydroxybenzoate than to control treatments.

The anogenital investigation data suggest no difference in interest in the three treatments (Table 2).

TABLE 1. EXPERIMENT 1: MOUNTING ATTEMPTS AND ANOGENITAL (A-G) INVESTIGATION (SNIFFING AND LICKING) BY FIVE MALE BEAGLES IN RESPONSE TO FIVE FEMALE BEAGLES WHOSE VAGINAS WERE NOT TREATED (C), TREATED WITH SALINE (S), OR TREATED WITH SALINE AND METHYL *p*-HYDROXYBENZOATE (SM)

Animal pairs M-F	Time (sec) spent in mounting			Number of mounting attempts			Time (sec) spent in A-G investigation		
	C	S	SM	C	S	SM	C	S	SM
10-min tests									
C-K	71	84	122	11	15	17	57	49	7
R-H	1	159	73	1	22	13	50	54	68
Mc-Mo	0	0	0	0	0	0	19	22	1
R-H	2	2	5	2	1	2	98	29	20
Mc-H	0	0	0	0	0	0	20	20	10
5-min tests									
C-K	6	14	14	2	6	3	75	67	53
R-MT	0	27	27	0	4	3	63	10	1
E-K	0	0	0	0	0	0	1	2	0
D-V	0	0	0	0	0	0	0	2	1
D-MT	0	0	0	0	0	0	4	0	0
E-MT	0	0	0	0	0	0	5	0	3
E-V	0	0		0	0		51	33	
C-K	100	225		15	21		56	66	
R-H	23	63		4	5		87	55	
D-V	0		0	0		0	42		22
C-MT	0		0	0		0	8		9
E-V	0		0	0		0	8		3
D-Mo	0		0	0		0	0		0

In experiment 3, the behavior exhibited by male beagles in response to the various odor stimuli consisted mainly of sniffing, licking, and urine-marking. The combined time spent in these activities was used in the analysis (Table 3).

As shown in Table 3, swabs treated with saline (S) or saline + methyl *p*-hydroxybenzoate (SM) elicited a low degree of interest. Swabs that had been treated with saline and then inserted into the vagina of an anestrous female (S-VA) or treated with saline + methyl *p*-hydroxybenzoate before vaginal insertion (SM-VA), elicited twice the response either compound (S or SM) did when not inserted. The response to saline vaginal swabs (S-VA) and the methyl *p*-hydroxybenzoate vaginal swabs (SM-VA) was of similar magnitude.

TABLE 2. EXPERIMENT 2: MOUNTING ATTEMPTS AND ANOGENITAL (A-G) INVESTIGATION (SNIFFING AND LICKING) BY FOUR MALE BEAGLES IN RESPONSE TO FOUR FEMALE BEAGLES WHOSE FLANKS WERE NOT TREATED (C), TREATED WITH SALINE (S), OR TREATED WITH SALINE AND METHYL *p*-HYDROXYBENZOATE (SM)

Animal pairs M-F (5-min tests)	Time (sec) spent in mounting			Number of mounting attempts			Time (sec) spent in A-G investigation		
	C	S	SM	C	S	SM	C	S	SM
C-K	1	4	10	1	1	3	69	52	26
R-MT	0	12	10	0	1	2	7	10	12
C-K	0	0	0	0	0	0	2	4	1
R-MT	0	0	0	0	0	0	8	9	0
E-V	0	0	0	0	0	0	13	16	33
D-H	0	0	0	0	0	0	10	3	8
E-H	0	0	0	0	0	0	3	2	0
D-V	0	29	28	0	4	1	15	1	7

Estrous urine (EU) and estrous vaginal swabs (S-VE) elicited 2.6 times the interest of the anestrous vaginal inserted swabs (S-VA and SM-VA), and six times the interest of saline (S) and saline + methyl*p*-hydroxybenzoate (SM) alone. The estrous urine (EU) and the saline-estrous vaginal (S-VE) swabs elicited a similar response.

TABLE 3. TOTAL TIME (SEC) FOUR MALE BEAGLES SPENT SNIFFING, LICKING, AND URINE-MARKING AT ODOR TEST DEVICES CONTAINING TREATED SWABS[a]

Males	S	SM	S-VA	SM-VA	S-VE	EU
Chevy	3	4	5	11	37	52
Renee	4	3	6	11	33	29
Endless	6	2	8	19	48	15
Douglas	3	4	6	11	28	19
Chevy	5	11	12	3	21	24
Renee	2	2	4	2	16	27
Endless	1	4	21	8	15	50
Douglas	6	6	8	16	12	18

[a]Swabs were treated with: S = saline; SM = saline plus methyl *p*-hydroxybenzoate; S-VA = saline (swab) inserted in vagina of an anestrous female; SM-VA = SM (swab) inserted into the vagina of an anestrous female; S-VE = saline (swab) inserted in vagina of an estrous female (EU = fresh estrous urine).

DISCUSSION

In experiment 1, factors responsible for the mounting attempts observed in response to the three treatments by two dogs are not known and might include novelty of an encounter with a female, play behavior, dominance behavior, moisture of the vulva, or behavioral cues given by the female. In addition bacterial action in the vagina or the mechanical stimulation of the vagina might be important factors causing the release of an unknown attractive compound.

We believe the sporadic mounting data do not represent a normal sexual response. After all olfactory studies had been completed, the four male beagles were exposed individually to an estrous female (Hula) with whom they had lived for one year when she was in anestrous periods. They each mounted the female within 5 sec, with intromission occurring within 2 min. In another test, an anestrous female (Val) that had been housed with the males was removed and housed for three days in a small kennel with an estrous female. When she was then brought into contact with four individual males they all exhibited excited, persistent mounting attempts with erections, despite strong resistance from the anestrous female. Therefore, the close familiarity of the dogs did not prevent a normal sexual response in either sex. This robust mounting behavior did not occur in the tests with any of the "treated" anestrous females, rather the males exhibited more "play" behavior, and the attempted mounts and dominance stand-overs were more subdued.

In experiment 2, the low mounting response again indicates that factors other than the compounds themselves might elicit mounting attempts. It was necessary, therefore, to separate the behavioral or visual cues from the olfactory signal, on the assumption that interest elicited by the odor alone was a valid measure of its attractant qualities. Experiment 3 addressed this question.

In experiment 3, the small number of animals used and the nonrandom presentation of odors prevents a formal statistical evaluation of the data. However, these tests suggest that vaginal insertion of saline and saline + methyl *p*-hydroxybenzoate elicits a higher degree of interest than either compound does without additional vaginal odors. Whether the males responded to odors occurring naturally in the vagina of an anestrous female, or if the stimulation of the vaginal walls causes the release of an attractive compound is unclear, but these tests indicate that methyl *p*-hydroxybenzoate is not a prime attractant. Natural estrous secretions are far more attractive than any of those produced by treating anestrous females, and identification of the attractive estrous compound(s) remains to be undertaken.

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Announcement

THE ESTABLISHMENT OF THE INTERNATIONAL SOCIETY OF CHEMICAL ECOLOGY

Following the charge presented at the January, 1983 Ventura Gordon Research Conference, Lincoln Brower, Jean Langenheim, Michael Martin, Gerald Rosenthal, Robert Silverstein, and John Simeone met in Lexington, Kentucky, on July 12–14 to formulate policy and draft a preliminary constitution for a new society of chemical ecology. It was decided that:

1. The Society shall be called The International Society of Chemical Ecology (ISCE).
2. The purpose of the ISCE shall be to promote the understanding of the origin, function, and significance of natural chemicals that mediate interactions within and among organisms. The goals are to broaden the scope of chemical ecology and to foster interdisciplinary cooperation among a diversity of scientific fields.
3. The official publication organ of the ISCE shall be the *Journal of Chemical Ecology*, published by Plenum Publishing Corporation.
4. ISCE members will receive a special subscription rate for the *Journal of Chemical Ecology:* Volume 10, 1984 (12 issues) $35.00 (outside the U.S., $42.00).

The Society's first meeting in 1984, tentatively set for June 11–14 in Austin, Texas, will be of the greatest importance in setting the stage for its intellectual vitality, influence, and effectiveness.

Membership forms are available from:

Dr. Gerald A. Rosenthal
Secretary Pro Tem.
International Society of Chemical Ecology
Thomas Hunt Morgan School of Biological Sciences
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EFFECTS OF MULTILURE COMPONENTS ON TWIG-CROTCH FEEDING BY EUROPEAN ELM BARK BEETLES[1]

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Abstract—The three components of the European elm bark beetle pheromone dispensed from polyethylene vials attached to the boles of healthy juvenile elms affected the rates of beetles landing and twig feeding on the baited trees. Maximum attraction to the tree occurred when all three pheromone components were presented together in a ratio of 1:1:8 for 4-methyl-3-heptanol (H), α-multistriatin (M), and α-cubebene (C). M released either alone or in excess of its natural ratio with H and C induced twig-crotch feeding. H presented alone had no effect on attraction or twig-crotch feeding, but in combination with M it induced landing on and boring into the tree bole. We concluded that the ratio of M and H being released influenced incoming beetles either to land on and colonize the bole or to feed in twig crotches. M in excess of H, known to occur when most females are mated, terminates colonization and deflects incoming beetles to crowns of elms.

Key Words—Coleoptera, Scolytidae, *Scolytus multistriatus*, European elm bark beetle, pheromone, epideictic pheromone, twig-crotch feeding.

INTRODUCTION

The European elm bark beetle, *Scolytus multistriatus* Marsham, transmits the Dutch elm disease (DED) fungus, *Ceratocystis ulmi* (Buisman) C. Moreau, by feeding in the twig crotches of healthy elms. Both sexes of the beetle may bore a hole into the center or slightly to one side of a twig crotch. If this feeding hole penetrates to the xylem and fungal spores are deposited, infection may occur.

[1]*Scolytus multistriatus* (Coleoptera: Scolytidae).

