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EDITORIAL

Journal of Chemical Ecology began as a quarterly publication in 1975 with a total of 500 pages and has grown steadily from the beginning, becoming a bimonthly publication in 1977. Throughout this period, we have been gratified by the quality of manuscripts and by the rigorous, constructive reviews they have received. The Editorial Board surely deserves the bulk of the credit on both counts.

As we enter our fifth year of publication, we are projecting the need for 1000 pages, double the original number. With this growth have come increased production costs, and these costs are amplified by the effects of inflation. Not unnaturally, the publisher has been forced to increase the subscription rate but in our opinion has done well to minimize increases, while at the same time meeting our demands for excellent technical production.

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SEX PHEROMONE COMPONENTS OF THE OBLIQUEBANDED LEAFROLLER MOTH, *Choristoneura rosaceana*[1,2]

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Abstract—Previously, (*Z*)-11-tetradecen-1-yl acetate was reported as the sex pheromone of the obliquebanded leafroller, *Choristoneura rosaceana* (Harris). A mixture of 92% (*Z*)-11-tetradecen-1-yl acetate and 8% (*E*)-tetradecen-1-yl acetate has been reported as an attractant for this insect. (*E*)-11-tetradecen-1-yl acetate and (*Z*)-11-tetradecen-1-ol have now been isolated and identified from female tip extracts and shown to be behaviorally active; in addition, there is some evidence for the presence of (*E*)-11-tetradecen-1-ol in these extracts. The *Z*:*E* ratio for both the acetates and the alcohols in these tip extracts was about 98:2; the proportion of alcohols was rather variable, but the acetates always predominated. Maximum trap catches in the field during the course of this study were obtained with 5 mg of 95:5 (*Z*):(*E*)-11-tetradecen-1-yl acetates containing 0.5–10% (*Z*)-11-tetradecen-1-ol (approx. 1% *E* isomer) in polyethylene caps. No evidence was found for the presence of (*Z*)-11-tetradecenal in female tip extracts, and this compound was found to be ineffective in increasing trap catches.

Key Words—Sex pheromone, sex attractant, *Choristoneura rosaceana*, Lepidoptera, Tortricidae, obliquebanded leafroller, (*Z*)-11-tetradecen-1-yl acetate, (*E*)-11-tetradecen-1-yl acetate, (*Z*)-11-tetradecen-1-ol, (*E*)-11-tetradecen-1-ol, (*Z*)-11-tetradecenal.

INTRODUCTION

The obliquebanded leafroller moth, *Choristoneura rosaceana* (Harris) is widely distributed in the temperate regions of North America (Chapman

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et al., 1968). It is one of a complex of sympatric Tortricinae moths that feed on apple in the northeastern United States and southeastern Canada (Chapman and Lienk, 1971) and has been of moderate economic importance. It has been increasingly difficult to control in New York orchards and has become the most important leafroller species in terms of crop damage in western New York.

In 1970 the sex pheromone of *C. rosaceana* was reported to be (*Z*)-11-tetradecen-1-yl acetate (*Z*11–14:Ac) (Roelofs and Tette, 1970). This compound, containing varying amounts of (*E*)-11-tetradecen-1-yl acetate (*E*11–14:Ac) (approx. 2–10%), attracts male *C. rosaceana* in the field in modest numbers. A mixture of 92:8 *Z*11–14:Ac to *E*11–14:Ac has been reported as an attractant for this insect (Cardé et al., 1977). Also, 1–10% of (*Z*)-11-tetradecenal added to *Z*11–14:Ac has been reported to increase trap catches of this insect over those obtained with *Z*11–14:Ac alone (Weatherston et al., 1976). This paper describes the results of a search for other *C. rosaceana* pheromone components in addition to *Z*11–14:Ac, in an effort to define more accurately the sex pheromone system of this insect and to develop a better lure for it in the field.

METHODS AND MATERIALS

GLC columns (glass, 1.8 m × 2 mm or 1.8 m × 4 mm) were packed with 3% OV-1 (methyl silicone) on 100–120 mesh Gas Chrom Q and 10% XF-1150 (50% cyanoethyl methyl silicone) on 100–120 mesh Chromosorb W-AW-DMCS. Hydrogen flame ionization detection was used. Mass spectra were recorded with a Finnigan 3300 dual quadrupole EI mass spectrometer interfaced with an OV-101 column (Cornell University Mass Spectometry Center). (*Z*)-11-tetradecen-1-yl acetate (*Z*11–14:Ac), (*E*)-11-tetradecen-1-yl acetate (*E*11–14:Ac), (*Z*)-11-tetradecen-1-ol (*Z*11–14:OH), and (*E*)-11-tetradecen-1-ol (*E*11–14:OH) were purchased from Farchan Chemical Company. The *Z* isomers contained about 1% of the corresponding *E* isomer, and the *E* isomers appeared to be isomerically pure. *Z*11–14:Ac was freed of the *E* isomer by preparative TLC on 40% $AgNO_3$-silica gel. (*Z*)-11-tetradecenal (*Z*11–14:ALD) was prepared from *Z*11–14:OH using pyridinium chlorochromate (Corey and Suggs, 1975).

Insects were reared in a greenhouse on fava bean plants (Glass and Hervey, 1962) and after eclosion were collected and sexed. Female abdominal tips were extracted with redistilled methylene chloride. This procedure and the microchemical reactions used have been described previously in detail (Hill et al., 1977).

Pherocon® 1C traps (Zoecon Corp., Palo Alto, California) were used in the field tests and baited with the chemicals in polyethylene caps (OS-6

natural polyethylene closures, Scientific Products). Mean trap catches of male moths (x) were transformed to $\sqrt{x + 0.5}$, submitted to an analysis of variance, and ranked using Duncan's new multiple range test or Waller and Duncan's 1967 BSD rule; in the tables, means followed by the same letter are not significantly different at the 5% level.

RESULTS

Identification of Additional Pheromone Components. A crude *C. rosaceana* female tip extract on OV-1, 190°, showed peaks at 3.4 min and 5.25 min corresponding to the 11-tetradecen-1-ols (11–14:OH's) and the 11-tetradecen-1-yl acetates (11–14:Ac's), respectively. The ratio of 11–14:Ac's to 11–14:OH's in seven different samples of tip extract varied from 2.3 to 46; for five of these samples the ratio ranged from 2.3 to 15. On XF-1150, 170°, the 11–14:Ac's (previously collected from OV-1) showed peaks at 6.55 min, peak A, and 7.2 min, peak B, corresponding to *E*11–14:Ac and the previously identified *Z*11–14:Ac, respectively. A was 1.7% and B was 98.3% of the mixture.

Peak A was further characterized by ozonolysis, as follows. The 11–14:Ac's from a crude female tip extract were collected from OV-1, then collected from XF-1150 at the retention of *E*11–14:Ac, before elution of *Z*11–14:Ac. This sample was free of *Z*11–14:Ac, as determined by examination on XF-1150, and was ozonized in the usual way (−80°, CS_2) to yield 11-oxoundecan-1-yl acetate (retention time on OV-1, 150°, of 11.95 min, compared to 11.90 min for a sample generated from synthetic *E*11–14:Ac).

The 11–14:OH's from a crude female tip extract showed peaks on XF-1150, 160°, at 5.2 min, peak C, and 5.65 min, peak D, corresponding to *E*11–14:OH and *Z*11–14:OH, respectively. Treatment with acetyl chloride of the 11–14:OH's, after collection from OV-1, produced a material showing no peak on OV-1, 190°, at the retention of the 11–14:OH's (3.3 min), but with a peak at the retention time of the 11–14:Ac's (5.2 min). On XF-1150, 170°, the 11–14:Ac's produced by acetylation of the 11–14:OH's from *C. rosaceana* females showed peaks corresponding to *E*11–14:Ac (6.55 min) and *Z*11–14:Ac (7.2 min), in the relative quantities 1.9% and 98.1%, respectively. The original 11–14:OH's were regenerated by treatment of the acetylated 11–14:OH's with hot ethanolic NaOH for 1 hr, as determined by the reappearance of peaks on XF-1150, 170°, at 7.3 min and 7.9 min, corresponding to the 11–14:OH's, *E*11–14:OH and *Z*11–14:2OH, respectively.

Peak C was further characterized as *Z*11–14:OH by mass spectrometry and ozonolysis. An EI mass spectrum of the 11–14:OH's from *C. rosaceana* females collected from OV-1 matched that of a synthetic sample of *Z*11–14:OH. Ozonolysis of the acetylated 11–14:OH's from *C. rosaceana* females

produced 11-oxoundecan-1-yl acetate, as determined by its GLC retention times on OV-1, 160° (10.65 min, identical with that of a sample prepared by ozonolysis of synthetic Z11-14:Ac) and on XF-1150, 180° (14.25 min, compared to 14.20 min for the ozonolysis product of synthetic Z11-14:Ac). A search for the presence of (Z)-11-tetradecenal (Z11-14:ALD) in the female tip extract revealed no evidence for the presence of this compound.

Field Tests. The results of two field trials, in which the effect of varying amounts of E11-14:Ac in Z11-14:Ac on trap catches of *C. rosaceana* males was tested, are presented in Table 1. These two tests demonstrate that 5–8%, and possibly as high as 12%, of E11-14:Ac in Z11-14:Ac is the best range of mixtures for luring males of this species into traps at the release rate used. In test 1, 5% E11-14:Ac in Z11-14:Ac was a significantly better lure than the other percentages tested, but in test 2 a broader range of *E* isomer content

TABLE 1. EFFECT OF VARYING *Z*:*E* RATIOS OF 11-14:AC'S ON TRAP CATCHES OF *C. rosaceana* MALES

11-14: Ac's (5 mg) % *E*	$\bar{x}$ males/trap
Test 1[a]	
3	12.0 b
5	21.0 a
9	15.0 b
20	4.5 c
30	1.5 d
40	.6 d
60	.3 d
Unbaited	0 d
Test 2[b]	
0	0.2 g
2	2.4 fg
3	3.6 f
5	5.2 ef
6	6.2 ef
8	7.8 e
10	4.2 f
12	5.8 ef
17	0.6 g
Unbaited	0.0 g

[a] Conducted June 17–28, 1973, near Highland, New York; 5 replicates, rerandomized 3 times.

[b] Conducted June 29, to July 18, 1973, near Geneva, New York; 5 replicates, rerandomized 2 times.

TABLE 2. EFFECT OF VARYING RATIOS OF Z11-14:OH TO 97:3 (*Z*:*E*) 11-14:AC'S ON TRAP CATCHES OF *C. rosaceana* MALES[a]

Treatment			
11-14:Ac's (mg)	Z11-14:OH[b] (mg)	% of mix	$\bar{x}$ males/trap
5	0.025	0.5	67.4 ab
5	0.050	1	50.0 bcd
5	0.125	2.5	74.4 a
5	0.250	5	54.4 abc
5	0.500	10	45.6 cd
5	1	20	41.2 cd
5	2.5	50	39.2 cd
5	—		33.2 d
Zoecon® OBLR "beem" cap			48.2 bcd
Unbaited			3.2 e

[a] Conducted June 15, to July 7, 1977, near Geneva, New York; 5 replicates, re-randomized 10 times.
[b] Contained 1% *E*11-14:OH.

was optimal. Using 3% *E* in Z11-14:Ac, which approximates the natural ratio, the effect of added Z11-14:OH on trap catches was tested (Table 2), showing that small added quantities (0.5-5%) of the alcohol significantly increased trap catches. A lure available commercially from Zoecon Corp. (Palo Alto, California), presumably formulated with 8% *E* isomer but without the alcohol, was included for comparison and, even though it caught higher numbers of male moths than the other reference lure without alcohol, the best lure in the series (2.5% Z11-14:OH) was still significantly better than either of the two reference lures formulated without the alcohol. Another trial (Table 3), designed to test the effect of added Z11-14:OH on a number of *Z*:*E* ratios of the 11-14:Ac's, showed that the alcohol, added at the 5% level, significantly increased catches with 4.7, 8.2, and 11.5% *E* in Z11-14:Ac. Curiously, addition of the alcohol at this level to 3% *E* in Z11-14:Ac did not result in any significant increase in trap catch, as it had in the previous trial at the 2.5% level (Table 2). In this test, the best lures in the series without Z11-14:OH were the 1.4% and 3% *E*-isomer mixtures; in the series with Z11-14:OH, the best lures were the 4.7% and 8.2% *E*-isomer mixtures. The alcohol did not increase trap catches with 0.5, 1.4 and 15.7% *E* in Z11-14:Ac.

An additional trial (Table 4) tested the effect of Z11-14:ALD added to Z11-14:Ac, since this aldehyde has been reported to increase trap catches of *C. rosaceana* males when added at the 1 and 10% level to 100 μg of Z11-

TABLE 3. EFFECT OF Z11-14:OH AND VARYING Z:E RATIOS OF 11-14:AC'S ON TRAP CATCHES OF *C. rosaceana* MALES[a]

Treatment			
11-14:Ac's (5 mg) % *E*	Z11-14:OH[b] (mg)	% of mix	$\bar{x}$ males/trap
0.6			5.2 efg
1.4			6.8 cde
3.0			12.6 bc
4.7			5.6 def
8.2			2.4 fg
11.5			1.8 fg
15.7			1.2 g
0.6	0.25	5	9.2 cde
1.4	0.25	5	11.4 bcd
3.0	0.25	5	13.0 bc
4.7	0.25	5	25.2 a
8.2	0.25	5	16.2 ab
11.5	0.25	5	9.2 cde
15.7	0.25	5	2.0 fg
Unbaited			2.8 fg

[a] Conducted August 11, to September 19, 1977, near Geneva, New York; 5 replicates, rerandomized 6 times.
[b] Contained 1% *E*11-14:OH.

14:Ac in a polyethylene vial cap (Weatherston et al., 1976). The data in Table 4 show that the added aldehyde did not significantly change the trap catches from those obtained with a 91:9 mixture of *Z*:*E*11-14:Ac, whereas Z11-14:OH added to this mixture at the 3% and 10% levels resulted in significantly increased trap catches. The apparent discrepancy between our results and those of Weatherston et al. might be due to the presence of the third component, *E*11-14:Ac, in our test mixtures.

DISCUSSION

In addition to the one sex pheromone component previously reported for *C. rosaceana*, Z11-14:Ac (Roelofs and Tette, 1970), two additional compounds, *E*11-14:Ac and Z11-14:OH, have been isolated and identified from *C. rosaceana* female tip extracts using chemical and physical tests, and they have been shown to affect trap catches in the field. The presence of a fourth compound, *E*11-14:OH, was indicated. No evidence for the presence of Z11-14:ALD was found.

TABLE 4. EFFECT OF Z11-14:ALD AND Z11-14:OH ADDED TO Z11-14:AC ON TRAP CATCHES OF *C. rosaceana* MALES[a]

11-14:Ac's[b] (mg)	Z11-14:ALD[c] (mg)	% of mix	Z11-14:OH[d] (mg)	% of mix	x males/trap
5					11.0 bc
5	0.005	0.1			11.4 bc
5	0.050	1			6.6 cd
5	0.500	10			13.8 ab
0.1	0.001	0.1			0.8 e
5			0.005	0.1	5.2 d
5			0.015	0.3	10.8 bc
5			0.050	1	9.6 bc
5			0.150	3	20.0 a
5			0.500	10	18.2 a
Unbaited					0.4 e

[a] Conducted June 23, to July 20, 1976, near Geneva, New York; 5 replicates; rerandomized 4 times.
[b] Contained 9% *E*11-14:Ac.
[c] Contained 1% *E*11-14:ALD.
[d] Contained 1% *E*11-14:OH.

The *Z*:*E* ratios of the 11–14:Ac's most consistently found in the field to be the best in luring *C. rosaceana* males into traps were those containing 3–8% of the *E* isomer, with the 5% *E* isomer being the best in some of the field tests. This is somewhat different from the 2% *E*-isomer composition found consistently in the females. This type of discrepancy has been reported for other insects (Roelofs et al., 1974; Baker et al., 1978) and could be due to an unnatural release rate of the chemicals from the lures. Most of these release rates are probably different from the rate at which the corresponding female moth releases her pheromone when calling, and this can affect the induced male moth flight responses. For example, it is possible for unnaturally high release rates of the natural pheromone mixture to capture fewer males than the same high release rate of an unnatural ratio. A discussion of the possible importance of the flight activation and disruption thresholds involved in orientation to a lure is given elsewhere (Roelofs, 1978).

The blend described here for *C. rosaceana* is unique among the sex pheromones reported to date. One other Tortricinae, the redbanded leafroller, *Argyrotaenia velutinana* (Walker), is known to produce a similar but slightly different *Z*:*E* blend of the 11–14:Ac's (8% of the *E* isomer, compared to 2% of the *E isomer for C. rosaceana*) (Roelofs et al., 1975). Another Tortricinae, the dusky-back leafroller, *Archips mortuanus* (Kearfoot), is known to be attracted to a mixture of 2–10% *E* in *Z*11–14:Ac in larger numbers than to other tested mixtures of these two acetates; these trap catches are markedly increased by addition of (*Z*)-9-tetradecen-1-yl acetate and dodecyl acetate (Cardé et al., 1977). *A.velutinana* is trapped in largest numbers with 6–10% *E* in *Z*11–14:Ac when combined with dodecyl acetate. *C. rosaceana* is trapped best with 3–8% *E* in *Z*11–14:Ac with added *Z*11–14:OH. These three insects occur sympatrically in New York, where the field trapping tests were carried out, and are not known to interbreed. Although the best *Z*:*E* 11–14:Ac mixtures for trapping these three species overlap, the additional components found in each case can be expected to result in species specificity for the blend of each insect (Cardé et al., 1977). Additional components could be involved in any of these pheromones, and if so, would be expected to add further to the species specificity of each sex pheromone blend. In field tests, such as those described here, the unnatural release rates from the synthetic lures could decrease the species specificity of the blends tested, even though blend compositions may match the natural blends closely.

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ISOLATION AND IDENTIFICATION OF VOLATILES IN THE FOLIAGE OF POTATO, *Solanum tuberosum*, A HOST PLANT OF THE COLORADO BEETLE, *Leptinotarsa decemlineata*

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Abstract—The volatile compounds of *Solanum tuberosum* L., a host plant of the Colorado beetle, *Leptinotarsa decemlineata* Say, were isolated by successive vacuum steam distillation, freeze concentration, and extraction. The main components are *trans*-2-hexen-1-ol, 1-hexanol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, and linalool. The distribution of these compounds in a variety of plant families and their biosyntheses are reviewed. These leaf volatiles constitute a general green leaf volatile complex, being active in the olfactory orientation of the Colorado beetle and as such are probably of importance to various phytophagous insects.

Key Words—*Solanum tuberosum, trans*-2-hexen-1-ol, 1-hexanol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, biosynthesis, host plant selection, olfactory orientation, *Leptinotarsa decemlineata*, Colorado beetle, Coleoptera, Chrysomelidae, potato.

INTRODUCTION

As contrasted with the well-studied actions of nonvolatile primary and secondary plant substances (Schoonhoven, 1968; Staedler, 1976), our knowledge is incomplete on the role of plant "odors" in host selection by phytophagous insects, in regard to host plant orientation, and the initiation and continuation of feeding. The reported information forms a range of possibilities concerning plant odors: (1) consisting of one or a group of chemically related, host-plant-specific "key" components, e.g., organic sulfur compounds to *Hylemya antiqua* (Matsumoto and Thorsteinson, 1968a,b) and isothiocyanates of cruciferous species to *Plutella maculipennis* (Gupta and

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Thorsteinson, 1960a,b); (2) being composed of substances less specific to host plants like the large chemical class of terpenes and their derivatives acting on a variety of forest pest insects (see, e.g., Werner, 1972; Staedler, 1974; Selander et al., 1974); (3) existing as a complex, a mixture of generally distributed unspecific components (Rodriguez et al., 1976). In this case, the total "essence" is required in performing the particular sequence of behavioral steps in host selection by phytophagous insects.

Visser and Nielsen (1977) have shown that adult Colorado beetles are attracted by the volatiles of their host plant, potato. Besides this, other members of the Solanaceae are attractive, while most of the nonsolanaceous plant species elicit neutral or repellent responses in Colorado beetles, except for *Apium graveolens* (de Wilde et al., 1969) and *Tropaeolum majus*, which enhance positive anemotaxis. Host plant selection by this oligophagous insect is a catenary process, in which the initial olfactory orientation confines this selection process mainly to solanaceous plant species. Eventually, when contact results, the aim is not achieved by a sole "odd" substance, but is attained by a combination of feeding incitants, feeding stimulants, feeding cofactors, and inhibitors (Ritter, 1967; Hsiao, 1969). Analogously, a complex of volatiles composing the attractive plant odor probably exists. The present study elucidates the chemical complexity of host plant odor acting in the initial attraction of Colorado beetles.

METHODS AND MATERIALS

The chemical analysis of potato plant odor started with the isolation of the volatile components in sufficient quantities to allow identification with a mass spectrometer. Extraction can be applied as a first-step procedure, using relatively large quantities of low-boiling organic solvents. The extraction process entails two major disadvantages: impurities from the solvent may accumulate in the aroma concentrate, and nonvolatile components are isolated along with the volatiles. Therefore, we preferred distillation as a first-step procedure for the isolation of all volatile material (see review of isolation procedures: Weurman, 1969). For the isolation of potato plant volatiles, a series of methods were employed: vacuum steam distillation followed by freeze concentration in order to reduce the amount of solvent used in the final extraction.

One kilogram of fully grown potato plants (cultivar Pimpernel), i.e., mainly leaves, stems, some flowers and fruits equivalent to the overground parts of two plants, were harvested from the field and homogenized in a mixer with 5 liters of demineralized water at 3°C. The resultant slurry was passed through glass wool, and the filtrate was steam distilled in two halves (Ahrenst-Larsen and Hansen, 1964) by the apparatus shown in Figure 1. Nitrogen was passed through the flask containing the potato plant filtrate for 4 min to minimize

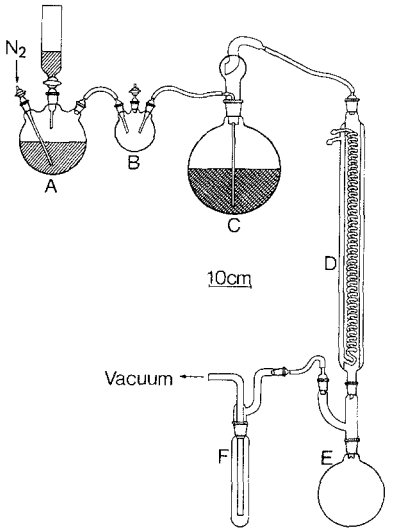

FIG. 1. Vacuum steam distillation apparatus. A: steam generator, filled with 1 liter of bidistilled water; B: steam drier; C: sample flask, containing 2.5 liters of potato plant filtrate; D: condenser; E: receiving flask, set in ice-water bath; F: cold trap, cooled in liquid nitrogen. Preceding the distillation, nitrogen was passed into the steam generator.

artifact formation during the distillation process (Nye and Spoehr, 1943). The steam distillation was carried out at reduced pressure (10–15 torr near the vacuum pump, 20–40 torr in the distillation system). At this reduced pressure, the water in the steam generator, set in a water bath kept at 42° C, was boiling, and the generated steam was bubbled through the potato plant filtrate, at a constant 39° C. The volatiles were transferred by the steam, condensed, and trapped into the receiving flask and a cold trap in liquid nitrogen. One liter of distillate was produced in one run. This amount was precooled to 2° C and transferred to the freeze concentration arrangement (see Figure 2). This technique had been reported to give high recoveries (>90%) after 20-fold concentration (Kepner et al., 1969). After a tenfold concentration the combined concentrates of two distillation runs (200 ml) were extracted three times with an equal volume of a mixture of diethylether and *n*-pentane, 1:2 by volume (Merck analytical grade, distilled before use). The extract was dried with sodium sulfate, followed by calcium sulfate at 4° C (Nursten and Williams, 1966). The solvents were carefully removed by distillation.

Analytical GLC separations were made on a Becker gas chromatograph (with flame ionization detector) fitted with packed polar (Carbowax 20M) and nonpolar (Apiezon L) columns. Mass spectra were recorded on the gas chromatograph (Varian 2700, fitted with a WCOT SP 2300 column)–mass

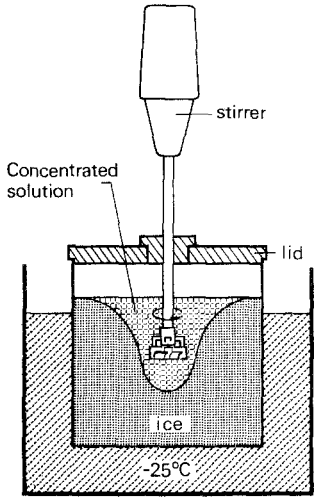

FIG. 2. Freeze concentration arrangement. A stainless-steel jar containing one liter of precooled distillate is inserted in a methanol bath of -25°C. While the mixture is cooled and stirred, the water is selectively frozen out, leaving the volatiles concentrated into a conical hole.

spectrometer (Varian MAT CH4) computer system (Varian Spectrosystem 100) of the Central Institute for Nutrition and Food Research TNO.

Authentic samples were obtained from commercial sources: the hexenols (98–99%) from Roth, *trans*-2-hexenal (99%) from Koch-Light Lab., linalool (99%) and 1-hexanol (99%) from Fluka.

RESULTS AND DISCUSSION

The main components of the oil (40 μl/kg)(Figure 3) were identified by coinjection with authentic samples on Carbowax 20M and Apiezon L columns, and by comparison of their mass spectra with those of authentic samples (Figures 4 and 5). The identity was further confirmed by analysis on the SP 2300 column. The mass spectra of *cis*-2- and *trans*-2-hexen-1-ol exhibited a high degree of similarity, as did *cis*-3- and *trans*-3-hexen-1-ol (Figure 4). However, GLC analyses of authentic samples showed consistent differences in their retention indices (Table 1) and allowed discrimination between geometrical isomers. The composition of the oil is shown in Figure 3, in decreasing order of magnitude: *trans*-2-hexen-1-ol, 1-hexanol, *cis*-3-hexen-1-ol, *trans*-2-hexenal, and linalool. At retention times >55 min only small quantities of compounds were detected. The most volatile components were lost in the final removal of the extractive solvents.

No evidence could be obtained that *cis*-2-hexen-1-ol, identified by Murray et al. (1972) as one of the components of *Solanum campylacanthum* oil, or *trans*-3-hexen-1-ol were present in the oil of potato plants. It is not clear to what extent the identification of *cis*-2-hexen-1-ol by Murray et al. (1972) had been based on mass spectral data only (see Table 1).

The main components of the potato plant oil are also present in other solanaceous plant species. They have been identified in *Solanum campylacanthum* leaves, potatoes, tomatoes, bell and tobasco peppers, and in the flowers of *Nicotiana alata* (see Table 2). These compounds are widely distributed in fresh foliage, vegetables, and fruits (Gildemeister and Hoffmann, 1960, 1963; Van Straten, 1977). The well-known leaf aldehyde 2-hexenal and the leaf alcohols 1-hexanol, 2-hexen-1-ol, and 3-hexen-1-ol have been reported as volatile components of numerous plant species belonging to a variety of plant families (Table 3).

The straight-chain, saturated and unsaturated aldehydes and alcohols are formed by oxidative degradation of plant lipids, as illustrated in Figure 6. Lipolytic acyl hydrolases liberate free fatty acids from the endogenous membrane lipids. The polyunsaturated fatty acids, linoleic and linolenic acid, are oxidized by the action of lipoxygenase to, respectively, hexanal and *cis*-3-hexenal.