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Although beetles may feed on healthy trees, they are generally able to breed only in stressed, diseased, or dying trees. Once such a tree is located by a female, she begins boring into the bark of the bole or limbs. At this time she releases an aggregation pheromone that, in combination with a host-produced synergist, attracts both males and females (Pearce et al., 1975). After mating, the females lay eggs in the phloem–cambial region of the tree. Upon hatching, the larvae construct feeding tunnels in a characteristic gallery pattern. Mature adults emerge and disperse until they encounter other diseased trees in which to breed, or healthy trees in which to feed. In most of North America there are two generations a year, with the first adults emerging in late spring and the second generation emerging in midsummer. Some of the progeny of the second generation emerge in the late summer or fall, but the majority overwinter as larvae in the bark.

It had been known for many years that beetles aggregate on diseased elms (Martin, 1936; Meyer and Norris, 1967). Peacock et al. (1971) demonstrated that mass attraction was governed by a pheromone produced when virgin females bore into elm bark. In 1975 the pheromone was found to consist of three components: 4-methyl-3 heptanol (H), α-multistriatin (M), and α-cubebene (C) (Pearce et al., 1975). The presence of isomers other than the active ones does not effect trap catch (Lanier et al., 1977; Elliott et al., 1979); however, it is not known what effect they may have on other aspects of beetle behavior. Synthetic H exists as a pair of diastereomers, each with two enantiomers; only the (−)-threo enantiomer is produced by virgin female beetles (Mori, 1977). M, a bycyclic ketal, occurs as four diastereomers (α, β, γ, and δ), each consisting of two enantiomers. Both α- and β-multistriatin have been found in aerations of beetle-infested logs, but only the (−)-α-isomer is attractive (Elliott et al., 1979). Cubeb oil was used as the source of C for which the (−)-α-isomer is active.

Lab and field tests indicate that individual components or a mixture of any two components were much less attractive than the three-component mixture (Pearce et al., 1975; Lanier et al., 1977). Gore et al. (1977) demonstrated that H is produced by only virgin females, M is produced by both mated and unmated females, and C is emitted from elm tissue as either sex bores into the tree. After mating, females continue to release M but not H; mating has the net effect of increasing the concentration of M relative to H. A decline in attraction following mating thus results from termination of release of the attractant (Elliott et al., 1975). When H was added to a log containing mated females, the attractiveness was restored (Lanier et al., 1977). On the other hand, when Cuthbert and Peacock (1978) increased M relative to H and C, the catch of beetles on sticky traps decreased sharply. In their tests, increasing H and/or C relative to M slightly enhanced trap catches.

Most DED control programs are aimed at reducing the population of

beetles, thereby decreasing the amount of feeding on healthy trees. The original purpose of this study was to see if high amounts of M would decrease twig-crotch feeding in the manner that it had decreased trap catches. Our initial tests showed the opposite result. During the following two field seasons, we made a detailed investigation of the effects on twig-crotch feeding when each of the beetle-produced pheromone components was varied. We report the result of our 3-year field study.

METHODS AND MATERIALS

During 1979, 1980, and 1981, we studied plots with populations of juvenile elms in or near the city of Syracuse, New York. Each plot selected met the following criteria: (1) plots were 200 m or further apart; (2) there were five (or six in 1979) healthy elms 3.4–10 m tall; and (3) there were no dying or diseased elms in the plot. All trees within a plot received the same treatment—various mixtures and concentrations of the three pheromone components—and there were three plot replicates of each treatment. H and M were synthetic (not isomerically pure); C was isolated from cubeb oil. In 1979 the chemicals were released from Conrel® (Albany International, Controlled Release Div., Needham Heights, Massachusetts) hollow fibers and Hercon® (Health-Chem Corp., Hercon Div., New York, New York) laminated plastic dispensers. To avoid possible contamination between fibers containing the different components, in 1980 and 1981 the chemicals were dispensed from 2-dram polyethylene vials hung in the bole of the tree approximately 2.5 m above ground level. The vials were loaded with 1×10^7 beetle hours (BH) of one of the three components. (One beetle hour is the amount of pheromone one virgin female released in one hour and equals 0.5 ng of H, 0.38 ng of M, and 1 ng of C.) Under controlled lab conditions, vials with 1×10^7 BH of one of the components were placed in 7.6-liter glass desiccators with dried air flowing through at 0.5 liters/min. After 6 weeks of daily weighings, the vials were gravimetrically determined to have an approximate release rate of: H, 672 μg/day (270,000 BH of active isomer/day); M, 35 μg/day (300,000 BH of active isomer/day); and C, 820 μg/day (575,000 BH of α-cubebene/day).

The trees were baited in mid-May just prior to the emergence of the overwintering beetle generation. In 1980 and 1981 beetle landing was monitored by placing 10-cm-wide white cardboard sticky bands at breast height on the boles of two of the five trees within each treatment plot. In early August, after the first-generation flight was complete, sticky bands were changed, and trees were rebaited. To monitor feeding, a sample consisting of ten current-year twig crotches of a dominant branch (leader) was taken from the top of each tree, following the sampling method developed by

Rabaglia and Lanier (1983). In September and October, after the second-generation flight was complete, the trees were sampled again and nine of the 15 trees per treatment were felled so that all feeding injuries within their crowns could be counted. (In 1979 all the trees were felled.) Previous research has shown that beetles feed most often in the twig crotches in the upper and outer portions of the tree (Wolfenbarger and Buchannan, 1939; Riedl and Butcher, 1975), especially in the twig crotches formed at the junction of the current and previous years' growth, i.e., "new-old" (N-O) type twig crotches (Rabaglia and Lanier, 1983). Limiting observations to N-O twig crotches and leaf petioles avoided possible confusion between current and previous year feeding injuries. For each of the felled trees, all the N-O twig-crotches within the top third of the crown were counted so that the percentage of crotches with feeding injuries could be compared for trees with different sizes and densities of crowns. The data were analyzed by single-classification analysis of variance and the Student-Newman-Kuells test of multiple comparison.

RESULTS AND DISCUSSIONS

Our 1979 results showed that baiting trees with M increased twig-crotch feeding rather than reducing it as we had hypothesized (Table 1). Both the spring and summer generation beetles fed more in the trees baited with M than those baited with HMC or the unbaited control trees. Although sticky bands were not placed in the trees to monitor beetle landing, we did note the occurrence of bole attacks (attempts to establish breeding galleries) on some

TABLE 1. MULTILURE COMPONENTS AND TWIG-CROTCH FEEDING, 1979

Treatments[a]	1st generation, May–July	2nd generation, July–September		
	Sampled feedings	Sampled feedings	Average feeding per tree	N-O with feeding (%)[b]
4M	1.19a[c]	3.25a	238.0a	53.2a
HMC	0.06b	1.18b	95.3b	21.7b
Blank	0.06[b]	0.33b	5.3b	3.0c

[a]Treatments: 4M—4 Conrel baits containing multistriatin. Total release rate of 400 μg/day = 3×10^6 beetle hours (BH) day. HMC—1 Hercon bait of multilure with a multistriatin release rate of 50 μg day = 4×10^5 BH/day.

[b]Percent of new-old twig crotches in the top third of the crown with evidence of feeding.

[c]Means followed by the same letter are not significantly different at $P = 0.05$ using the Student-Newman-Keuls test of multiple comparisons.

TABLE 2. MULTILURE COMPONENTS AND TWIG-CROTCH FEEDING, 1980

Treatments[a]	1st generation, May–July		2nd generation, July–September			
	Sampled feedings	Beetles trapped	Sampled feedings	Average feeding per tree	N-O feeding (%)[b]	Beetles trapped
HMC + 3M	3.64a[c]	24.67b	4.73a	311.67a	63.95a	59.0b
HMC	1.14b	187.33a	3.08b	150.71b	43.14b	302.3a
4M	0.57b	2.67b	1.53c	57.89c	18.06c	21.0b
Blank	0.36b	0.67b	0.91c	14.40c	4.64cd	2.0b
H	0.00b	0.00b	0.53c	14.78c	2.57d	0.0b

[a]Treatments: HMC—Approximate release rate of H:M:C of 1:1:2 (1 = 2.7×10^5 BH/day). HMC + 3M—Approximate release rate of H:M:C of 1:4:2 (1 = 2.7×10^5 BH/day). 4M—Release M only at 1.1×10^6 BH/day. 4H—Release of H only 1.1×10^6 BH/day.
[b]Percent of new-old twig crotches in the top third of the crown with evidence of feeding.
[c]Means followed by the same letter are not significantly different at $P = 0.05$ using the Student-Newman-Keuls test of multiple comparisons.

of the trees. Only one M-baited tree had a substantial number of attacks, and this tree had sustained DED from an early summer twig feeding injury (diseased elms are normally colonized for breeding, and this tree contained beetle brood). In contrast, 10 of the 15 trees given the HMC treatment were attacked on the bole, although most of these breeding attempts did not result in broods.

In 1980 we tested the hypothesis that M alone attracts large numbers of beetles that proceed to feed in twig crotches but are not particularly stimulated to attack and colonize the bole of the tree. To further explore these apparent behavioral shifts in response to pheromone components, we placed sticky bands on boles of the trees, increased the number of treatments, and included several combinations of the chemicals that would simulate different beetle–host situations. Multilure plus extra multistriatin (HMC ± 3M) was tested to simulate a tree heavily infested with mated females that would release a high M:H ratio compared to the 1:1 ratio released by virgin females (Gore et al., 1977). During the first generation, as expected, sticky bands on the multilure-baited (HMC) trees caught more beetles than the other treatments, even though the HMC + 3M-baited trees had significantly more feedings than any other treatment (Table 2).