Alcohol dehydrogenase converts hexanal to 1-hexanol, whereas *cis*-3-hexenal easily isomerizes to *trans*-2-hexenal and is converted to *cis*-3-hexen-1-ol. *Trans*-2-hexen-1-ol is formed from *trans*-2-hexenal. Possibly *trans*-3-hexen-1-ol and *cis*-2-hexen-1-ol originate from isomerization during processing and storage of plant products. These biosyntheses are operative in several

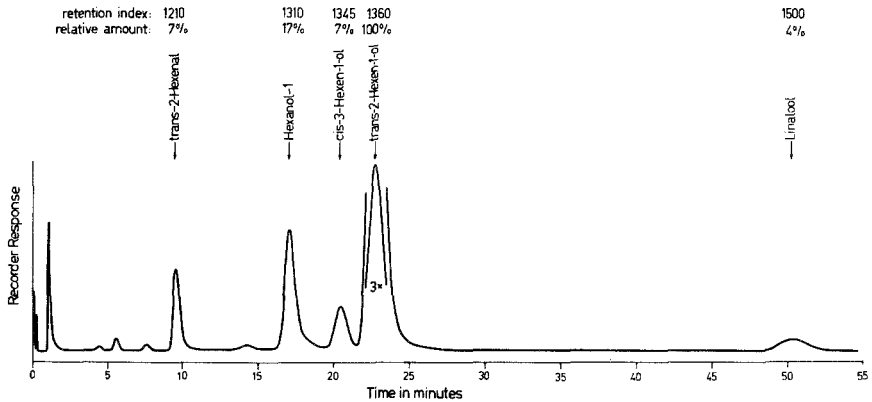

FIG. 3. Gas chromatogram of the potato plant oil: 0.1 μl of oil injected on a stainless-steel column, 3 m long and 4.4 mm ID, filled with 10% Carbowax 20M on Chromosorb P-AW-DMCS, 60–80 mesh. Chromatogram run isothermally at 100°C, carrier gas nitrogen at 30 ml/min. Retention indices were calculated according to Kováts (1961).

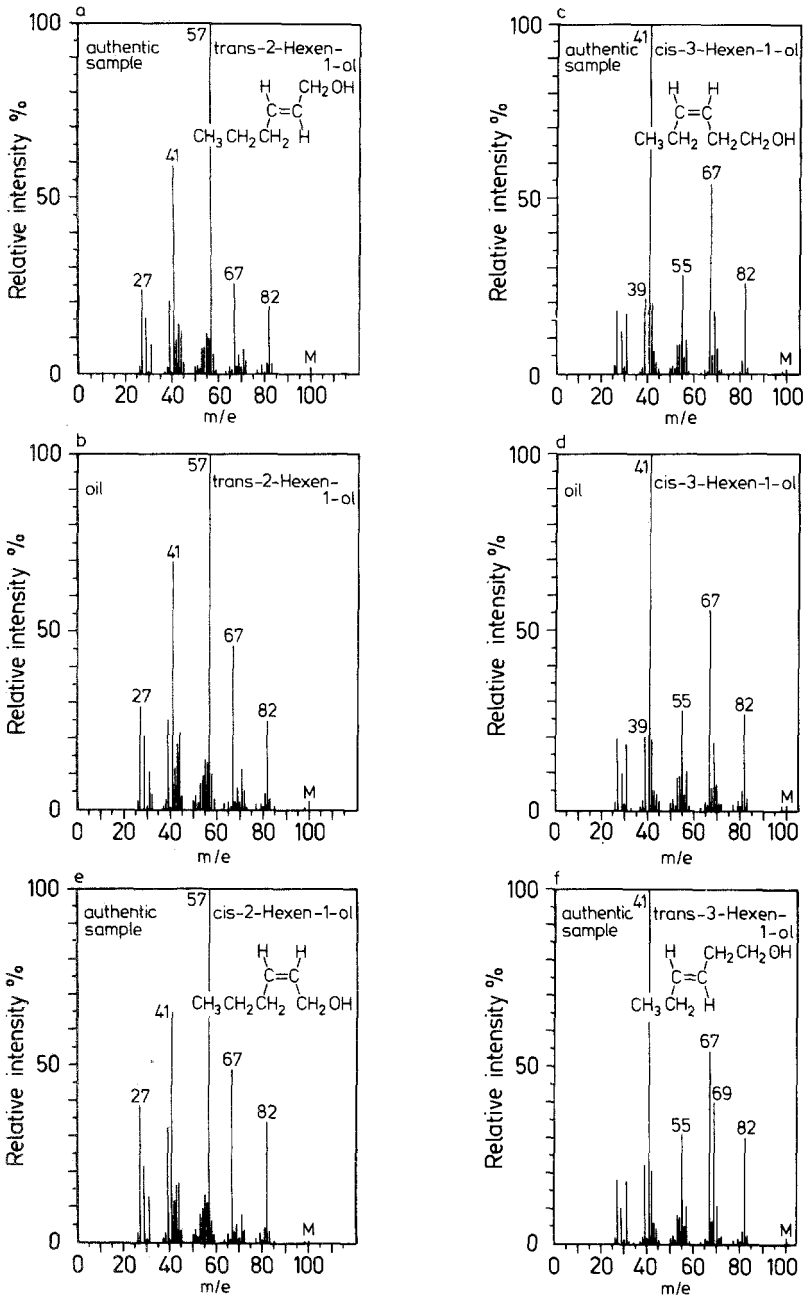

FIG. 4. Mass spectra of the main volatiles and of authentic samples.

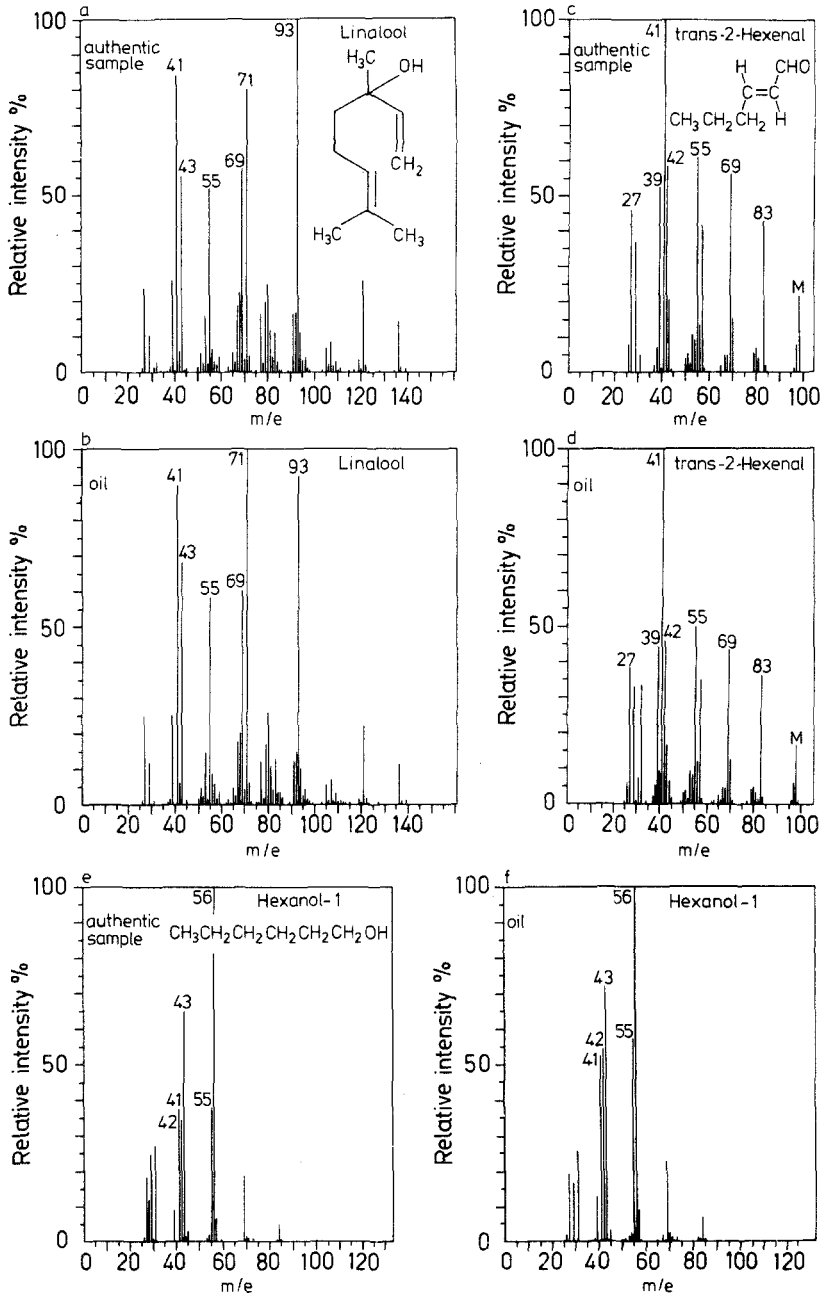

FIG. 5. Mass spectra of the main volatiles and of authentic samples.

TABLE 1. RETENTION INDICES[a]

	Present investigation 10% Carbowax 20M[b]		Murray et al. (1972) 8% Carbowax
	Authentic samples	Potato plant oil	*Solanum campylacanthum* oil
trans-2-Hexenal	1210	1210	1200
1-Hexanol	1310	1310	1312
trans-3-Hexen-1-ol	1320		
cis-3-Hexen-1-ol	1345	1345	1343
trans-2-Hexen-1-ol	1360	1360	
cis-2-Hexen-1-ol	1370		1360
Linalool	1500	1500	1500

[a] Retention indices were calculated according to Kováts (1961)
[b] See legend of Figure 3 for GLC conditions.

TABLE 2. DISTRIBUTIONS OF IDENTICAL COMPOUNDS IN THE OIL OF SOLANACEOUS PLANT SPECIES

Capsicum annuum
Bell peppers: *trans*-2-Hexenal,[1,21] *cis*-3-hexen-1-ol,[1,21] linalool[1,21]

Capsicum frutescens
Tobasco peppers: 1-Hexanol,[9] *cis*-3-hexen-1-ol[9,21]

Solanum campylacanthum
Leaves[12]: 1-Hexanol, 2-hexenal, *cis*-2-hexen-1-ol, *cis*-3-hexen-1-ol, linalool

Solanum lycopersicum
Tomatoes: Hexanol,[2,19,21] 1-hexanol,[4,5,7,11,13,14,17,22–24] 2-hexenal,[7,15,16] *trans*-2-hexenal,[2,6,11,14,18,21–24] *trans*-2-hexen-1-ol,[19,22] 3-hexen-1-ol,[20] *cis*-3-hexen-1-ol,[2,10,11,14,18,21–24] linalool[2,11,21,23]

Solanum tuberosum
Potatoes: 2-Hexenal,[8] *trans*-2-hexenal,[21] 2-hexenol[15]; Leaves[25]: 1-Hexanol, *trans*-2-hexenal, *trans*-2-hexen-1-ol, *cis*-3-hexen-1-ol, linalool

Nicotiana alata
Flowers[3]: 1-Hexanol, linalool

[1]Buttery et al., 1969; [2]Buttery et al., 1971; [3]Chang and Collins, 1972; [4]Dalal et al., 1967; [5]Dalal et al., 1968; [6]Galliard and Mattew, 1977; [7]Grosch, 1963; [8]Grosch et al., 1976; [9]Haymon and Aurand, 1971; [10]Johnson et al., 1968; [11]Kazeniac and Hall, 1970; [12]Murray et al., 1972; [13]Nelson and Hoff, 1969; [14]Pyne and Wick, 1965; [15]Ryder, 1966; [16]Schormüller and Grosch, 1962; [17]Schormüller and Grosch, 1964; [18]Schormüller and Kochmann, 1969; [19]Seck and Crouzet, 1973; [20]Shah et al., 1969; [21]Shankaranarayana et al., 1975; [22]Sieso et al., 1976; [23]Viani et al., 1969; [24]Wick, 1965; [25]present investigation.

TABLE 3. DISTRIBUTIONS OF LEAF ALDEHYDE 2-HEXENAL AND LEAF ALCOHOLS 1-HEXANOL, 2-HEXEN-1-OL, AND 3-HEXEN-1-OL IN VARIOUS PLANT FAMILIES

2-Hexenal:	*Polypodiaceae*[a] *Lauraceae*[a] *Piperaceae*[b] *Saxifragaceae*[b] *Rosaceae*[a,b] *Mimosaceae*[a] *Papilionaceae*[a,b] *Theaceae*[a,b] *Betulaceae*[a] *Fagaceae*[a] *Ulmaceae*[a] *Polygonaceae*[a] *Moraceae*[a] *Cruciferae*[a,b] *Myrtaceae*[b] *Umbelliferae*[a,b] *Caprifoliaceae*[a] *Oleaceae*[a,b] *Apocynaceae*[a] *Rutaceae*[a,b] *Aceraceae*[a] *Hippocastanaceae*[a] *Ericaceae*[b] *Vitaceae*[a,b] *Cucurbitaceae*[b] *Solanaceae*[b] *Labiatae*[a,b] *Salicaceae*[a] *Juglandaceae*[a] *Musaceae*[b]
1-Hexanol:	*Lauraceae*[a] *Saxifragaceae*[b] *Rosaceae*[a,b] *Papilionaceae*[b] *Theaceae*[a,b] *Violaceae*[a] *Passifloraceae*[b] *Caricaceae*[b] *Cannabinaceae*[b] *Cruciferae*[b] *Myrtaceae*[b] *Umbelliferae*[b] *Oleaceae*[b] *Rubiaceae*[b] *Geraniaceae*[a] *Rutaceae*[a,b] *Ericaceae*[b] *Vitaceae*[b] *Cucurbitaceae*[b] *Buettneriaceae*[b] *Solanaceae*[b] *Labiatae*[a,b] *Musaceae*[b] *Palmae*[b]
2-Hexen-1-ol:	*Saxifragaceae*[b] *Rosaceae*[a,b] *Papilionaceae*[b] *Theaceae*[b] *Cannabinaceae*[b] *Umbelliferae*[b] *Oleaceae*[b] *Rutaceae*[b] *Ericaceae*[b] *Vitaceae*[b] *Solanaceae*[b] *Labiatae*[b] *Musaceae*[b]
3-Hexen-1-ol:	*Piperaceae*[b] *Saxifragaceae*[b] *Rosaceae*[a,b] *Mimosaceae*[a] *Papilionaceae*[a,b] *Theaceae*[a,b] *Violaceae*[a] *Passifloraceae*[b] *Betulaceae*[a] *Fagaceae*[a] *Moraceae*[a] *Cannabinaceae*[b] *Cruciferae*[a,b] *Myrtaceae*[b] *Umbelliferae*[b] *Oleaceae*[b] *Geraniaceae*[a] *Rutaceae*[a,b] *Ericaceae*[a,b] *Vitaceae*[a,b] *Cucurbitaceae*[b] *Solanaceae*[b] *Labiatae*[a,b] *Gramineae*[a] *Musaceae*[b]

[a]Gildemeister and Hoffmann (1960, 1963).
[b]Van Straten (1977).

plant species: potatoes (Grosch et al., 1976), tomatoes (Galliard and Mattew, 1977; Kazeniac and Hall, 1970; Sieso et al., 1976), tea leaves (Hatanaka and Harada, 1973; Hatanaka et al., 1976a), peas (Grosch, 1968, 1969), apples and other fruits and leaves (Drawert et al., 1965, 1966), legumes like soybeans, and some cereal grains (Tappel, 1961), and are regarded as widely distributed.