Compared to the 1979 results (Table 1), the relative rates of twig-crotch feeding in the HMC and 4M treatments were reversed (Table 2). This incongruity is probably a result of many more beetles being attracted to HMC-baited trees. The ratio of beetles trapped on HMC and 4M is approximately 90:1 for the first generation and 15:1 for the second generation, while twig

TABLE 3. MULTILURE COMPONENTS AND TWIG-CROTCH FEEDING, 1981

Treatments[a]	1st generation, May–July		2nd generation, July–September			
	Sampled feedings	Beetles trapped	Sampled feedings	Average feeding per tree	N-O with feeding (%)[b]	Beetles trapped
HMC + 3M	1.40a[c]	3.5a	2.47a	142.2a	28.07a	16.8a
HMC	0.79b	5.0a	1.13b	118.0ab	20.00ab	36.3a
hMc + M	0.71b	3.7a	1.20b	50.6b	12.71bc	14.2a
HMC + 3H	0.47b	3.5a	0.73b	73.9bc	15.47b	38.3a
4H	0.07b	0.0a	0.20b	27.1c	4.60cd	0.0a
Blank	0.00b	0.0a	0.67b	11.9c	2.13cd	0.0a

[a]Treatments: HMC—Approximate release rate of H:M:C of 1:1:2 (1 = 2.7×10^5 BH/day). HMC + 3M—Approximate release rate of H:M:C of 1:4:2 (1 = 2.7×10^5 BH/day). hMc + M—Approximate release rate of H:M:C of 0.25:1:0.50 (1 = 2.7×10^5 BH/day). HMC+3H—Approximate release rate of H:M:C of 4:1:2. 4H—Release of H only at 1.1×10^6 BH/day.
[b]Percent of new-old twig crotches in the top third of the crown with evidence of feeding.
[c]Means followed by one or more of the same letters are not significantly different at $P = 0.05$ using the Student-Newman-Keuls test of multiple comparisons.

feeding parameters for both generations range from approximately 2 to 3:1. The twig feeding rates given on Table 2 were also affected by the exclusion from the second generation data of four 4M-baited and three HMC + 3M-baited trees that were colonized after they sustained Dutch elm disease infections. This elimination was necessary because pheromone released by colonizing beetles confounded the treatments.

In 1981 we concentrated on studying the effect of H on the twig-feeding and bole-attacking behaviors of *S. multistriatus*, while once more testing varying proportions of M (Table 3).

Results in 1981 were consistent with the previous observation that extra M stimulated twig-crotch feeding. The HMC + 3M treatment sustained the highest twig feeding rate, although differences from HMC data are significant only in the "sampled-feedings" category (Table 3). H alone (H vs. blank) or extra H (HMC + 3H vs. HMC) had no discernible effect on attraction or twig feeding. Difference in sticky band catches were not significant for any treatment comparisons.

Reduced rates of twig feeding and sticky band catches in 1981 reflect a first generation depressed by wintering mortality and a second generation dispersed by an abundance of attractive potential breeding sources resulting from an outbreak of elm phloem necrosis (elm yellows disease). In addition to colonizing some of the diseased trees, millions of beetles were absorbed by several thousand "weed" elms that we had killed with cacodylic acid in

an effort to stem the elm phloem necrosis outbreak; treated trees are often colonized by beetles, but their broods fail (O'Callaghan et al., 1980).

Based on the data collected during the three years of this project, along with the knowledge of the modulation of H as virgin females are mated (Gore et al., 1977) and the demonstration of the effects on trap catches of varying M, H, and C (Cuthbert and Peacock, 1978), we propose the follow-

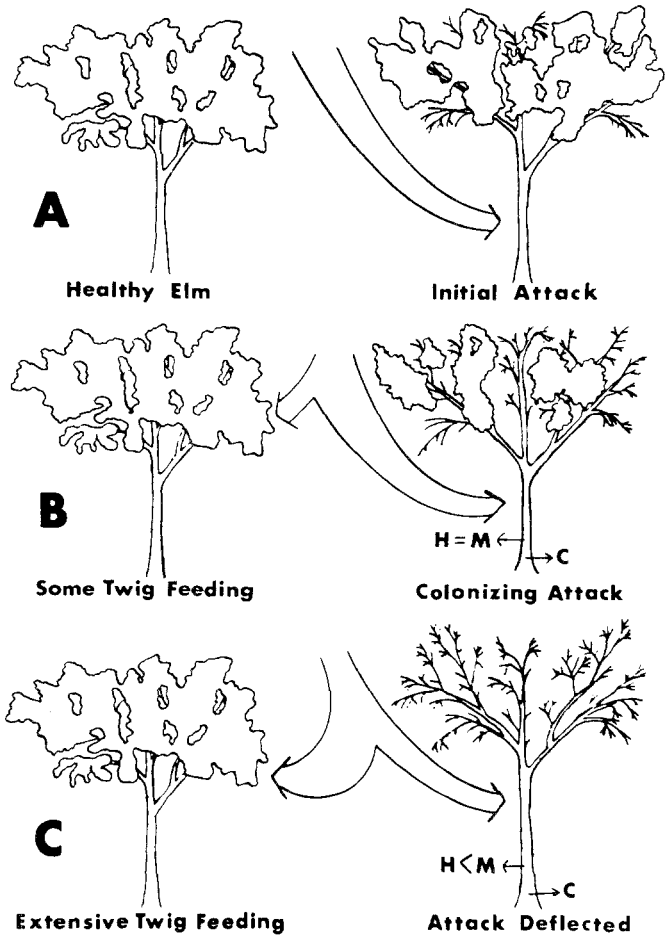

FIG. 1. Attack phases of *Scolytus multistriatus:* (A) pioneers attack diseased or weakened elm; (B) males and females mass attack tree in response to pheromone and some attacking beetles land in crown of nearby elms and feed on twig crotches; (C) after females are mated production of H decreases deflecting most of incoming beetles into nearby elm crowns (see text for further explanation).

ing scenario. When a diseased tree is initially colonized, virgin females boring into the bark release H and M in approximately equal amounts. The amount of C produced by the tree increases with the degree of boring activity of the beetles. The incoming population responds to this message by orienting to the bole, where the beetles locate mates (males) and/or oviposit (females). The increased number of beetles around the tree will result in some twig feeding. As established females are mated, the release of H ceases. Eventually the available breeding space is utilized so that there are few new attacks by virgin females and the production of H diminishes. Thus, the ratio of M:H increases. Beetles continue to be attracted but the altered M:H ratio inhibits landing on the area of highest concentration: the colonized bark. Instead, the incoming beetles are deflected into the crowns of healthy elms where they feed in twig crotches (Figure 1).

Twig-crotch feeding is a means by which beetles gain moisture and energy reserves when breeding space is not available. Most of the *S. multistriatus* captured at pheromone sources by Lanier et al. (1976) had not fed on twig crotches. Wolfenbarger and Buchannan (1939) found that beetles held without food or water lived for an average of 2.2 days, while those allowed to feed on twig crotches lived for 7.6 days. In addition to securing food reserves, the beetles may mate (Bartels and Lanier, 1974; Švhira and Clark, 1980) or infect the tree with the Dutch elm disease fungus, which provides future generations with suitable breeding material. After the beetles have fed, they may once again disperse to locate a breeding site.

Collins (1938), who studied the distribution of twig-crotch feeding in elms of southeastern New York State in the 1930s, found that "in every instance trees located relatively near what might be termed 'beetle wood' . . . had the largest number of feeding wounds. . . ." He also mentioned that the presence of "dead wood alone" was not in itself related to feeding activity; the wood must either be producing or attracting beetles. In considering DED control, it is important to underscore the notation that diseased or moribund elm wood that may be colonized represents a hazard to nearby healthy elm before any brood adults emerge.

Before the pathogenic DED fungus was brought to North America, *S. multistriatus* would breed in stressed trees or parts of trees, as do many other members of the genus. Having a mechanism to deflect an attack from a fully utilized area to healthy parts of the same or adjacent trees prevents continued colonization that would not increase the brood output owing to intensified intraspecific competition for a limited resource. Collins (1938) cites an instance where half of an elm tree damaged by lightning contained *S. multistriatus* larvae and pupae, while half of the tree that was still living contained an extraordinarily high number of twig-crotch feedings. Collins' observation can be explained in terms of the pheromone modulation reported by

Gore et al. (1977) and our results: initial colonization, stimulated by HMC, was deflected by reduction of H after the breeding space was filled and females were mated. An odorant that has the effect of spacing the population in this way has been termed an epideictic pheromone (Prokopy 1981). These are common in insects and among the Scolytidae have been intensively studied in the genus *Dendroctonus*. The Douglas-fir beetle, *D. pseudotsugae* Hopkins, and the southern pine beetle, *D. frontalis* Zimmerman, both utilize a pheromone message to deflect attack from a utilized resource (Rudinsky, 1973; Rudinsky et al., 1973).

In addition to confirming the importance of elm wood colonization to DED incidence, our results are very relevant to programs for destroying in-flight *S. multistriatus* on multilure (HMC) -baited sticky traps. HMC in the concentrations being used stimulates some twig-crotch feeding in nearby healthy elms. If extra M is contributed by twig feeding beetles or if the relative concentration of H is low due to differential elution or incorrect formulation, twig feeding will be stimulated. Thus, the destruction by pheromone-baited traps of a substantial proportion of the extant beetle population can be countered by increasing the rate of twig feeding by the surviving population. Furthermore, high concentrations of HMC, resulting from overly dense spacing or high-dosage baits, may interrupt the flight of beetles toward the baited traps and induce landing and twig feeding in healthy elms. Finally, it is clear that an abundance of H minimizes the twig-feeding behavior stimulated by M.

These results provide potential explanations for differences in degree of the contribution of mass trapping to DED control programs. In elm groves surrounded with traps, a decline in DED almost always accompanied the application of the technique (Lanier, 1979, 1981; Peacock et al., 1981). In these cases traps were few (7–20), they were positioned at least 30–100 m from the nearest elm, and seldom was any diseased wood present within the trapping zone. Large-scale trapping programs in Detroit, Michigan, Evanston, Illinois, and Ft. Collins, Colorado, differed from the "grove" test by employing a larger number of traps relatively densely spaced and positioned without particular regard to the presence of elm (Peacock et al., 1981). Although millions of beetles were killed on these traps and the number of extant beetles was reduced (Ft. Collins study), there was no concomitant decline in DED rates in these areas. Lack of an identifiable impact of DED in these large areas does not preclude a contribution of mass trapping to DED control in large areas. In Syracuse, New York, where scattered clusters of traps were sparsely spaced through an area of about 2500 hectares (10 mi^2), DED declined dramatically (Lanier, 1981). Furthermore, sampling of twigs showed that the feeding rate was substantially lower within, compared to outside, the treatment area where Dutch elm disease rates were

higher (Rabaglia and Lanier, 1983). Results of this study warrant articulation of the following recommendations for utilization of multilure for control of DED.