Because enzyme inhibitors were not employed except for nitrogen in the present study, these products might be formed during the steam distillation of potato plant leaves and for that reason be regarded as biologically insignificant. However, vapor sampling of the air over potato plant leaves showed that these leaf alcohols and aldehydes are present (Visser and Schaefer, unpublished data).

These compounds are smelled by man as a grass-like odor. However, the ratio between the several products of this biosynthesis—the relative proportions of the different components—varies in and over different plant species. Within the same plant species, the proportions are modified seasonally (Hatanaka et al., 1976b) as caused by the expressions and/or the shift in the expressions of the several enzymes involved, owing to plant aging and injury (Buttery et al., 1971; Kazeniac and Hall, 1970; Sayo and Takeo, 1975). Consequently, unbalanced mixtures containing overdoses of one or more com-

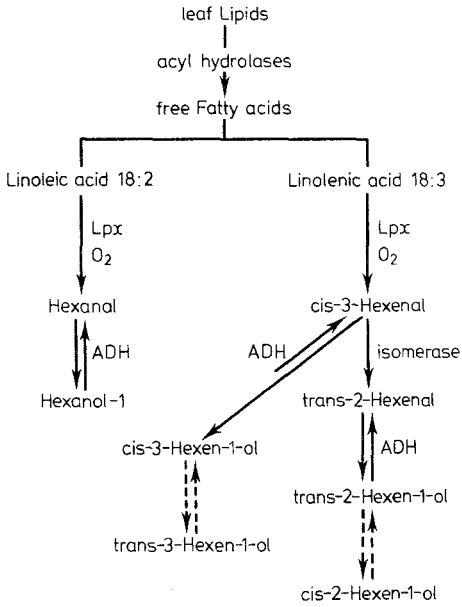

FIG. 6. The biosyntheses of the leaf aldehydes and alcohols, constituting a general green leaf volatile complex. Lpx: lipoxygenase (linoleate: oxygen oxidoreductase; EC 1.13.1.13); ADH: alcohol dehydrogenase (Alcohol: NAD oxidoreductase; EC 1.1.1.1). (According to: Drawert et al., 1966; Galliard and Mattew, 1977; Grosch, 1968, 1969; Hatanaka and Harada, 1973; Hatanaka et al., 1976a; Kazeniac and Hall, 1970; Sieso et al., 1976; Wardale and Galliard, 1977).

ponents, give an off flavor to the human sense, the haylike odors of several food products like deteriorated beans and peas (Whitaker, 1972).

In the same way, these leaf aldehydes and alcohols, constituting a general green leaf volatile complex, act in the olfactory orientation of the adult Colorado beetle. Electroantennogram recordings show the olfactory sensilla of the Colorado beetle to be mainly responsive to these types of compounds (Visser, 1979). In behavior tests none of these components, when applied singly, are attractive, whereas some of them, in minute quantities with potted potato plants, mask the attractive host plant vapors; that is, the beetles no longer react with an odor-conditioned positive anemotaxis (Visser and Avé, 1978).

The natural potato plant odor, attractive to Colorado beetles, appears to be the result of complex interactions between these leaf components. Detailed information of this system, probably operative to a variety of phytophagous insects, will be presented in subsequent papers.

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IDENTIFICATION OF THE SEX PHEROMONE OF THE YELLOW SCALE[1,2]

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Abstract—The sex pheromone of the yellow scale, *Aonidiella citrina* (Coquillett), was isolated from airborne collections and found to be (*E*)-3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate.

Key Words—Sex pheromone, yellow scale, *Aonidiella citrina*, Homoptera, Diaspididae.

INTRODUCTION

The yellow scale, *Aonidiella citrina* (Coquillett), is nearly a worldwide pest of citrus crops, although it is neither as abundant nor as serious a pest as the California red scale, *A. aurantii* (Maskell). From external morphology the two species are almost indistinguishable, and they can occur sympatrically. The existence of sex pheromones for the two species was demonstrated separately (Tashiro and Chambers, 1967; Moreno et al., 1972a) and then Moreno et al. (1972b) used live females and female extracts to show that the sex pheromonal systems were species specific.

The identification of the two components of the sex pheromone of the California red scale (Roelofs et al., 1977, 1978) generated interest in the yellow scale pheromone. Red scale males are attracted to sources emitting the individual pheromone components, 3-methyl-6-isopropenyl-9-decen-1-yl acetate and (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate, as well as the two compounds together. Had the yellow scale males been attractive to either individual compound, the redundant nature of the red scale system

[1] *Aonidiella citrina* (Coquillett) (Homoptera: Diaspididae).
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offered an interesting possibility for monitoring the population levels of both species. This could have been done by comparing the male catch of a trap emitting the one chemical attractive to both species with the red scale male catch of the trap emitting both chemicals. However, when the individual compounds extracted from female red scale were used in a greenhouse bioassay no yellow scale were caught. A program was then initiated to identify the sex pheromone of the yellow scale to compare with that of the California red scale and to eliminate the need for live female traps or time-consuming visual inspections that would be necessary to monitor natural population levels.

METHODS AND MATERIALS

Pheromone-containing extracts were obtained from airborne collections of yellow scale reared on lemons and wild gourd, *Cucurbita foetidissima* (H.B.K.), using Porapak Q in the same way as with the California red scale (Roelofs et al., 1978). Greenhouse bioassays using a turntable olfactometer were also performed in a fashion similar to that reported earlier.

The crude extract obtained by washing the Porapak Q with Skelly B, a hydrocarbon solvent, was chromatographed first on a 1.5- × 50-cm column of florisil (60–80 PR mesh, Floridin Co.) eluted with 900 ml of a 0–50% gradient of diethyl ether in Skelly B. Fractions were collected (15–20 ml each), portions of every second one were bioassayed, and the active fractions combined. Aliquots of this material were applied to each of the following GLC columns: 3% OV-101 (methyl silicone on 100–120 mesh Gas-Chrom Q; 2-m × 4-mm glass column with carrier flow of 40 ml/min) at 160° or 10% XF-1150 (50% cyanoethyl methyl silicone on 100–120 mesh Chromosorb W-AW-DMCS; 2-m × 2-mm glass column with carrier flow of 25 ml/min) at 165°. Eventually, the pure pheromone was obtained by collecting the florisil-active fractions from OV-101 and XF-1150 sequentially.

A mass spectrum of the pheromone was obtained at 20 eV on a Hitachi RMU-6E interfaced with an OV-1 column. An aliquot of the pheromone was hydrogenated at atmospheric pressure with platinum oxide in Skelly B, and a mass spectrum of this material was also obtained.

In order to determine if the compound was an acetate, a portion of the purified material was reduced with $LiAlH_4$ in diethyl ether. The product was collected from the GLC (OV-101), and half of the collected sample was treated with acetyl chloride. The original compound, the reduction product, and the acetylated material were simultaneously bioassayed.

Microozonolyses were performed in CS_2 according to Beroza and Bierl (1967), except that it was not necessary to add triphenylphosphine to complete the degradation of the ozonide. One synthetic standard, 3-oxo-4 methyl-

pentanal, was prepared by oxidizing 2-methyl-5-hexen-3-ol to the corresponding ketone using pyridinium chlorochromate buffered with sodium acetate (Corey and Suggs, 1975) and ozonizing to the desired product. The second standard, 3-methyl-5-oxopentan-1-yl acetate, was prepared by acetylating 3-methyl-5-hexen-1-ol with acetyl chloride and ozonizing the product to form the desired compound. Mass spectra were obtained from both synthesized materials to confirm their identity.

A proton magnetic resonance (PMR) spectrum of the pheromone was obtained on a Varian XL 100 (Fourier Transform) spectrometer (College of Environmental Science and Forestry, Syracuse, New York) in C_6D_6 using C_6H_6 as an internal standard.

RESULTS AND DISCUSSION

Table 1 shows the bioassay results at the three stages of the pheromone purification. Crude extract from the airborne collections of yellow scale reared on lemons was first applied to florisil and the active fractions combined. This material was then collected from OV-101 and XF-1150. This procedure yielded one compound that had a carbon number, relative to

TABLE 1. BIOASSAY RESULTS DURING PHEROMONE PURIFICATION: CATCHES OF MALE YELLOW SCALE, *Aonidiella Citrina* (COQUILLETT)

Florisil[a]		OV-101[b]		XF-1150[c]	
Fraction number	Number of males caught	Fraction number	Number of males caught	Fraction number	Number of males caught
2-	0	1-	2	1-	0
4-	0	2-	0	2-	0
6-	0	3-	0	3-	0
8-	2	4-	0	4-	0
10-	2	5-	0	5-	1
12-	0	6-	0	6-	1
14-	0	7-	0	7-	26
16-	10	8-	1	8-	5
18-	43	9-	51	9-	2
20-	38	10-	2	10-	1
22-	22	11-	0	11-	1
24-	0	12-	0	12-	2
26-44	6 (total)	13-16	2 (total)	13-24	3 (total)

[a]Crude pheromone extract from airborne collection.
[b]Active fractions (16-22) from florisil.
[c]Active fraction (9) from OV-101.

saturated straight-chain acetates, of 12.90 on both of the GLC columns used. The pheromone was shown to be an acetate as no male yellow scale were attracted to the compound after treatment with $LiAlH_4$, but acetylation of the reduced material restored the biological activity to that of the original pheromone.

Mass spectra of the attractant (YS) and of the hydrogenated material (YS-H_2) (Figure 1) indicated that the compound was a branched 15-carbon acetate with 2 units of unsaturation. Also, the presence of a strong M-60-43 peak in both spectra indicated the facile loss of 3 carbons after initial loss of acetate. To ensure that the attractant was not a host volatile, some yellow scale were reared on wild gourd. The active material was collected and purified in the same manner as above and a mass spectrum that was identical to that of the pheromone produced by scales reared on lemons was obtained. All further steps were performed on extracts from lemon-reared scales.

Ozonolysis produced two products visible on the GLC that eluted after the CS_2 solvent. The compounds eluted in the same order from both polar and nonpolar GC columns with retention times on OV-101 (initial column temperature of 50° held for 7 min, then programmed 10° / min to 120°) of 6.35 min and 15.05 min and on XF-1150 (initial column temperature of 60° held for 8 min, then programmed 10° / min to 120°) of 6.20 min and 15.05 min. The retention times of synthetic 3-oxo-4-methylpentanal and 3-methyl-5-oxo-pentan-1-yl acetate were identical to those of the ozonolysis products on both GLC columns as determined by coinjection. An attempt was made to obtain mass spectra of the two pheromone ozonolysis products. The compound with the longer retention time produced a spectrum essentially identical to that of the aldehyde-acetate synthetic. However, the more volatile ozonolysis product could not be sufficiently separated from the solvent to yield a useful spectrum.

The PMR spectrum of YS (Figure 2) is consistent with the proposed structure with δ values as follows: 5.12–5.36 (2H, m) [vinylic]; 4.12 (2H, t) and 1.72 (3H, s) [$—CH_2—C\underline{H}_2—O—CO—C\underline{H}_3$]; 2.83 (2H, d) [$R_2C{=}CH—C\underline{H}_2—CR'{=}CHR''$]; 2.3 (1H, m) and 2.0 (2H, m) [allylic]; 1.65 (3H, s) and 1.70 (3H, s) [$RCH{=}C(C\underline{H}_3)_2$]; 1.2–1.6 (3H, m) [saturated]; 1.08 (6H, d) [$RCH(C\underline{H}_3)_2$]; and 0.88 (3H, d) [$R,R'CH—C\underline{H}_3$].

These data show the structure of the sex pheromone of the yellow scale to be 3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate. To confirm this, a racemic synthetic sample of the *E* and *Z* isomers (4:1 ratio, respectively) of the pheromone was prepared by R.J. Anderson and C.A. Henrick (Zoecon Corp.). The isomers were separated by GLC collection from the XF-1150 column. The *E* isomer, which eluted first, had the same retention time as the natural material and had an identical PMR. When 30 ng of the *E* and *Z* isomer were tested individually against blanks in the greenhouse bioassay, the mean catch of 4 replicates was 39.3, 6.5, and 3.5 males, respectively. The physical

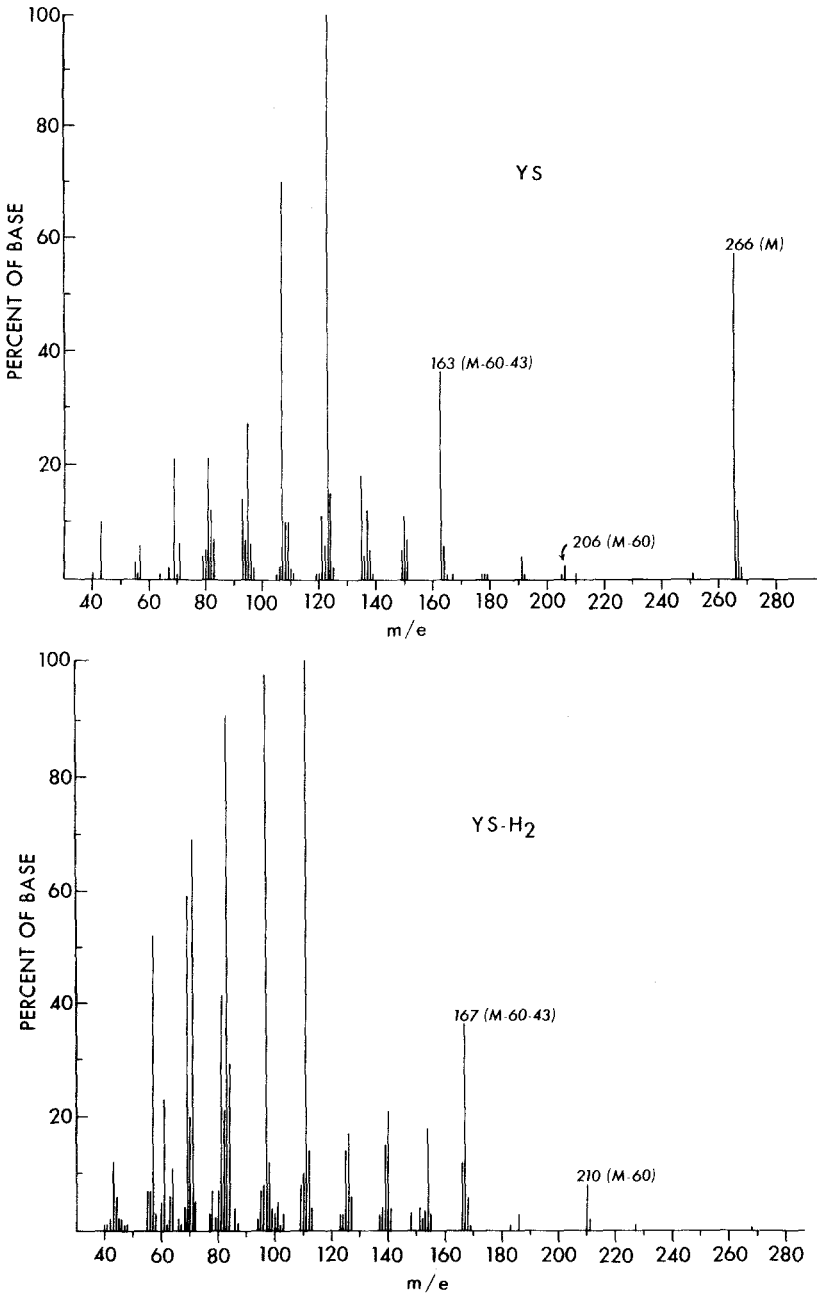


FIG. 1. Mass spectra (20 eV) of the yellow scale pheromone (YS) and its hydrogenation product (YS-H_2).

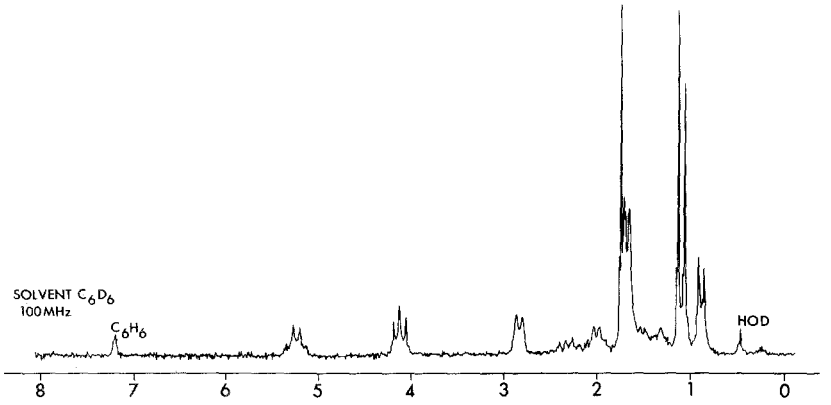
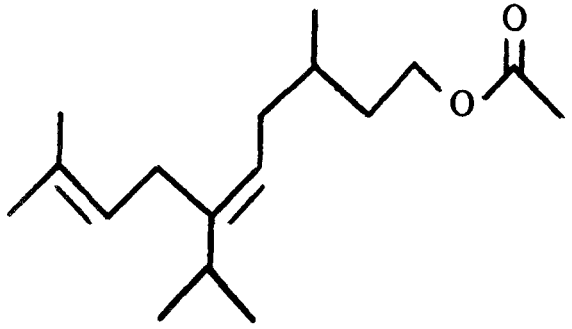


FIG. 2. PMR spectrum of the yellow scale pheromone.

data and the specificity of the yellow scale males for the *E* isomer clearly indicate that the natural pheromone has the structure of I.



I

The configuration of the 3-methyl group is presently unknown.

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TANDEM CALLING PHEROMONE IN THE GENUS *Leptothorax* (HYMENOPTERA: FORMICIDAE): BEHAVIORAL ANALYSIS OF SPECIFICITY

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Abstract—Ants of the genus *Leptothorax* recruit to new nest sites and newly discovered food sources by tandem running. This involves one ant directly leading a nestmate to the target area. Pheromones from the poison gland have proved the most important signal employed during this recruitment behavior. Comparative behavioral analysis with 11 species of *Leptothorax* demonstrated a subgenus specificity of the tandem calling pheromone between the subgenera *Leptothorax* and *Mychothorax*.

Key Words—Hymenoptera, Formicidae, *Leptothorax*, *Mychothorax*, *Harpagoxenus americanus*, recruitment, pheromone, poison gland, specificity, comparative behavioral analysis.

INTRODUCTION

Tandem running is a recruitment technique used by different ant species during foraging and nest emigration (Wilson, 1971; Hölldobler, 1978). One nestmate at a time is led by a successful scout to a newly discovered food source or nest site.

In the three subfamilies where tandem running has been reported so far, the basic pattern of this recruitment technique appeared to be quite similar. However, analysis of the behavior and communication signals involved reveals some basic differences in the organization of this recruitment technique (Hölldobler et al., 1974; Maschwitz et al., 1974; Möglich et al., 1974).

In the subfamily Myrmicinae (*Leptothorax acervorum*) we discovered a new kind of communication signal which we named tandem calling. Recruiting workers invite nestmates to tandem following by extruding the

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sting and releasing a secretion from the poison gland. This substance attracts nestmates, and one of them is then led to the target area. The pheromone also "binds" the following ant to the leading ant (Möglich et al., 1974).

It has been suggested that tandem running could have served as a preadaptation for the evolution of chemical mass recruitment (Wilson, 1971; Möglich et al., 1974; Hölldobler, 1978). To learn more about possible steps in the evolution of ant recruitment systems, one has to concentrate on the detailed analysis of differences in recruitment techniques among closely related taxonomic units. This article focuses on differences found within the genus *Leptothorax*, and the closely related genus *Harpagoxenus*. The genus *Leptothorax* is especially promising because we know that elements of both conceivable steps to chemical mass recruitment (tandem running and group recruitment) occur even within individual species, such as *Leptothorax longispinosus, L. curvispinosus,* and *L. ambiguus* as hosts of *L. duloticus,* (Alloway, personal communication).

METHODS AND MATERIALS

Mature colonies of *Leptothorax* are relatively small (30–200 workers) and therefore can be collected as a whole in the field and housed in laboratory nests. I used artificial nest chambers built out of plaster of Paris or constructed out of cardboard covered with slide-glass plates ($2 \times 6 \times 0.2$ cm). Some colonies were collected by T.M. Alloway [*Leptothorax muscorum* (New World), *L. duloticus*, *Harpagoxenus americanus*] and were kept in small petri dish nests as described by Alloway (1978). Both the glass and petri dish chambers allowed observation without disturbing the behavior inside the nest. These nests could also easily be transfered into larger plaster of Paris arenas (75×29 cm) where bioassays were conducted. The ants were fed with honey water and insect prey (cockroaches) and with a special ant diet (Bhatkar and Whitcomb, 1970).

To analyze the recruitment signals, colonies were first starved for a period of 3–20 days (typically about 2 weeks to elicit best frequency of tandem recruitment). Then they were placed in the test arena. There a watch glass with honey water was offered 60 cm from the nest entrance. Soon scouts would discover the food source and would start recruiting nestmates by the tandem running technique. Leader and follower ants could now be tested for their responses to various stimuli in a series of dummy experiments. To prevent the premature release of glandular secretions, workers were killed by deep freezing. Differently treated body parts obtained from these ants were attached to find needles with which they could be handled during the dummy tests.

Furthermore, full and undamaged sting glands were dissected out of the

ants by slightly squeezing the tip of the gaster and pulling out the whole sting apparatus. Only those Dufour's glands and poison glands were used which showed no sign of depletion after the procedure. The glands were prepared on the same day the experiment was conducted and were stored in Ringer's solution (0.75% NaCl; 0.038% KCl; 0.028% $CaCl_2:2H_2O$). When testing individual glands, they were separated from the sting and attached to the tip of very fine dissecting forceps (Dumont, Swizerland, #5). Then the gland was carefully crushed with an applicator stick. The forceps with the glandular preparation were then used as pheromone dummies during the bioassays. During pauses in the experiments, the forceps with the attached glands were stored in chambers with humid air to prevent fast evaporation of the secretion. Further experimental details will be given with the results of individual experiments.

RESULTS

Behavior during Tandem Recruitment. The first investigations were conducted with three palearctic species: *Leptothorax acervorum*, *L. muscorum,* and *L. nylanderi.*[2] When a successful scout of *Leptothorax acervorum* returns to the nest, she rapidly antennates several nestmates. Food exchange is frequently observed. Then the scout turns around, raises her gaster into a slanting position (Figure 1a). Simultaneously the sting is extruded and a substance is released. Nestmates are obviously attracted by this calling behavior and approach the scout ant. When the caller is touched on the gaster or hind legs by the antennae of a nestmate, she lowers her gaster and starts to lead the nestmate to the food source by the tandem running technique (Figure 1b and c). In following, the nestmate keeps close contact to the leader ant while continuously antennating the leader's hind legs. The sting of the leader ant remains slightly extruded (Figure 1d) but is not dragged over the surface as is observed in most species which lay chemical trails with sting gland secretions. If the tactile contact is interrupted, the leader ant immediately assumes the calling position. This can last for several minutes during which the substance is being discharged continuously. Usually the follower finds the calling ant quickly and the tandem continues as described.

The other palearctic species investigated (*Leptothorax muscorum, L. nylanderi*) as well as the studied species of the North American continent (see Table 2) showed a similar tandem calling behavior except that some species raise their gaster less conspicously than does *L. acervorum.*

Analysis of Communication Signals. Very simple tactile signals on hind legs and gaster provide the information for the leader ant that a nestmate is following. We were able to imitate the signals with a hair, and could induce the

[2]A preliminary report of these studies has been published by Möglich et al. (1974).

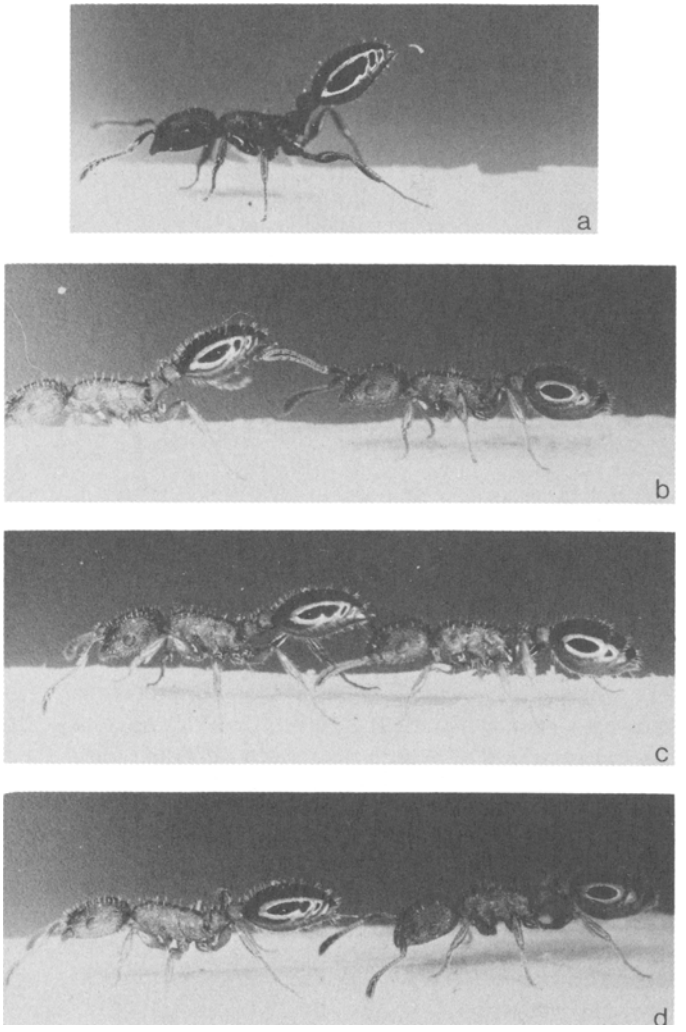

FIG. 1. Tandem running in *Leptothorax acervorum*.
(a) Tandem calling worker (sting is extruded and substance is discharged); (b) nestmate responds to tandem calling and touches calling worker at gaster; (c) calling worker lowers gaster and starts tandem running; (d) during tandem running sting remains slightly extruded.

calling ant to continue her way, after she had lost her follower (Möglich et al., 1974).

To find out which signals are important to attract nestmates to follow in tandem, we conducted a series of dummy experiments. After the leader ant was removed, we offered a test dummy to the searching follower ant and

recorded whether or not she would follow. The dummy was considered to be effective (positive response), if we were able to lead the ant for at least 4 times her body length in randomly changing directions. To control for a possible bias in handling the dummies differently, "double-blind" pilot experiments were conducted. The different dummies had code numbers so that the experimenter was unaware of the identity of the dummy he was testing.