1. Position traps away from elms (50 m or more if possible).
2. Maintain release of H in excess of M.
3. Minimize potential sources of natural pheromone.
4. Give prophylactic treatments (insecticide and/or fungicide) to elms near natural or artificial sources of pheromone.

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CHEMISTRY AND FUNCTION OF MANDIBULAR GLAND PRODUCTS OF BEES OF THE GENUS *Exoneura* (HYMENOPTERA, ANTHOPHORIDAE)[1]

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Abstract—Female *Exoneura richardsoni*, *E. bicolor*, and *E. bicincta* (Hymenoptera: Anthophoridae) release a pungent, staining liquid from their mandibular glands upon disturbance. This secretion is primarily composed of ethyl dodecanoate, with lesser amounts of homologous ethyl and methyl esters, salicylaldehyde, and 1,4-benzoquinone. The secretion elicits vigorous grooming when topically applied to antennae of *Formica* ants. The shared, unique combination of mandibular gland lipids of these three *Exoneura* species supports their monophyletic classification, while the presence of salicylaldehyde may associate *Exoneura* (Allodapini) with *Pithitis* (Ceratinini).

Key Words—*Exoneura*, Allodapini, Hymenoptera, bees, defense, mandibular glands, ethyl decanoate, salicylaldehyde, 1,4-benzoquinone, ester, aldehyde, quinone.

INTRODUCTION

It has long been suspected that the Australian genus *Exoneura* and probably other allodapine bees secrete materials from the mouth region that repel ants and perhaps other predators. When held between the fingers, a female *Exoneura* often exudes a rather copious, brown, yellowish, or reddish liquid onto the skin. This liquid stains white paper or one's skin yellowish-brown, and has an odor suggestive of that of carabid or tenebrionid beetles (C.D.M.)

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with overtones of raisins (J.H.C.). [The sweetish odor may result from an admixture of regurgitated crop contents but ethyl esters such as ethyl decanoate in the secretion may account for it (Amerine and Roessler, 1976; Heath and Pharin, 1978).] Michener (1965) wrote that the females "emit this material either at the nest or, if captured or greatly disturbed, away from the nest. On one occasion [he] found females of *E. bicolor* so common . . . visiting flowers . . . that when the bushes bearing the flowers were beaten with a net, the odor could be perceived several feet away. The effectiveness of adult females in defending their nests is shown by a series of nests of *E. variabilis* set up for observation in Brisbane. Within a day those containing no adults were robbed of all immature stages by ants. Those containing adults mostly survived for many days."

Because of the observations reported above, one of us (C.D.M.) suggested that the liquid from the mandibular gland serves to repel predators such as ants and that it probably contains quinones, well-known repellants produced by other arthropods (Eisner and Meinwald, 1966) but not previously recognized among glandular products of bees. [A similar function, however, has been shown for other mandibular gland products of some bees (Cane, 1982; Wheeler et al., 1977).] The present paper documents the mandibular gland lipid chemistry and its defensive function for three species of *Exoneura*.

METHODS AND MATERIALS

To properly relate this paper to previous work on *Exoneura* biology, the following notes on the specific names seem necessary: (1) *Exoneura richardsoni* Rayment is probably the same as *E. variabilis* Rayment, the name used in a study of a Queensland population (Michener, 1965). However, since *richardsoni* was described from the Dandenong range, the source of our material, we feel that until the group is studied taxonomically, it is appropriate to use that name even though it will probably ultimately be synonymized under *variabilis*. (2) *Exoneura bicolor* Smith is used in a traditional and possibly correct sense for a form similar to *E. hamulata* Cockerell but with the upper hooks of the yellow clypeal mark of the female usually reduced or absent, the clypeal yellow area of the male much reduced, and the abdominal pubescence of the male all black. *E. bicolor*, however, was described from West Australia and Tasmania, probably from specimens belonging to two species, and the proper application of the name remains in doubt. (3) *Exoneura bicincta* Rayment is similar to and likely to be a synonym of *E. angophorae* Cockerell. Our males appear to agree with the male type of *bicincta*, but are also similar to males of *E. angophorae* in the Snow Entomological Museum, University of Kansas. Voucher specimens of all

three species are placed in the Snow Entomological Museum and in the Australian National Insect Collection, Canberra.

Nests of *Exoneura bicolor*, *bicincta*, and *richardsoni* in dead blackberry (*Rubus*) stems were collected in the Dandenong Ranges, Victoria, Australia, in May 1982, by Mr. Michael Schwarz of Monash University. Immature forms were absent, males were scarce, and the bees were evidently in overwintering quarters. C.D.M. transferred the bees to glass tubes 5 mm in inside diameter (plugged with pipe cleaners), one for each nest. The tubes were refrigerated (about 4°C) when practical, and carried by air to Kansas in a plastic bag containing a little moist cotton for humidity. Every specimen arrived alive and was active when not refrigerated.

Tests with ants were made in Lawrence, Kansas, in July, using female *Exoneura bicolor* only. Workers of *Crematogaster lineolatus* were in a rather weak trail moving up and down a post. Workers of *Formica pallidefulva* were placed individually in 4-dram vials for topical treatments and observations. Each treatment consisted of touching the middle part of one flagellum a few times with a needle or closed fine forceps bearing the test material.

For the chemical studies, made in June, females that had been continuously chilled for at least four days were killed on Dry Ice; then 15–35 glands (depending on the species) were removed with their attached mandibles and placed in chilled methylene chloride. The chemical analysis was made on the day of dissection.

All spectral analyses were made with a Girdel gas chromatograph (GC) with split Ros injection, coupled to a Ribermag R-10-10 quadrupole mass spectrometer and PDP 8/A computer in the Department of Chemistry, University of Kansas. The GC oven was programed at 5°C/min steps from 70°C to 250°C, followed by a 10-min isothermal period at 250°C. Components were separated using a 12-m, 0.20-mm inside diameter methyl silicone wall coated (WCOT) fused silica capillary column (Hewlett-Packard). Total ion chromatograms were compiled from 1.2-sec scans from 40 to 500 amu at 70 eV, in EI mode. All spectra were taken from chromatogram peak maxima with background subtraction. Components of glandular extracts were positively identified by a combination of matching retention times (±2% range) and comparison of our spectra with published spectra (quinone) and authentic samples (esters). Individual injections contained between one half and one glandular equivalent of extracted secretion.

RESULTS

Defensive Function of Mandibular Gland Products. Preliminary observations were made with the ant *Crematogaster lineolatus*. Freshly macerated heads of females of *Exoneura bicolor* applied to the ant trail on a post

were not attractive; the trail was interrupted, but within 3–5 min (three trials) the ants resumed movement around the macerated material, which was ignored. Freshly macerated thoraces or abdomens, however, were immediately attractive; ants in the trail stopped and apparently fed from the material (three trials for each body part). Likewise, five macerated heads inside a glass tube 5 mm in diameter and 5 cm long placed among ants at the bottom of the post were not touched in an hour, although at least one ant wandered about inside the tube. Macerated thoraces and abdomens of two bees inside another tube were removed by the ants during the first half hour. These crude experiments suggest either that heads do not contain attractive materials present in thoraces and abdomens or that heads contain a repellant substance absent from thoraces and abdomens.

To get less equivocal information on possible repellance, a larger ant, *Formica pallidefulva*, was used for topical application of materials. Secretion of the mandibular glands, taken on a needle or closed fine forceps directly from the mouth area of live *Exoneura*, was applied (in invisible amounts) to one ant antennal flagellum. As controls, there is the untreated flagellum of the same ant and other ants similarly treated but with water or raw egg white to see whether sticky material might elicit the same response as the *Exoneura* secretion.

As Table 1 shows, the cleaning response to the *Exoneura* secretion was intense and lengthy. The antenna was repeatedly (up to 46 times per minute) drawn through the strigilis on the foreleg for up to 15 min. Moreover, after every two or three antennal cleaning movements, some individuals cleaned the foreleg involved by drawing it through the mouthparts. No such cleaning of the other foreleg was observed. It was as though an irritant affected not only the antenna but the leg used to clean the antenna.

Egg white often elicited short bouts of cleaning. The movements were

TABLE 1. ANTENNAL CLEANING BY *Formica pallidefulva* AFTER MATERIAL FROM MANDIBULAR GLANDS OF FEMALES OF *Exoneura bicolor* WAS PLACED ON ONE FLAGELLUM, WITH EGG WHITE AND WATER FOR COMPARISON

	Exoneura secretion	Egg white	Water	Untreated antenna
Duration of response (min)[a]	2,5,12,12,15	0,1,1,2,3	0,0,0,1,1	0 ($N = 15$)
No. of cleaning movements per min[b]	$\bar{X} = 37$ (20–46, $N = 15$)	$\bar{X} = 8$ (0–12, $N = 10$)	$\bar{X} = 5$ (0–8, $N = 10$)	$\bar{X} = 1$ (0–5, $N = 35$)

[a]Based on five different ants for each treatment. The numbers given are minutes after treatment until cleaning of the treated antenna either ceased or decreased to the low level at which the untreated antenna was cleaned.
[b]Based on counts for full minutes.