Since the behavioral observations suggested that some part of the sting might be responsible for the tandem calling effect, we first dissected the whole sting apparatus with glands attached (Dufour's and poison gland) and tested it against control dummies (empty forceps). As the results in Table 1 (A and B) show, the leader ant could successfully be replaced by the dummy. Subsequently we discovered that we did not even have to remove the leader ant but could "compete" directly with her by trying to insert the dummy between her and the follower ant. We were even able to "take over," provided the tested dummy was effective.

When we finally tested Dufour's gland against poison gland, the poison gland clearly was revealed as the source of the tandem calling pheromone (Table 1, C and D). Figure 2 shows a *Leptothorax* worker (in this case *L. rugatulus*) following an odor dummy with poison gland secretion.

Next we investigated whether or not the tandem calling pheromone produced in the poison gland is the only chemical communication signal

TABLE 1. SUMMARIZED RESULTS OF DIFFERENT DUMMY EXPERIMENTS[a]

Test	Species tested	Signal Quality tested	No. of tests	Positive response (%)
A	ACE	Empty forceps	10	0
B	ACE	Sting apparatus with glands attached	56	100
C	ACE	Dufour's gland	69	0
D	ACE	Poison gland	312	100
E	ACE	Gaster without sting glands	126	5.6
F	ACE	Same as E, but extracted	90	1.1
G	NYL	Poison gland	183	100
H	NYL	Gaster without sting and attached glands	141	90.1
I	NYL	Same as H, but from different colony	189	97.4
J	NYL	Head	23	100
K	NYL	Thorax with legs	25	100
L	NYL	Same as H, but extracted	90	100

[a]Species abbreviations see Table 2.

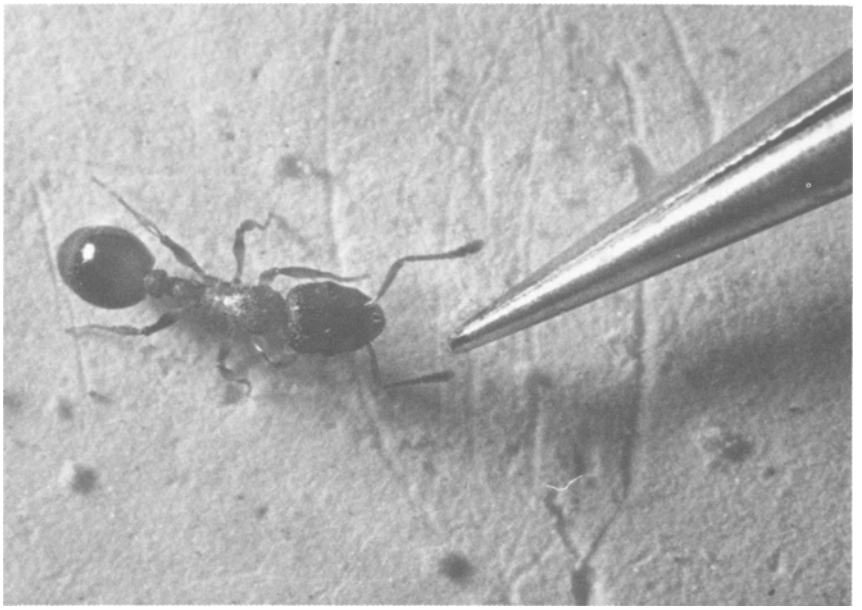

FIG. 2. Worker of *Leptothorax rugatulus* is following an odor dummy (forceps with dissected poison gland attached)

involved in tandem running. To answer this question we tested the whole gaster, out of which the sting with the attached glands had been removed. We also tested gasters (sting apparatus removed) which were extracted in a series of different solvents (methanol, acetone, ethyl ether). These experiments demonstrated (Table 1, E and F) that in *Leptothorax acervorum* the chemical poison gland signal is of principal importance for releasing and maintaining the tandem contact. For the following ant other chemical or mechanical signals are neither necessary nor more effective in maintaining contact.

In *Leptothorax nylanderi*, however, we obtained different results. Although the poison gland secretion showed the same effect in releasing and maintaining tandem contact (Table 1, G), other dummies without the tandem calling pheromone also proved to be effective. We tested gasters without sting apparatus of workers from the test colony and from other colonies, heads, thoraces with legs and extracted gasters (Table 1, H–L). With all these dummies we obtained a positive response.

Thus unlike *Leptothorax acervorum*, in *L. nylanderi* the tandem follower responds to several signals: (1) the tandem calling pheromone from the poison gland which is involved in initiating tandem recruitment and also in providing continuous stimulation during tandem running, and (2) a mechanical signal which the follower receives by pushing with its antennae against the dummy

object. This mechanical signal seems to be sufficient in maintaining the tandem contact. Since this mechanical signal is not bound to a specific part of the body and since extracted body parts are effective, we can assume that no chemical signal is involved.

These were the first behavioral differences we detected in these two species which belong to different subgenera: *Mychothorax* (*Leptothorax acervorum*) and *Leptothorax* s.str. (*L. nylanderi*). In the next series of experiments I therefore investigated the behavioral specificity of the tandem calling pheromone of several species belonging to three different subgenera.

Specificity of Tandem Calling Pheromone. The species, their respective subgenera, and the locality where they were collected are given in Table 2. Using basically the same experimental techniques described above, I tested the workers' responses to poison gland secretion prepared from other species. In the first series I included *Leptothorax acervorum*, *L. muscorum* (both subgenus *Mychothorax*), and *L. nylanderi* (subgenus *Leptothorax*).

As shown in Table 3, tandem followers of *L. acervorum* could be led with pheromone preparations from *L. muscorum* and from conspecifics, but not with the poison gland secretion of *L. nylanderi*. Conversely, tandem followers of *L. nylanderi* do not respond to dummies of species of the subgenus *Mychothorax*.

Although I had compared only three species, the results strongly suggested that the tandem calling pheromone might be subgenus specific, at least with respect to the two subgenera *Leptothorax* and *Mychothorax*. Nevertheless, to confirm this it was neccessary to compare more species of the two subgenera and of other subgenera. This was achieved by extending these tests to North American species.

It has to be mentioned here that the test procedure contained one small uncertainty in the cases where pheromone dummies showed no effect. Although the dummies were prepared very carefully and double checked by inspection before the gland was crushed, there was still a slight chance that the preparation of the dummy was a failure, thus being ineffective even for the species from which the gland was taken (e.g., the gland could have been accidentally removed while being crushed). The only way to exclude this uncertainty would be to conduct experiments with two (or even more) colonies at the same time. However, it would have been extremely difficult to achieve simultaneous food recruitment (to provide ant tandems for the tests) in two or more colonies of different species. Quite frequently I did not even have large enough colonies of the two test species at the same time, but had only some workers which could be used as pheromone donors.

Nevertheless, I tried to conduct a double-control experiment at least once to strengthen the conclusions. After many trials I succeeded in inducing food recruitment with one colony each of the species *Leptothorax crassipilis* and *L. rugatulus* which had been starving for the same time and which were

TABLE 2. SPECIES OF *Leptothorax* INVESTIGATED IN THIS STUDY; SUBGENUS, NEST SITE, AND LOCALITY ARE INDICATED[a]

Species	Species abbreviation	Subgenus	Nest site	Locality
L. pergandei(Emery)	PER	*Dichothorax* (Emery)	Under rocks	Big Pine Key, Florida, U.S.A.
L. nylanderi (Foerster)	NYL O	*Leptothorax* (Mayr)	Hollow twigs	Oak forest near Frankfurt, West Germany
L. ambiguus (Emery)	AMB	*Leptothorax* (Mayr)	Acorns	Estabrook Woods, near Concord, Mass., U.S.A.
L. curvispinosus (Mayr)	CUR	*Leptothorax* (Mayr)	Acorns	Estabrook Woods, near Concord, Mass., U.S.A.
L. longispinosus (Roger)	LON	*Leptothorax* (Mayr)	Acorns	Estabrook Woods, near Concord, Mass., U.S.A
L. rugatulus (Emery)	RUG	*Leptothorax* (Mayr)	Under rocks	Chiricahua Mountains, Portal, Arizona, U.S.A.
L. acervorum (Fabr.)	ACE O	*Mychothorax* (Ruzsky)	Hollow twigs	Nürnberger Reichswald, West Germany
L. muscorum (Nyl.)	MUS O	*Mychothorax* (Ruzsky)	Hollow twigs	Nürnberger Reichswald, West Germany
L. muscorum (New World)	MUS N	*Mychothorax* (Ruzsky)	?	(probably: Rouyn-Noranda, Quebec, Canada)
L. crassipilis (Wheeler)	CRA	*Mychothorax* (Ruzsky)	Under rocks	Chiricahua Mountains, Portal, Arizona, U.S.A.
L. duloticus (Wesson)	DUL	*Mychothorax* (Ruzsky)	Hollow milk-weed stem	White Hall, Illinois, U.S.A.
Harpagoxenus americanus (Forel)	HAR	—	Acorns	White Hall, Illinois, U.S.A.

[a] The species abbreviations listed are used in the other tables and figures.

TABLE 3. TEST OF PHEROMONE SPECIFICITY AMONG 3 PALEARCTIC SPECIES OF *Leptothorax*[a]

Test series	Pheromone source	Test colony	No. of gland preparations	No. of workers tested	No. of responses		Positive response (%)	Effectiveness of the pheromone
					Positive	Negative		
A	ACE	ACE	7	20	188	0	100	+
	MUS	ACE	7	9	61	0	100	+
	NYL	ACE	9	11	4	123	3.5	–
B	NYL	NYL	5	22	172	0	100	+
	ACE	NYL	5	10	0	88	0	–
	MUS	NYL	5	12	0	84	0	–

[a]Species abbreviations see Table 2.

simultaneously placed in two large foraging arenas to induce food recruitment. With the tandems appearing, I was then able to test each preparation with workers from the donor colony and from the test colony and vice versa. Thus the effectiveness of a preparation could be tested with the donor species, and possible failure in the test species would demonstrate the specificity of the pheromone.

The results are shown in Table 4. In all four preparations which did not show any tandem leading effect in a species of another subgenus, the preparation itself proved to be effective when tested with the donor species. Since it proved to be very unlikely that a negative response was due to a failure in preparation, all negative results were accepted as valid if at least two different preparations showed no positive response in all tests. However, in all cases, where possible, I tested many more preparations.

In general the ants reacted quite clearly. This is reflected in the absolute differences of most results: 0 or 100%. This illustrates an all-or-none reaction to the chemical releaser. If the dummy was presented in the right behavioral context and handled correctly, the ants reacted in almost all trials positively, provided the pheromone was effective for that species. Sometimes, however, single workers exhibited different behavioral reactions. Either they did not follow a dummy at all (control with effective pheromone of own species) or they reacted aggressively towards the dummy. Since both cases are believed to be artifacts caused by experimental deficiencies, the worker was not tested further and not included in the results. Some of the few positive responses to dummies which were otherwise totally ineffective might have been due to a slight aggressive reaction not detected by the observer. Usually the aggressive behavior started with gaping of the mandibles. Then in most cases the ant ran towards the dummy and, if the experimenter failed to remove it quickly, grasped the tip of the forceps and showed stinging movements. Whether the aggressive behavior was caused by incorrect handling of the dummy or by its contamination could not be decided. Therefore the dummy was also eliminated from further test series if it had been attacked by an ant.

The final results are given in Table 5 and summarized according to the subgenera tested in Figure 3. The tandem calling pheromone of *Leptothorax* proved to be specific between the subgenera *Leptothorax* and *Mychothorax*. As the shaded sections in Figure 3 demonstrate, there is no further specification down to the species level. Tandem followers of every species within the subgenus can be led with poison gland secretion of any other species of the same subgenus.

Across these two subgenera, however, I was able to demonstrate a strict specificity, which holds true for all combinations tested, except one: the tandem calling pheromone of the dulotic species *Leptothorax duloticus* is responded to by tandem followers of three species of the sub-

TABLE 4. SIMULTANEOUS TESTS OF PHEROMONE SPECIFICITY BETWEEN 2 NEARCTIC SPECIES OF *Leptothorax*[a]

No. of preparation	Source of pheromone	Control in donor colony				Test colony			
		No. of workers tested	No. of responses		Positive responses (%)	No. of workers tested	No. of responses		Positive responses (%)
			Positive	Negative			Positive	Negative	
1	CRA	7	39	0	100	6	0	27	0
2	RUG	6	18	0	100	7	0	21	0
3	RUG	6	18	0	100	7	0	21	0
4	RUG	6	18	0	100	7	0	21	0

[a]Both colonies were simultaneously tested. Species abbreviations see Table 2.

TABLE 5. TEST OF PHEROMONE SPECIFICITY AMONG 8 SPECIES OF *Leptothorax*[a]

Pheromone Source	Test colony	No. of glands tested	No. of workers tested	No. of responses		Positive responses (%)	Effectiveness of pheromone
				Positive	Negative		
MUS N	MUS N	3	11	67	0	100	+
	RUG	3	20	0	105	0	−
CRA	CRA	3	23	165	0	100	+
	CUR	3	13	2	79	2.5	−
	AMB	2	4	0	36	0	−
	RUG	2	9	0	49	0	−
CUR	CUR	6	18	111	0	100	+
	MUS N	2	5	0	14	0	−
	LON	3	4	12	0	100	+
	AMB	3	4	42	0	100	+
	RUG	1	6	42	0	100	+
	PER	1	1	2	5	28.6	?
LON	LON	6	30	95	0	100	+
	MUS	2	10	0	27	0	−
	CUR	1	4	9	0	100	+
	RUG	2	4	30	0	100	+
AMB	AMB	2	4	27	0	100	+
	CUR	3	6	36	0	100	+

	LON	3	4	9	0	100	+
	PER	1	3	12	3	80	+
RUG	RUG	9	38	196	2	99	+
	MUS N	2	10	0	21	0	−
	CRA	5	23	0	421	0	−
	CUR	2	4	11	0	100	+
	LON	3	8	21	0	100	+
	PER	3	3	3	21	12.5	?
DUL	CUR	1	3	9	0	100	+
	LON	4	35	123	0	100	+
	RUG	1	3	7	0	100	+
	PER	1	2	9	0	100	+
PER	PER	4	7	42	0	100	+
	MUS N	2	7	0	36	0	−
	CUR	1	4	0	17	0	(−)
	LON	4	35	123	0	100	+
	RUG	4	25	95	20	82.6	+
H.AM	CUR	2	4	15	0	100	+
	LON	4	31	125	0	100	+
	RUG	1	3	8	0	100	+
	PER	2	6	10	9	52.6	?

[a](Including additionally: *Harpagoxenus americanus*). Species abbreviations see Table 2.

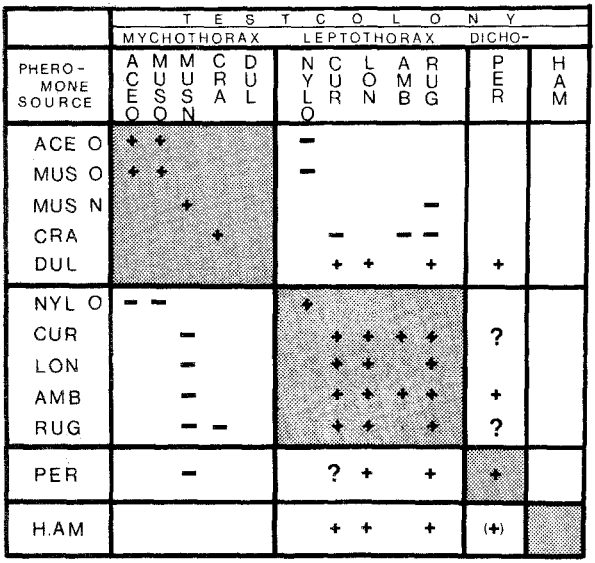

FIG. 3. Summarized results of all tested combinations of pheromone specificity. Combinations with no symbols have not been tested. The shaded areas in the graph represent test combinations within subgenera. Species abbreviations see Table 2. N: New World; O: Old World.

genus *Leptothorax*. Whether this case is a true exception will be discussed below.

The specificity of the tandem calling pheromone of *Leptothorax pergandei*, a species of the third subgenus (Dichothorax), appeared less clear. The pheromone was effective in two species of *Leptothorax* but released no response in *Mychothorax*. Because of the very small numbers of testable tandem followers (*L. pergandei* colonies were extremely small and showed very low recruitment rates), the results on the response of *L. pergandei* followers are not conclusive. The preliminary results, however, suggest a very broad response spectrum. A positive intersubgeneric response of *L. pergandei* workers has been demonstrated with *L. ambiguus* (subgenus *Leptothorax*). Furthermore, although the tests with the pheromone of *L. curvispinosus* and *L. rugatulus* are inconclusive, it is noteworthy that five positive responses were recorded without any aggressive behavioral actions exhibited by the ants against the foreign pheromone. Finally the poison gland secretion of *Harpagoxenus americanus*, a species which is closely related to the genus *Leptothorax*, was tested in several combinations. The pheromone proved to be effective in all species of the subgenus *Leptothorax*. It was also effective in *L. pergandei*, but somewhat less markedly.

DISCUSSION

All *Leptothorax* species investigated use the tandem running technique to recruit nestmates to new food sources as well as to new nest sites. During nest emigration, however, tandem running is a specialized technique to recruit secondary recruiters and potential carriers (Möglich, 1978).

Tandem running recruitment is initiated by a specific chemical display behavior, which we named tandem calling behavior (Möglich et al., 1974). The calling ant releases the secretion of the posion gland containing a pheromone which proved to be highly effective in initiating and maintaining tandem following behavior.

The behavioral analysis of the tandem calling pheromone showed a highly significant specificity at the subgenus level between *Leptothorax* and *Mychothorax*. This result was obtained in all combinations tested, except one: the tandem calling pheromone of *L. duloticus*, a slavemaking species. In an investigation of the recruitment signals between slavemaking species and their hosts (conducted with T.M. Alloway, 1978) we were able to show that even unparasitized colonies of *L. longispinosus* and *L. curvispinosus* responded to the calling pheromones of the slavemakers *L. duloticus* and *Harpagoxenus americanus*. Not only potential slave species but even workers of *L. rugatulus* followed *L. duloticus* pheromone dummies.[3]

All three species mentioned are clearly members of the subgenus *Leptothorax* and show the pheromone specificity between *Leptothorax* and *Mychothorax*. *L. duloticus*, however, is considered to be a *Mychothorax* (Wesson, 1937; Creighton, 1950; Wilson, 1975). Wilson (1975) especially describes several morphological characters which distinguish *L. duloticus* from *L. ambiguus*, a member of the subgenus *Leptothorax*. Alloway (personal communication), on the other hand, found other traits which would generate some doubts of *L. duloticus* being a *Mychothorax*. I am not able to discuss these different morphological findings here. Nevertheless, the results on the pheromone specificity show an important behavioral trait which would be difficult to explain in the case of *L. duloticus* being a *Mychothorax*. The tandem releasing properties of the pheromone composition resemble those of the subgenus *Leptothorax*.

Results on the pheromone specificity of the third subgenus under investigation (*Dichothorax*) were not as clear. I could only test one species from this subgenus whose colonies and thus recruitment frequencies were very small. However, it could be demonstrated that the pheromone of *L. pergandei* is effective in the subgenus *Leptothorax*, whereas it showed no tandem leading quality within the subgenus *Mychothorax*.

[3] Unfortunately we have not yet had the chance to test the complementary case, that is, the response of workers of the subgenus *Mychothorax* to the *L. duloticus* calling pheromone.

Although I could record positive responses of *L. pergandei* to pheromone preparations of *L. curvispinosus*, *L. rugatulus*, and *L. ambiguus*, only the latter case showed a conclusive result. Nevertheless, it supports the findings in the reciprocal test (*L. pergandei* pheromone vs. *Leptothorax* test colonies), demonstrating the intersubgenus effectiveness between *Leptothorax* and *Dichothorax*. The results obtained with *L. pergandei* as the pheromone donor and as the test colony support the notion that the tandem calling pheromone of the subgenus *Dichothorax* is ineffective for *Mychothorax*, but is effective across borders with *Leptothorax*. Thus, at least with regard to the composition of the tandem calling pheromones, the subgenus *Dichothorax* seems to be phylogenetically closer to *Leptothorax* than to *Mychothorax*.

Even here *L. duloticus* plays the same exceptional role. Since its pheromone is not effective with *Mychothorax*, *L. pergandei* is not supposed to respond to *L. duloticus* pheromone; but it does, thus indicating again that *L. duloticus* has similar chemical tandem releasers as species of the subgenus *Leptothorax*.

Bushchinger (1970) discussed the notion that phylogenetic relationships between hosts and their social parasites should be closer than between the nonparasitic species of different subgenera. He found that many morphological characteristics of social parasitic ants and their hosts are similar. Our specificity tests of the tandem calling pheromone support this notion.

Recently Buschinger and Winter (1977) examined the recruitment of *Harpagoxenus sublaevis* during slave raids. They found the same behavioral pattern of tandem recruitment in the slavemaking species as we have described it for *Leptothorax*. Using an experimental technique similar to ours for *Leptothorax*, they were able to demonstrate that even the same communication signals are involved. Moreover, mixed tandems (slave raider and slave) could be observed.

Previously Buschinger (1968) described a display behavior ('Lock-sterzeln') in ergatoid virgin females of *Harpagoxenus sublaevis*, by which they chemically attract males. This behavior resembles precisely the tandem calling; even the same chemical signals seem to be involved. Thus the same behavioral pattern is employed in two different contexts: as a recruitment signal in the organization of slave raids and for chemical sexual calling during during reproductive activities. This discovery supports the hypothesis that, in at least some myrmicine ants, sex attractants and recruitment pheromones had the same evolutionary origin (Hölldobler, 1971).

Wilson (1971) suggested that tandem running might be a phylogenetic precursor of the chemical mass recruitment techniques first analyzed in *Solenopsis invicta* (*S. saevissima*) (Wilson, 1962). The analysis of the signals involved in tandem calling of *Leptothorax* species supports this hypothesis. The behavior as well as the signals involved can be considered as

preadaptations for the evolution of the chemical mass recruitment system used in many myrmicine species. Nevertheless, the only way to find actual phylogentic steps is to investigate the recruitment strategies of very closely related species. In this study I attempted a first step in this direction.

In this respect the finding that *L. duloticus* seems to have the same recruitment pheromone(s) as its host appears to be important. When *L. duloticus* conducts slave raids, it recruits nestmates in "processions" of up to 25 workers (Wesson, 1940; Alloway, 1978). This recruitment technique closely resembles the so-called group recruitment, which was first analyzed in *Camponotus socius* (Hölldobler, 1971). We considered group recruitment to present an intermediate step of the evolutionary development from tandem running to chemical mass recruitment (Möglich and Hölldobler, 1975; Hölldobler, 1978). One of Alloway's striking observations is that even within one species a response to both tandem calling (tandem running within normal colonies of the slave species *L. curvispinosus*, *L. longispinosus*, and *L. ambiguus*) as well as to group recruitment (in mixed processions to the odor trail laid down by *L. duloticus* the slavemaker species) is possible. Although the analysis of the signals involved is not yet completed, observations suggest that the pheromone released from the sting during tandem running is also used to lay a trail on which slavemakers as well as slaves can follow a leading scout during a raid. Alloway's findings of two recruitment responses in one species and my results that their pheromones have the same specificity strongly support the ideas that tandem running could have served as preadaptation for the evolution of group recruitment. Since it is believed that slavemaking species have evolved from ancestors similar to the host species, it appears that some slavemaking species have given up tandem running and replaced it with group recruitment.

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SPECIES-SPECIFIC NATURAL PRODUCTS OF ADULT MALE LEAF-FOOTED BUGS (HEMIPTERA: HETEROPTERA)

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Abstract—The 7–8th ventral abdominal gland secretions from 6 adult male leaf-footed bugs, *Leptoglossus* spp., and a related species, *Euthochtha galeator*, were chemically analyzed by GC-MS. Of the 11 volatile compounds identified, all but one of the compounds (*n*-octanol) were aromatic, including compounds with the familiar odors of cherries, vanilla, cinnamon, and roses. The preponderance of aromatics in the adult male ventral abdominal gland secretions contrasts sharply with the aliphatic compounds which comprise the metathoracic gland defensive secretions of adult males and females. Also, the male-specific secretions are species-specific, both qualitatively and quantitatively, whereas the metathoracic gland secretions of Coreoidea are only distinctive at the generic level. It is proposed that males were favored as the emitters of attractive signals by sexual selection, whereas the specificity of the signal is the result of natural selection against hybridization.

Key Words—Coreidae, Heteroptera, Hemiptera, sex attractant, pheromone, sexual selection, *n*-octanol, benzyl alcohol, vanillin, 2-phenylethanol, leaf-footed bugs, chemotaxonomy, allomome.

INTRODUCTION

It has been recently demonstrated that adult males of a common leaf-footed bug, *Leptoglossus phyllopus* (L.) (Heteroptera: Coreidae), produce a blend of volatile aromatic compounds in a bilobed ectodermal abdominal gland opening midventrally in the 7–8th intersegmental membrane (Aldrich et al., 1976). Adult male and female *Leptoglossus* spp. also possess a large exocrine gland in the metathorax, but previous chemical analyses have revealed no obvious differences in the secretions of males and females (Aldrich and

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Yonke, 1975). Furthermore, there was no indication that the metathoracic gland secretions were species specific. In order to determine if the adult male 7–8th ventral abdominal gland secretions are species specific, we have chemically analyzed the secretions from five more species of *Leptoglossus* plus one species from a related genus, *Euthochtha galeator* (Fab.)

METHODS AND MATERIALS

Insects. Adult males of *L. phyllopus*, *L. fulvicornis* (Westwood), *L. corculus* (Say), *L. oppositus* (Say), and *E. galeator* were field collected in Georgia and Florida. *Leptoglossus clypealis* Heideman adult males were collected near Austin, Texas, and *L. gonagra* (Fab.) adult males were obtained from a laboratory culture which originated from specimens that were collected in Texas.

Preparation of Extracts. The 7–8th ventral abdominal gland could be easily removed from a CO_2-anesthetized male, under tap water, by pulling out the genital segments (8 and 9), grasping the cuticle around the mouth of the gland with forceps, and gently pulling the gland out through the intersegmental membrane. The excess water was then drawn off the gland with tissue paper while the gland orifice was pinched shut, and the gland was macerated in 100 μl of solvent (CS_2 or *n*-pentane). The extract thus prepared was then flame sealed in a 50- or 100-μl capillary for cold storage at $-20°C$ until analysis, or the process was repeated in order to obtain a pooled sample before it was sealed in a capillary.

Chemical Analysis. All gas chromatographic–mass spectrometric (GC-MS) analyses were conducted with an LKB-9000 mass spectrometer at 70 eV, with a source temperature of 270°C, separator at 260°C, and 60 μA ionizing current. The best separations were achieved with a 1% OV-17 column, programed from 50 to 225°C at 10°C/min. Compounds were identified by comparison to spectra of standards on file in the computerized mass spectral search system (Heller, 1972) or to published spectra (Stenhagen et al., 1969). Identifications were cross-checked by comparison of the retention time of each standard to that of each natural product on a Tracor 222 GC with a flame ionization detector coupled to a Varian CDS 111 automatic peak area integrator, using a 3% OV-1 column programed from 80 to 205°C at 5°C/min.