TABLE 2. LIPID COMPONENTS OF MANDIBULAR GLAND PRODUCTS OF *Exoneura*

Compound	Proportional integreated peak areas from GC (%)[a]		
	E. richardsoni	*E. bicolor*	*E. bicincta*
p-Benzoquinone (1,4-benzoquinone)	0.2	2.5	1.5
Salicylaldehyde (2-hydroxybenzaldehyde)	0.5	0.2	0.6
Ethyl decanoate	5.5	3.2	1.0
Methyl dodecanoate	4.6	2.4	1.2
Unknown[b]	0.1	10.0	3.2
Ethyl dodecanoate	100.0	100.0	100.0
Dodecanoic acid	0.7	0.7	0.8
Methyl tetradecanoate	1.5	0.9	0.3
Ethyl tetradecanoate	34.4	31.6	23.1
Tetradecanoic acid	0.5	0.1	1.2
Ethyl hexadecanoate	4.5	—	1.8
Approx. quantity[c] of lipids gland (μg)	20–30	30–40	30–40

[a]Calculated as percent of largest peak (ethyl dodecanoate).
[b]Spectrum resembling a dimethyl naphthol.
[c]Calculated from GC integrated peak area of ethyl stearate standard.

often slow, as though considerable force was required to draw the sticky antenna through the strigilis. It is not clear that water elicited any cleaning movements.

Chemistry of Mandibular Gland Products. The secretion is red in dissected gland sacs; when exposed it is yellowish, gradually browning, presumably as a result of an unknown oxidation.

Table 2 lists the lipid components in the glands of three species of *Exoneura.*

DISCUSSION

Evidence is strong that the mandibular gland products in *Exoneura* serve for defense against ants and perhaps other natural enemies. The mixture of materials in these glands is similar in three sympatric species that are not particularly closely related to one another. Probably the most recent common ancestor of these species possessed the defensive mixture, which has been retained with little modification in the descendant species. The mixture may, of course, have other functions also.

The mandibular gland product of *Exoneura* contains a quinone as well as salicylaldehyde and various ethyl and methyl esters of aliphatic acids. Quinones have not previously been recorded as exocrine products of bees, although they are well known in defense secretions of other arthropods

(Eisner and Mainwald, 1966). It is noteworthy that the mandibular glands do not contain the mevalonic derivatives (e.g., citral, geranyl acetate, etc.) that are present in these glands of *Ceratina* (including *Pithitis*) (Hefetz et al., 1979; Wheeler et al., 1977). The differences in chemistry of gland products between the allodapine bee *Exoneura* and *Ceratina* support the suggestion that the allopadines might well be placed in a separate tribe, Allodapini, rather than within the Ceratinini as has been the recent custom. However, among all bees so far examined, *Pithitis* and *Exoneura* are the only ones producing salicylaldehyde in addition to some of the ethyl esters. If production of these compounds is synapomorphic, *Pithitis* and *Exoneura* may have a common ancestor not shared by *Ceratina*, a conclusion not supported by known morphological features. The large amouint of ethyl dodecanoate is interesting; traces of the same compound are found in mandibular glands of males of an unrelated anthophorid, *Centris adani* (Vinson et al., 1982).

Of course the mandibular gland product is not the only defense by *Exoneura* at nest entrances. The bee at the entrance will bite at a disturbing object (ant or fiber held by an observer); then if the disturbance continues she will turn and firmly block the constricted nest entrance with the flattened dorsoapical part of the abdomen. Thus the construction of the nest entrance, as well as the behavior and secretions of the guard, contribute to nest defense. At least some guards of nests set up in Brisbane and heavily persecuted by *Pheidole megacephala* (?) ants seemed to have drops of brownish fluid that looked like the mandibular gland product around the edges of the abdominal area used to plug the nest entrance. Thus it is likely that *Exoneura*, like *Pithitis*, rubs the mandibular gland secretion over its body when disturbed (Hefetz et al., 1979).

North American ants were used to test the defense secretion of an Australian bee for practical reasons. However, the ant species *Pheidole megacephala*, noted by C.D.M. attacking *Exoneura* nests in Brisbane, is an introduced species there. Henry Hacker (personal communication, 1957, to C.D.M.) remarked on the great reduction in native bee populations (especially *Exoneura*) in southeastern Queensland, compared to his experience early in the century. Perhaps the introduction of *Pheidole* is a factor in this reduction. Even when the guards of *Exoneura* are able to keep ants out of the nest for many days, the colony is likely to succumb because the *Pheidole* are persistent and, whenever a bee is entering or leaving, an ant is likely to get in. Presumably defense against native ants is more effective.

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1-METHYLCYCLOHEX-2-EN-1-OL AS AN AGGREGATION PHEROMONE OF *Dendroctonus pseudotsugae*[1,2]

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Abstract—1-Methylcyclohex-2-en-1-ol (1,2-MCH-ol) was synthesized, identified as a compound found in volatiles of the female Douglas-fir beetle, and shown by bioassays to be an aggregation pheromone. 1,2-MCH-ol matches in both GC retention index and mass spectrum a compound released by the female after feeding. 3,3-MCH-ol was also synthesized as a candidate compound; its mass spectrum is presented because published mass spectra are incorrect for this compound. Synthetic 1,2-MCH-ol increased arrestment and stridulation of males in olfactory walkways and increased trap catches of flying beetles. Males were more responsive to 1,2-MCH-ol than females.

Key Words—Aggregation pheromone, attractant, 1-methylcyclohex-2-en-1-ol, *Dendroctonus pseudotsugae*, Coleoptera, Scolytidae, bark beetle, Douglas-fir beetle, 3-methylcyclohex-3-en-1-ol, *Pseudotsuga menziesii*.

INTRODUCTION

The female Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopkins (Coleoptera: Scolytidae), releases numerous aggregation pheromones, frontalin, 3-methylcyclohex-2-en-1-one, verbenone, 3-methylcyclohex-2-en-1-ol (3,2-MCH-ol), *trans*-verbenol, and pent-3-en-1-ol (Rudinsky et al., 1974; Ryker et

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al., 1979 and references therein). 1-Methylcyclohex-2-en-1-one also functions as an antiaggregation pheromone (Furniss et al., 1974).

Renwick and Hughes (1975) obtained 1,2-MCH-ol, 3-methylcyclohex-2-en-1-one, 3,2-MCH-ol, and other derivatives from *D. frontalis* exposed to vapors of 1-methylcyclohex-1-ene and concluded that bark beetles may have a general metabolic system for oxidation of hydrocarbons. They also cited unpublished data of G.B. Pitman than an unidentified GC peak elutes at the same retention time (on FFAP), as 1,2-MCH-ol in analyses of female *D. pseudotsugae* hindguts.

An unidentified compound that superficially resembled 3,2-MCH-ol was reported from fed female Douglas-fir beetles by Ryker et al. (1979). We now report the synthesis, identification, and bioassay of 1-methylcyclohex-2-en-1-ol, a previously unidentified pheromone.

METHODS AND MATERIALS

Chemical Synthesis. 3-Methylcyclohex-3-en-1-one was prepared by reaction of 3-methylcyclohex-2-en-1-one with methylmagnesium bromide and ferric chloride in ether according to the procedure of Meinwald and Hendry (1971). The prescribed workup yielded 60% of a mixture, bp 40–45°C (4 mm Hg), which GLPC analysis revealed consisted of 1,3-dimethylcyclohex-2-en-1-ol (90%), 3-methylcyclohex-3-en-1-one (10%), and starting material (3%). GLPC analysis was performed on a 30-m × 0.21-mm-ID glass capillary column coated with Carbowax 20 M at 125°.

The mixture from this reaction was reduced with sodium borohydride in ethanol (80% yield) to give a mixture which GLPC analysis, as above, revealed to consist of 1,3-dimethylcyclohex-2-en-1-ol (90%), 3-methylcyclohex-2-en-1-ol (10%), and 3-methylcyclohex-3-en-1-ol (3%). The desired product, 3-methylcyclohex-3-en-1-ol, was isolated from the mixture by preparative GLPC at 100° on a 1/8-in. × 10-ft column packed with Chromosorb G and coated with 5% SP1000. NMR ($CDCl_3$) δ 1.65 (3H, d, $J = 4$ Hz), 1.73–2.3 (5H, m), 3.98 (1H, m, C<u>H</u>OH), 5.48 (1H, m, vinyl).

1-Methylcyclohex-2-en-1-ol was prepared by addition of methyl lithium (Aldrich) to cyclohex-2-en-1-one. The isolated product exhibited the following: bp 68–69°C (9 mm Hg); NMR ($CDCl_3$) δ 1.28 (3H, s, CH_3), 1.6–2.2 (7H, m), 5.60 (2H, m, vinyl).

GLPC analysis of both 3-methylcyclohex-3-en-1-ol and 1-methylcyclohex-2-en-1-ol by capillary GLPC, as above, revealed these compounds to be >95% pure.

Identification of Unknown. Volatile compounds of female *D. pseudotsugae* fed in fresh logs of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, were trapped on Porapak-Q® from air passed over living beetles and analyzed

by GC-MS in an earlier study (Ryker et al., 1979), which provided a reference mass spectrum of the unknown. Synthesized candidate compounds were injected into the GC-MS along with the marker compounds ethyl pentanoate and ethyl hexanoate, which were used to define I_E values (Van Den Dool and Kratz, 1963) for comparison between synthetic compounds and the unknown. The unknown eluted at I_E 5.03, which is rather early for an unsaturated cyclic alcohol, on our nonpolar SF-96 column. In comparison, 3,2-MCH-ol elutes at I_E 5.88 (Ryker et al., 1979). We used a stainless-steel capillary column 152 m × 0.76 mm ID with a helium flow of 15 ml/min, programed after 5 min at 2°/min from 80 to 160°C. The column was coupled to a Finnigan 1015C mass spectrometer interfaced to a Riber 400 data system. Electron impact mass spectra were obtained with approximately 475 μA ionizing current and 70 eV electron energy. Mass scans were taken every 5–6 sec, covering the range m/z 10–250. Data were acquired using IFSS (integration as a function of signal strength), a program that improves sensitivity on weak ions while avoiding electronic saturation on intense ions.

Laboratory Bioassay. 1,2-MCH-ol was screened for possible pheromone function by a bioassay of walking male beetles. The olfactory walkway (Jantz and Rudinsky, 1965) simulated the male approach to the female gallery. "Arrestment" over the vial of test compounds (Rudinsky and Michael, 1972) and stridulation of "simple" or "interrupted" chirps (Rudinsky and Ryker, 1976) were considered positive responses. The walkway was equipped for acoustic monitoring (Rudinsky and Ryker, 1976). 1,2-MCH-ol was tested in combination with 100 ppm frontalin and 100 ppm alpha-pinene in 95% ethanol, a standard attractant solution for *D. pseudotsugae* (Rudinsky and Ryker, 1980). Four concentrations of 1,2-MCH-ol, from 1 to 1000 ppm, were tested initially. The concentration that elicited the greatest response was then tested alone and in combination with alpha-pinene. Tests were run at 20–22°C and 30–40% relative humidity in a room darkened except for window light beyond the terminus of the walkway. Beetles stored at 4°C on moist paper toweling were warmed to room temperature several minutes before being tested individually, and each test lasted about 1 min. Three samples of 20 males each were observed for each treatment, June 5–19, 1981. Beetles were used within 5 days of emergence in the greenhouse. Sex of *D. pseudotsugae* was determined by the presence of minute tubercles on the elytral declivity of females and their absence in males (Jantz and Johnsey, 1964). Two-way ANOVA and Scheffé's test (Snedecor and Cochran, 1967) were utilized for multiple comparisons of means. Racemic frontalin used in this study was of 99% chemical purity (Albany International, Columbus, Ohio) and D-alpha-pinene was of 95% chemical purity, optical purity unknown (K & K Laboratories, Inc., Plainview, New York). The impurities in the alpha-pinene were determined by GC-MS to be monoterpene hydrocarbons.

Field Bioassay. 1,2-MCH-ol was tested for its effect on flying *D. pseudotsugae* in the Oregon Coast Range, 540 m elevation on Marys Peak, Siuslaw National Forest, April–May, 1982. Test compounds were evaporated from open 2-ml glass vials (½ dram) placed inside aluminum 35-mm film cans with a perforated bottom. The cans were placed 1 m high on a pole, inside cylindrical screen traps coated with Stikem Special® (Bedard et al., 1969; Furniss and Schmitz, 1971). Vials of compounds were weighed before and after testing to obtain evaporation rates of the attractants. The six treatments (see Table 2) were placed in traps in a rectangle formed by two rows of three traps, with a minimum of 25 m between traps. One to three replications were deployed for four different daily collections, for a total of ten replications. Treatments were assigned by random numbers to traps on each test day. Wilcoxon's matched-pairs, signed-ranks, nonparametric test, one-tailed, was used for statistical comparison to accomodate the large variations in flight due to changing temperature and other conditions on different test days.

RESULTS AND DISCUSSION

The similarities between the mass spectra of the unknown eluting at I_E 5.03 and that of 3,2-MCH-ol suggested an isomer with the methyl group, double bond, or both in different positions. The early elution of the unknown suggested a tertiary alcohol, perhaps the allylic isomer of 3,2-MCH-ol (1,2-MCH-ol), which was implicated as a candidate Douglas-fir beetle pheromone by the studies of Renwick and Hughes (1975) on *Dendroctonus frontalis*

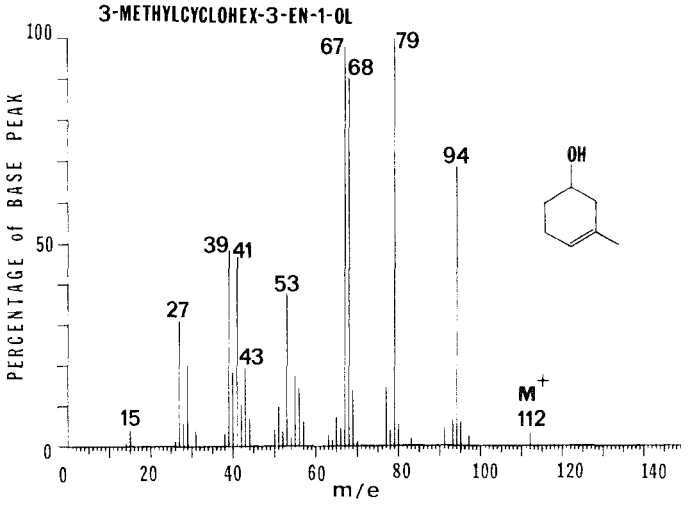

FIG. 1. Mass spectrum of synthetic 3-methylcyclohex-3-en-1-ol.

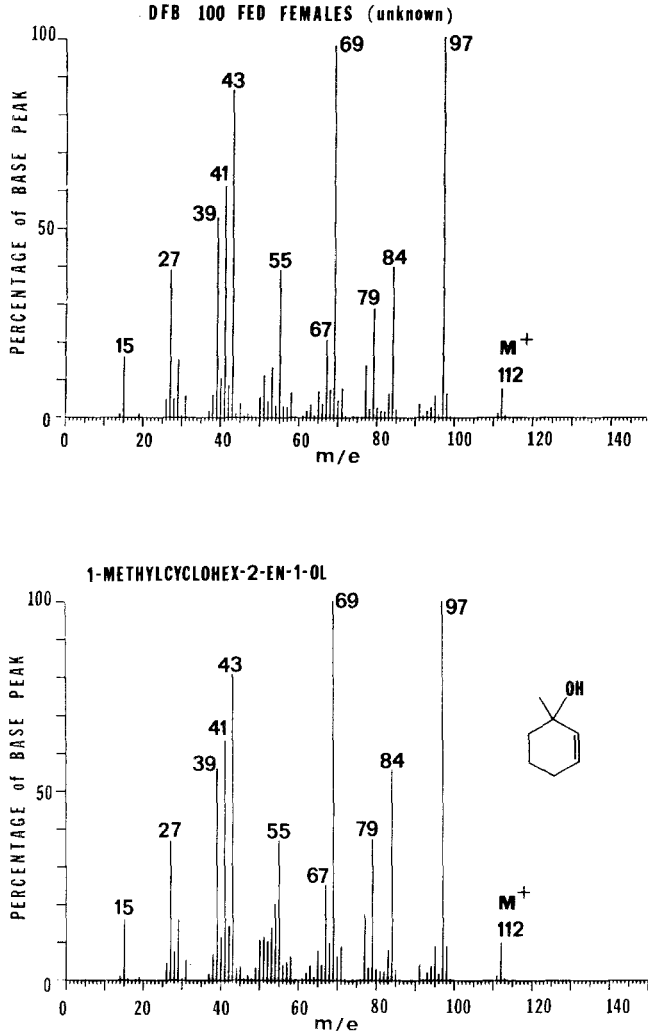

FIG. 2. Mass spectra of "unknown" compound with I_E 5.03 (SF-96) emitted by fed female *Dendroctonus pseudotsugae* (top), and synthetic 1-methylcyclohex-2-en-1-ol, which has I_E 5.05 (bottom).

Zimm. However, we selected 3-methylcyclohex-3-en-1-ol for initial synthesis as the most likely isomer on the basis of large ions of mass 97, 84, 55, 69, and 79 present both in the mass spectrum of the unknown and in mass spectra of 3,3-MCH-ol published in the Registry of Mass Spectral Data (Stenhagen et al., 1974) and the EPA/NIH Mass Spectral Data Base (Heller and Milne, 1978). 3,3-MCH-ol also seemed likely due to the presence of the 3,2- and

3,3-ketone derivatives in male *D. pseudotsugae* and of the 3,2-MCH-ol in the female.

The I_E value (on SF-96) for synthesized 3,3-MCH-ol was 6.00 rather than 5.03, and the mass spectrum (Figure 1) was unlike that of the unknown. Ions of mass 97 were present at 2% in authentic 3,3-MCH-ol as compared to 100% in the unknown; similarly, none of the other major ions were in agreement between the two compounds. Apparently, the mass spectrum listed as 3,3-MCH-ol in standard references (Stenhagen et al., 1974; Heller and Milne, 1978) is mislabeled; it closely matches the mass spectrum of 3,2-MCH-ol (Rudinsky et al., 1974).

Synthesized 1,2-MCH-ol eluted at I_E 5.05 and has a mass spectrum closely similar to the unknown compound in all major ions (Figure 2). We conclude that the unknown compound from female *D. pseudotsugae* (Ryker et al., 1979) is 1,2-MCH-ol. The enantiomeric composition of natural 1,2-MCH-ol is not known.

Laboratory Bioassay. The 1,2-MCH-ol prepared for bioassays was optically racemic and 99% pure, with trace amounts of cycloheptadiene, tricycloheptane, and benzofuran present as impurities after preparative GC. 1,2-MCH-ol added at 100 and 1000 ppm significantly ($\alpha = 0.05$) increased the arrestment of male *D. pseudotsugae* over vials containing 100 ppm frontalin and 100 ppm alpha-pinene (Table 1). Males also were more likely to stridulate with 100 ppm 1,2-MCH-ol present, especially producing the simple chirps characteristic of attraction to female frass (Rudinsky and Ryker, 1976). When

TABLE 1. RESPONSE OF MALE *Dendroctonus pseudotsugae* TO SEVERAL CONCENTRATIONS OF 1-METHYLCYCLOHEX-2-EN-1-OL WITH AND WITHOUT OTHER ATTRACTANTS IN OLFACTORY WALKWAY, JUNE 5–19, 1981

Stimulus	Mean ± SE of 20 males responding, 3 replications		
	Arrested	Simple chirps	Interrupted chirps
Attractant[a] (control)	8.3 ± 0.67	1.0 ± 0.57	0.0
+1 ppm 1,2-MCH-ol	11.3 ± 0.67	1.0 ± 0.57	0.0
+10 ppm 1,2-MCH-ol	15.7 ± 0.67	3.7 ± 0.33	1.0 ± 0.57
+100 ppm 1,2-MCH-ol	17.7 ± 0.33*[b]	6.7 ± 0.66*	1.7 ± 0.66
+1000 ppm 1,2-MCH-ol	19.0 ± 0.00*	3.0 ± 2.24	1.3 ± 0.33
α-Pinene (control)	4.3 ± 1.77	0.0	0.0
+100 ppm 1,2-MCH-ol	9.7 ± 1.67 NS	2.0 ± 1.15	0.0
Ethanol (control)	0.0	0.0	0.0
+100 ppm 1,2-MCH-ol	1.3 ± 0.58	1.3 ± 1.42	0.0

[a]Attractant contains 100 ppm each frontalin and D-alpha-pinene in 95% ethanol.
[b]Treatment significantly different from control at *$\alpha = 0.05$ level according to ANOVA and Scheffé's test for multiple comparison of means. NS = not significantly different.

TABLE 2. FIELD RESPONSE OF *Dendroctonus pseudotsugae* IN THREE TESTS OF 1-METHYLCYCLOHEX-2-EN-1-OL WITH AND WITHOUT OTHER ATTRACTANTS AT 540 m ELEVATION ON MARYS PEAK, NEAR CORVALLIS, OREGON APRIL–MAY 1982, 22–25°C

Treatment	$\bar{X} \pm$ SE of beetles trapped[a]			Sex ratio (♂/♀)
	Males	Females	Both sexes	
Empty trap	0.1	0	0.1	---
1,2-MCH-ol[b]	5.6 ± 1.59**[c]	3.5 ± 0.75**	9.1 ± 2.06**	1.6
α-Pinene, resin	0.9 ± 0.52	1.0 ± 0.39	1.9 ± 0.91	0.9
+ 1,2-MCH-ol	14.1 ± 3.21**	5.7 ± 1.25**	19.8 ± 4.15**	2.5
α-pinene, resin, and frontalin	6.9 ± 2.02	1.3 ± 2.37	8.2 ± 2.12	5.3
+ 1,2-MCH-ol	20.4 ± 6.10**	3.9 ± 0.75[NS]	24.3 ± 6.27**	5.2

[a]Ten replications.
[b]Evaporation rates measured in the field were 300 μg/hr for 1,2-MCH-ol, 40 μg/hr for frontalin, and 20 μg/hr for a 1:1 mixture of α-pinene + Douglas-fir resin.
[c]Significantly different at **α = 0.01 by Wilcoxon's matched-pairs, signed-ranks test, one-tailed; NS = not significantly different.

presented with alpha-pinene, 1,2-MCH-ol did not significantly affect arrestment, although there was a greater tendency for males to chirp. 1,2-MCH-ol at 100 ppm in ethanol slightly increased arrestment and stridulation (Table 1) compared to the ethanol control.

Field Bioassay. On the basis of trap catches, 1,2-MCH-ol was found to be slightly attractive to both male and female *D. pseudotsugae* when presented by itself (Table 2). Trap catches of both sexes increased significantly with the addition of 1,2-MCH-ol to alpha-pinene and Douglas-fir resin. When frontalin was included in the control attractant, the addition of 1,2-MCH-ol provided increased catches of males but did not significantly alter the catches of females (Table 2). Males were more responsive to the odor of 1,2-MCH-ol than females, as is also true for 3,2-MCH-ol and frontalin (Dickens et al., 1983).

Based on a 7% recovery of 3,2-MCH-ol released by females in our pheromone collection apparatus and the amounts of 1,2-MCH-ol trapped per female (Ryker et al., 1979), we estimate that each female released 3 ng/hr under laboratory conditions. This is comparable to the amount of 3,2-MCH-ol released under the same conditions, but how this compares with natural pheromone release is unknown. Field responses of *D. pseudotsugae* to 1,2-MCH-ol and 3,2-MCH-ol cannot be compared as they were not tested together, so it is premature to offer comparisons. However, the responses of male and female beetles seem qualitatively very similar (cf. Rudinsky et al.,

1974). Degradation, derivatization, and/or NMR analysis of the unknown would additionally confirm the designation of the unknown female volatile as 1,2-MCH-ol. However, the nearly identical mass spectra of the natural and synthetic compounds, as well as the very close correspondence of their retention indices (I_E 5.03 versus 5.05 on SF-96), are for us sufficiently convincing. On the basis of laboratory and field bioassays, we conclude that 1,2-MCH-ol is a female aggregation pheromone that attracts both sexes of *D. pseudotsugae*, with males predominating.

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OCCURRENCE OF 2,6-DICHLOROPHENOL IN HARD TICKS, *Hyalomma dromedarii* and *Hyalomma anatolicum excavatum*, AND ITS ROLE IN MATING[1]

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Abstract—2,6-Dichlorophenol (2,6-DCP) is the major volatile sex pheromone component in the extracts of *Hyalomma dromedarii* (35 ng/female) and *Hyalomma anatolicum excavatum* (20 ng/female). The GC fraction containing essentially pure 2,6-DCP, as well as an equal amount of synthetic 2,6-DCP, elicits from the male of each species a hierarchy of responses culminating in attempted copulation.

Key Words—*Hyalomma dromedarii*, *Hyalomma anitolicum excavatum*, ticks, Acarina, Ixodidae, pheromone, sex pheromone, 2,6-dichlorophenol, phenol.

INTRODUCTION

Hard ticks of the genus *Hyalomma* Koch are hardy ectoparasites, widely distributed in desert, semidesert, and savannah biotopes throughout Asia and Africa. *Hyalomma dromedarii* Koch parasitizes a variety of domestic animals and wildlife but is especially common on camels and other ungulates. This species transmits an impressive variety of viral, rickettsial, and protozoan

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pathogens (reviewed by Khalil et al., 1981). *Hyalomma anatolicum excavatum* Koch is a closely related species occurring throughout much of western Asia and North Africa, often sympatric with *H. dromedarii* and parasitizing many of the same hosts.

Mating behavior has been studied in *H. dromedarii* (Khalil et al., 1981) and *H. asiaticum* Schulze & Schlottke (Leonovich, 1981); in both cases, biological evidence implicated a volatile pheromone emitted by the female. Khalil et al. (1981) demonstrated that *H. dromedarii* males respond to optimal concentrations of authentic 2,6-dichlorophenol (DCP) applied to unattractive, engorged females. The response was indistinguishable from that to partially fed virgin females. Although these findings implicate 2,6-DCP as the volatile pheromone in *H. dromedarii*, chemical studies are needed to provide confirmation. Other evidence of 2,6-DCP occurrence in *Hyalomma* ticks was reported by Wood et al. (1975), who found this compound in extracts of *Hyalomma truncatum* Koch.

This study describes chemical evidence demonstrating the presence of 2,6-dichlorophenol in *H. dromedarii* and *H. anatolicum excavatum* and its function as the most important female sex attractant pheromone component.

METHODS AND MATERIALS

Ticks. H. dromedarii and *H. anatolicum excavatum* were colonized from engorged females collected from camels in the Imbaba camel market, Giza Governorate, Arab Republic of Egypt. Both species were reared in the NAMRU-3 Medical Zoology Department in an incubator at 28° ± 1° C and 75% relative humidity. All life stages were fed on rabbits, *Oryctolagus cuniculus*. All animal care and manipulations were done in accordance with the Animal Welfare Act of 1976 (PL 94-279 with subsequent ammendments). The ticks supplied for extraction were unfed females 2–4 weeks postemergence and fed males attached 9–10 days to a rabbit.

Chemistry. The fractionation scheme is shown in Figure 1. The female ticks collected in cold, reagent-grade, double-distilled hexane (Krackeler Chemical Company, Albany, New York) were forwarded to the Department of Chemistry, State University of New York, Syracuse, New York, in flame-sealed glass ampoules. The separate ampoules containing 1000 *H. dromedarii* females and 1100 *H. anatolicum excavatum* females were subjected to repeated cycles of freezing in liquid nitrogen, thawing, and sonication with a Bransonic II ultrasonic probe (Branson Sonic Power Company, Danbury, Connecticut (Sonenshine et al., 1976). For each species, the hexane was decanted from the residue, and the procedure was repeated twice with distilled pentane.

The hexane–pentane solution for each species was concentrated to about

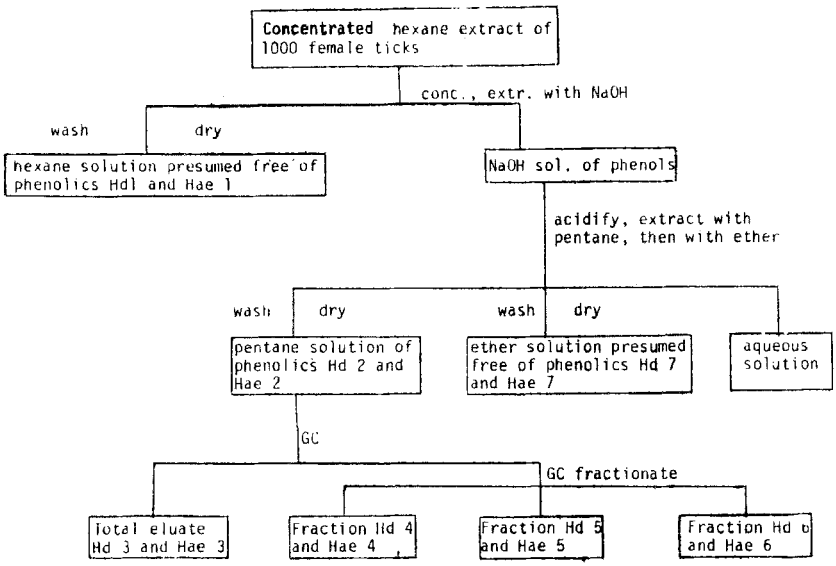

FIG. 1. Fractionation scheme for both species of ticks.

5 ml by solvent distillation through a packed column, and the concentrated solution was cooled and extracted three times with ice-cold 3% NaOH. The extracted hexane solution was washed with water and dried over Na_2SO_4 (extracts Hd 1 and Hae 1); these solutions were bioassayed to determine whether all the activity had been removed (see below). The cold NaOH solution was acidified with ice-cold 4% HCl to pH 2, and the acidified solution was extracted three times with pentane and once with diethyl ether. The pentane solutions from each species were each washed with water, dried over Na_2SO_4, and concentrated to 1 ml by solvent distillation through a packed column. An aliquot of each of the concentrated pentane solutions (phenolic extract Hd 2 and Hae 2) was injected on a gas chromatography (GC) column and the total eluates were collected without fractionation (Hd 3 and Hae 3). Another aliquot for each species was injected on the same GC column and three fractions were collected (Hd 4–6 and Hae 4–6). The ether solutions were dried, concentrated (extract Hd 7 and Hae 7), and bioassayed to determine whether all the activity had been removed by the pentane extraction of the acidified solution.

GC fractionation (flame ionization detector) was carried out as follows: 3.1-m × 4-mm-ID glass column, 5% OV-101 on Chromosorb G 50–80 mesh, 60 cm^3 N_2/min, 110° C for 26 min, then temperature programed to 200° C at 6 min. Under these conditions, 2,6-DCP eluted in 21 min. The presence of 2,6-DCP in fraction 2 (Hd 5 and Hae 5) (collected from 18 to 24 min) was verified

by means of an electron-capture detector (3.1-m × 1.5-mm-ID glass column, 4% FFAP on Chromosorb G 80-100 mesh, 20 cm^3 N_2/min, 170° C, retention time, 10 min.) Coinjection with an authentic sample of 2,6-DCP gave a single sharp peak. Capillary column chromatography (flame ionization detector) of fraction 2 (Hd 5 and Hae 5) gave a single sharp peak, as did coinjection with an authentic sample of 2,6-DCP (Varian 50-m × 0.21-mm-ID fused silica FFAP column).

Mass spectral electron impact data (70 eV), confirming the single peak in fraction 2 (Hd 5 and Hae 5) as pure 2,6-DCP, were obtained on a Finnigan Gc-MS-DS 4000 instrument interfaced with a Varian 50-m × 0.21-mm-ID fused silica FFAP column.

Bioassays. These were done as described by Khalil et al. (1981). Sexually active (SA) males were those allowed to feed on rabbits for 8 or 9 days and tested for their ability to respond to partially fed confirmed attractive virgin females (CAV females). The samples tested were the phenolic extracts (Hd 2 and Hae 2), the unfractionated gas chromatography (GC) collection of the phenolic extract (Hae 3), three GC fractions of the phenolic extracts (Hd 4–6 and Hae 4–6), and the material presumably free of phenolics (Hd 1 and 7, and Hae 1 and 7). Samples were diluted in hexane and deposited in aliquots of 1–5 μl onto attached, dead, fully engorged mated females, previously confirmed to be unattractive to SA males. The number of female equivalents (FE) in each sample was recorded. Several tests were repeated with different FEs to

TABLE 1. RESPONSES OF SEXUALLY ACTIVE MALE *Hyalomma dromedarii* TO CONSPECIFIC FEMALES AND EXTRACTS THEREOF[a]

Test material	Male responses (%)		
	O	M	AC
Live, partially fed unmated, females (CAV)	100	93	76
Dead, engorged, mated females	20	3	0
Dead, engorged, mated females + 1 μl hexane	30	13	0
Dead, engorged, mated females + 5 μl hexane*	30	16	0
Hd 1. NaOH-extracted hexane sol. (nonphenolic) 1 μl	20	3	0
Hd 1. NaOH-extracted hexane sol. (nonphenolic) 5μl	0	0	0
Hd 2. Pentane sol. of phenolics, 2 FE	73	36	26
Hd 2. Pentane sol. of phenolics, 1 FE	43	30	10
Hd 3. Total GC eluate of Hd 2	Sample lost		
Hd 4. GC fraction 1 of Hd 2 1 FE	56	20	0
Hd 5. GC fraction 2 of Hd 2 (contains 2,6-DCP) 1 FE	86	56	20
Hd 6. GC fraction 3 of Hd 2 1 FE	40	26	13
Hd 7. Ether extract of pentane-extracted aq. sol. (non phenolic)*	11	11	0

[a]Abbreviations: O, orientation; M, mounting; AC, attempted copulation. 10 SA males per test except for those marked*. *4 SA males.

TABLE 2. RESPONSES OF SEXUALLY ACTIVE MALE *Hyalomma anatolicum excavatum* TO CONSPECIFIC FEMALES AND EXTRACTS THEREOF[a]

Test material	Male responses (%)		
	O	M	AC
Live, partially-fed unmated females (CAV)	86	86	86
Dead, engorged, mated females	16	13	10
Dead, engorged, mated females + 1 μl hexane	10	10	6
Dead, engorged, mated females + 5 μl hexane	Sample lost		
Hae 1. NaOH-extracted hexane sol. (nonphenolic)	0	0	0
Hae 2. Pentane sol. of phenolics, 3 FE*	41	25	8
Hae 2. Pentane sol. of phenolics, 1.5 FE	66	50	33
Hae 3. Total GC eluate of Hae 2 1 FE	70	33	23
Hae 4. GC fraction 1 of Hae 2 1 FE	53	10	10
Hae 5. GC fraction 2 of Hae 2 (contains 2,6-DCP) 1 FE	56	26	20
Hae 5. GC fraction 2 of Hae 2 (contains 2,6-DCP) 0.5 FE	76	46	33
Hae 6. GC fraction 3 of Hae 2, 1 FE	90	50	13
Hae 7. Ether extract of pentane-extracted aq. sol. (nonphenolic)**	4	0	0

[a]Abbreviations: O, orientation; M, mounting; AC, attempted copulation. 10 SA females per test except for those marked * or **. *4 SA females; **8 SA females.

measure the effect of concentration. The positive control consisted of partly fed live females. The negative controls were one set of dead, engorged, mated females, and another set to which 1 μl or 5 μl of hexane was applied.

RESULTS

A hexane extract of 1000 unfed *H. dromedarii* females assayed by GC contained 35 ng of 2,6-DCP per tick. Grinding these residual tick remains with a mortar and pestle and reextracting with ether yielded only about 2% additional 2,6-DCP per tick. A hexane extract of 1100 unfed *H. anatolicum excavatum* females was found to have 20 ng of 2,6-DCP per tick when assayed by GC. No evidence of 2,6-DCP was found in the samples of fed *H. dromedarii* males (56 males).

Bioassay results for *H. dromedarii* are given in Table 1, and those for *H. anatolicum excavatum* in Table 2. Bioassays demonstrated highly significant orientation, mounting, and attempted copulation responses by SA males of each species to all samples containing 2,6-DCP: Hd 2 and Hae 2 (phenolic extracts), and Hd 5 and Hae 5 (GC fractions containing 2,6-DCP). However, significant responses were also obtained to the GC fractions that did not contain detectable amounts of 2,6-DCP (Hd 4, 6 and Hae 4, 6). Responses to

a dead engorged female with and without added hexane, and to presumably nonphenolic samples (Hd 1, 7 and Hae 1, 7), were much lower.

In tests with *H. dromedarii* males, 73% oriented, 36% mounted, and 26% attempted copulation when exposed to the phenolic extract (Hd 2) containing 2 FE; dilution to 1 FE resulted in 43% orientation, 30% mounting, and 10% attempted copulation. On the other hand, dilution of the phenolic extract from *H. anatolicum excavatum* (Hae 2) from 3 FE to 1.5 FE resulted in an increased response by males: from 41% to 66% orientation, from 25% to 50% mounting, and from 8% to 33% attempted copulation. Similarly, dilution of the GC fraction containing 2,6-DCP (Hae 5) from 1 FE to 0.5 FE resulted in an increased response by *H. anatolicum excavatum* males: 56% to 76% orientation, 26% to 46% mounting, and 20% to 33% attempted copulation.

The responses to the phenolic fractions were qualitatively similar to those elicited by a CAV female, but the percentage of males responding was higher in the three categories for the CAV female.

DISCUSSION

The identification of 2,6-DCP in *H. dromedarii* and *H. anatolicum excavatum* adds two more species to the list of ixodid ticks reported to contain this compound. Recently, Berger (1983) reported finding 2,6-DCP in both sexes of *Dermacentor albipictus* (Packard) and *Haemaphysalis leporis palustris* (Packard), while Rechav and Silverstein (unpublished) have identified this compound in *Hyalomma marginatum rufipes* (Koch). To date, 12 species, including those noted above, are now known to contain this compound. It is common to many species of the Metastriata, but it has not been found in any species of the Prostriata (genus *Ixodes* L.) or in species of the Argasidae. Moreover, Wood et al. (1975) did not find it in *Rhipicephalus compositus* Neumann, *R. simus* Koch, *R. pulchellus* Neumann, or *R. appendiculatus* Neumann. Males of the latter two species responded to mixtures of phenol and *p*-cresol, but not to 2,6-DCP. Clearly, it would be misleading to suggest that 2,6-DCP is the only sex pheromone in hard ticks or that its biological role is fully appreciated.

The differences in 2,6-DCP content in the two *Hyalomma* species probably reflect real differences rather than an artifact of sample preparation. Khalil et al. (1983) have observed maximum attraction of *H. dromedarii* SA males to concentrations of 2,6-DCP twice as high as that necessary to achieve maximum attraction of *H. anatolicum excavatum* SA males. Moreover, the latter were repelled by the same concentration of 2,6-DCP that was optimal for *H. dromedarii*. Similar trends were observed in the bioassays with the tick extracts described in this study. Indeed, the sensitivity of *H. anatolicum excavatum* males to very small quantities of 2,6-DCP may partially explain their high response to GC fraction 3 (Hae 6), which may have contained traces

of this compound carried over from the preceding fraction but undetected by GC. The ability of the ticks to modulate the secretion and perception of the volatile sex pheremone may provide an important means of discriminating between conspecific and heterospecific mates when the two *Hyalomma* species share a common host. In contrast, *D. variabilis* and *D. andersoni* males are unable to discriminate differences in 2,6-DCP concentration over a wide range (Sonenshine et al., 1976).

These findings confirm the role of 2,6-DCP as a sex pheromone in *H. dromedarii* and *H. anatolicum excavatum*. In addition, they indicate that other unidentified phenolic compounds also elicit responses in these species.

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Erratum

TRAIL PHEROMONE OF THE LEAF-CUTTING ANT, *Acromyrmex octospinosus* (REICH), (FORMICIDAE: MYRMICINAE)

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At top of p. 1120, add a CH_3 group to C-4 of structure I.

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