The percent area of each peak in the GC tracing of a species was determined either with the Tracor 222-Varian CDS 111 automatic peak area integrator system or with a Graphics Calculator™ (Numonics Inc.). The quantification data reported in Table 1 for *L. phyllopus*, *L. corculus*, and *L. fulvicornis* were calculated by means of the former system by analyzing several males of each species individually, and then calculating a weighted

TABLE 1. COMPOUNDS AND THEIR RELATIVE ABUNDANCES IDENTIFIED IN THE 7–8th VENTRAL ABDOMINAL GLAND SECRETIONS OF ADULT MALES FROM 6 SPECIES OF *Leptoglossus* AND *E. galeator*

N[a]	Species	*n*-Octanol	Benzyl alcohol	Guaiacol	*p*-Hydroxy-benzaldehyde	Methyl *p*-hydroxy-benzoate	Vanillin	Vanillyl alcohol	2-Phenylethanol	Cinnamyl alcohol	Syringaldehyde	Acetosyringone	Unidentified peaks (%)
9	*Leptoglossus phyllopus*[b]		74	14		5	<1				4	2	
7	*L. corculus*[b]	23	47	<1	3		15	5		7			
12	*L. fulvicornis*[b]		76	21									3
3	*L. oppositus*[c]		100										
2	*L. clypealis*[c]		67	26									7
1	*L. gonagra*[c]								100				
1	*Euthochtha galeator*[c]		37						57				6

[a]N = number of specimens used for quantification.
[b]Percent peak areas calculated with a Varian CDS 111 automatic peak area integrator.
[c]Percent peak areas calculated with a Graphics Calculator.

average (weighted according to the volume of secretion in the gland) for each compound in each species' blend. These data are undoubtedly the most accurate and biologically meaningful since the volume of secretion in the 7-8th ventral abdominal gland of a male is apparently positively correlated with sexual maturity (Aldrich et al., 1976). The quantification data for the remaining species were determined from GC tracings of samples containing 1–3 male glands using the Graphics Calculator and are not as accurate as the data obtained with the Varian CDS 111 integrator.

RESULTS

The secretions produced by adult male leaf-footed bugs in the 7-8th ventral abdominal gland were species specific, both quantitatively and qualitatively (Table 1). Some compounds had a widespread distribution in these glandular exudates. For example, guaiacol was found in four of the *Leptoglossus* species, but the percentage of this compound ranged from $<1\%$ in *L. corculus* to 26% in *L. clypealis*. Compounds unique to a species were found in three of the *Leptoglossus* species: methyl *p*-hydroxybenzoate, syringaldehyde, and acetosyringone in *L. phyllopus*; *n*-octanol, *p*-hydroxybenzaldehyde, vanillyl alcohol, and cinnamyl alcohol in *L. corculus*; and 2-phenylethanol in *L. gonagra*. 2-Phenylethanol was also a major constituent in the male specific secretion of *E. galeator*. Benzyl alcohol, which occurred in all but one of the *Leptoglossus* species investigated, was also a quantitatively important constituent in the glandular exudate of *E. galeator*.

DISCUSSION

The volatile exudates from the 7–8th ventral abdominal gland of male *Leptoglossus* adults differ considerably from the defensive scents of adults and nymphs, both chemically and in specificity. Nymphs of *Leptoglossus* spp., and many other Heteroptera as well, discharge (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal from capacious dorsal abdominal glands when disturbed (Aldrich and Yonke, 1975). The metathoracic gland secretions of male and female adults (hexanal, 1-hexanol, hexyl acetate, and acetic acid) are less uniform in chemical composition than the secretions of nymphs, but these blends are still neither sexually dimorphic nor species specific (Table 2). Qualitative interspecific variation in *Leptoglossus* metathoracic gland secretions is insignificant, and quantitative variations in composition may merely reflect the imprecision of the biosynthetic pathway for these compounds, since it appears that the major aldehydic constituent in the metathoracic gland secretion of *L. phyllopus* is produced in the gland reservoir by enzymatic hydrolysis of some of the available acetate ester

and oxidation of some of the alcoholic product (Aldrich et al., 1978b; Gilby and Waterhouse, 1967; Games and Staddon, 1973). Although some quantitative variation in the composition of the ventral abdominal gland secretions from sexually immature bugs was observed, the secretions from adult *Leptoglossus* males observed courting or mating were uniform and unequivocally species specific. It is interesting that the two species whose ventral abdominal gland secretions were most similar, *L. fulvicornis* and *L. clypealis*, are parapatric (Allen, 1969). Thus, these male abdominal secretions appear to be of chemotaxonomic value at the species level.

Based on the chemical analysis of the defensive secretions from 52 species of carabid beetles, Moore and Wallbank (1968) concluded that the defensive secretions of closely related species are generally the same. Similarly, the defensive secretions from 35 species of polyzosteriine cockroaches were chemotaxonomically useful only to the generic level (Wallbank and Waterhouse, 1970), and the defensive secretions of 147 species of tenebrionid beetles were of chemotaxonomic significance only as low as the subgeneric level (Tschinkel, 1975). Clearly, the metathoracic defensive gland secretions of coreoid Heteroptera (Table 2) conform to the pattern exhibited by these Coleoptera and Dictyoptera. At least two species have been analyzed in six different genera, but in no case were the metathoracic gland secretions species specific. Species from two or more genera have been analyzed in three coreid tribes (Colpurini, Dasynini, and Mictini). Only in the Mictini were different genera found to exhibit differing metathoracic gland chemistries; however, the Mictini will probably soon be split into at least three tribes (C.W. Schaefer, personal communication). Rothschild (1961) predicted that natural selection would cause the evolution of chemically similar defensive secretions in related species, classifying such chemical congruities as examples of Müllerian mimicry.

Since it is apparently adaptive for related species to have the same type of chemical defense, the fact that the 7-8th ventral abdominal gland secretions of male *Leptoglossus* adults are both species and sex specific implies a sexual role for these secretions. We have previously proposed (Aldrich et al., 1976) that heteropteran males have been favored as the emitters of attractive signals as an adaptation to the colonization of successional habitats. This male strategy may be the result of sexual selection, similar to the strategy evolved in male crickets competing for females (Cade, 1978). Male orthopterans acoustically call flying females at night. Courtship ensues immediately, with both acoustic and chemical signals being involved. Calling male crickets also attract other males, as well as the parasitic tachinid fly, *Euphasiopteryx ochracea* (Cade, 1975). The system evolved in the Heteroptera appears to be analogous except that the peak flight occurs at midday (Dingle, 1972), and males have evolved chemical signals, rather than acoustical signals, to call females. Male-specific volatiles have recently been identified from phytophagous and predaceous

TABLE 2. METATHORACIC GLAND SCENTS OF COREOIDEA (HEMIPTERA: HETEROPTERA)[a]

Family	Subfamily	Tribe	Species	Groups detected	Reference
Alydidae	Alydinae		*Alydus eurinus* (Say)	I, III	Aldrich and Yonke, 1975
			A. pilosulus Herrick-Schaeffer	I, III	Aldrich and Yonke, 1975
			Megalotomus quinquespinosus (Say)	III, IV	Aldrich and Yonke, 1975
Coreidae	Meropachydinae		*Merocoris distinctus* Dallas	III	Aldrich and Yonke, 1975
	Agriopocorinae		*Agriopocoris froggatti* Miller	I	Waterhouse and Gilby, 1964
	Coreinae	Colpurini	*Pachycolpura manca* Breddin	I	Waterhouse and Gilby, 1964
			Hygia opaca (Uhler)	I	Tsuyuki et al., 1965
		Amorbini	*Amorbus alternatus* Dallas	I	Waterhouse and Gilby, 1964
			A. rhombifer Westwood	I	Waterhouse and Gilby, 1964
			A. rubiginous (Guerin)	I	Waterhouse and Gilby, 1964
		Dasynini	*Amblypelta nitida* Stal	I	Baker et al., 1972
			Aulacosternum nigrorubrum Dallas	I	Waterhouse and Gilby, 1964
		Mictini[b]	*Mictis caja* Stal	I	Waterhouse and Gilby, 1964
			M. profana (Fab.)	I	Waterhouse and Gilby, 1964
			Mozena obtusa Uhler	I	McCullough, 1973

		M. lunata Burmeister	I	McCullough, 1974
		Pternistria bispina Stal	I, III	Baker and Kemball, 1967
		Euthochtha galeator (Fab.)	II	Aldrich and Yonke, 1975
		Archimerus alternatus (Say)	II	Aldrich and Yonke, 1975
	Gonocerini	*Plinachtus bicoloripes*	I	Tsuyuki et al., 1965
	Acanthocorini	*Acanthocoris sordidus* (Thumberg)	I, II	Tsuyuki et al., 1965
	Chelinideini	*Chelinidea vittiger* Uhler	I	McCullough, 1974
	Coreini	*Hypselonotus punctiventris* Stal	I	McCullough, 1971
	Acanthocephalini	*Acanthocephala declivis* (Say)	II	McCullough, 1970
		A. granulosa (Dallas)	II	McCullough, 1970
		A. femorata (Fab.)	II	Blum et al., 1961
	Anisoscelidini	*Leptoglossus phyllopus* (L.)	I	Aldrich et al., 1978b
		L. clypealis Heidemann	I	Aldrich and Yonke, 1975
		L. oppositus (Say)	I	Aldrich and Yonke, 1975
Hyocephalidae		*Hyocephalus* sp.	I	Waterhouse and Gilby, 1964

[a] In each species 1 or more compounds from the following groups have been found: I = hexanal, 1-hexanol, hexyl acetate, hexanoic acid; II = (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*E*)-2-hexenyl acetate, (*E*)-2-hexenoic acid; III = butanal, butanoic acid; IV = 2-methylbutanal, 2-methylpropanal, 2-methylbutanoic acid, 2-methylpropanoic acid.

[b] The Mictini are being revised and will probably be split into at least three tribes (C.W. Schaefer, personal communication).

Pentatomoidea (Ubik et al., 1975; Vrkoč et al., 1977; Aldrich et al., 1978a). In fact, in the southern green stink bug, *Nezara viridula*, the emission of male-specific volatiles has been correlated with the attraction of females and males in the laboratory (Brennan et al., 1977) and in the field (Mitchell and Mau, 1971; V. Harris and J. Todd, 1979). Interestingly, the tachinid fly, *Trichopoda pennipes*, parasitizes adult males of *N. viridula* significantly more than females (Mitchell and Mau, 1971; Todd and Lewis, 1976), and the same species of fly also parasitizes adult males of *L. phyllopus* significantly more than females (Aldrich, unpublished data). If females are strongly disposed toward obtaining energy, the use of food as an attractant by males ought to be particularly strongly favored (Ghiselin, 1974). Thus, male bugs combining the chemical attraction of females with the lure of abundant food resources may be able to mate with more females, and the likelihood of their offspring surviving may be better than would be the case were males merely to search for females directly. At the same time, natural selection against hybridization should select for specificity of the attractant signals (Mayr, 1972).

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PRODUCTION OF CARDIAC GLYCOSIDES BY CHRYSOMELID BEETLES AND LARVAE

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Abstract—Cardenolides were looked for in 17 chrysomelid beetles belonging to 11 genera from three subfamilies, and they were found only in *Chrysolina* and *Chrysochloa* species (Chrysomelinae, Chrysolinini). The food plants of these insects are not known to produce cardenolides. The *Chrysochloa* and most *Chrysolina* species secrete a complex mixture of cardenolides, but *Chrysolina didymata* secretes a single compound, and *Chrysolina carnifex*, none. Several quantitative and perhaps qualitative differences were observed in the patterns of cardenolides produced by far distant populations of both *Chrysolina polita* and *C. herbacea*, collected in either France and Belgium, or Greece. These differences remain constant from one generation to the other, whatever the food plant is, and appear to be genetic. In *C. polita* from Greece, the pattern is unchanged after four generations bred in the laboratory on *Mentha* × *villosa*, which is known to be without cardenolides. In adults, the cardenolides are released with the secretion of the pronotal and elytral defensive glands, but in the larvae which lack the defensive glands, cardenolides are also produced. The total amount of cardenolides and the complexity of their mixture increases through the life cycle of the insects. The six main cardenolides secreted by *C. coerulans* were identified as: sarmentogenin, periplogenin, bipindogenin, and their corresponding xylosides. *C. didymata* secretes only sarmentogenin.

Key Words—Chemical defense, exocrine secretion, cardenolides, *Chrysochloa, Chrysolina*, Coleoptera, Chrysomelidae.

INTRODUCTION

The chrysomelids constitute one of the major families of Coleoptera. These phytophagous insects are well known for their ravaging outbreaks: they include several agricultural pests, and two species, *Chrysolina brunsvicensis*

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and *C. quadrigemina*, were used to control the Klamath weed (*Hypericum perforatum*) (Holloway, 1964). Since they are often numerous and moreover slow moving and conspicuous, they ought to be chemically well protected. Indeed, defensive glands are described in several chrysomelid larvae and beetles, and reflex bleeding is also frequently observed in immatures and adults (Deroe and Pasteels, 1977, and literature therein).

We recently reported (Pasteels and Daloze, 1977) that several species of chrysomelid beetles secrete complex mixtures of cardiac glycosides from pronotal and elytral glands. These compounds are not sequestered from the food plants, which are devoid of cardenolides. We shall here extend the list of species which secrete cardenolides and discuss this ability, according to the taxonomic position of the species within the family, in relation to the quality of the food plants selected by or offered to the insects, as well as to genetic differences between far-distant populations. We shall also demonstrate that the larvae, although devoid of defensive glands which characterize the adults, do produce cardenolides. Finally the structure of the six main cardenolides secreted by *Chrysolina coerulans*, a common European beetle feeding on several *Mentha* species, will be fully described.

METHODS AND MATERIALS

The species studied here were collected in Europe and Africa. About half of the species were fed in the laboratory, "milked" several times with bits of filter paper at about one-week intervals, and the secretion was stored in methanol. The other species were collected directly in methanol. The list of species and the kind of material used for analysis (extracts of whole beetles or of secretion) are given in Table 1.

Two populations of *Chrysolina polita*, originating from Greece and France, were bred in the laboratory on *Mentha* × *villosa* or on other defined food plants (see Results, Geographic Variations in the Pattern of Cardenolides). Four to five generations of *C. polita* can be obtained in the laboratory in one year, without diapause.

A large sample of about 2000 *Chrysolina coerulans* was collected in Switzerland on *Mentha longifolia*. This sample, preserved in methanol, was used for further identification of the cardenolides secreted by this species.

Physical data were obtained with the following instruments: ultraviolet (UV) (CH_3OH): Unicam SP 800; infrared (IR) (KBr): Unicam SP 1000; mass spectrometry (MS): Hitachi-Perkin-Elmer RMU 6D; high-resolution MS: AEI MS 902; nuclear magnetic resonance (NMR) (270 MHz): Bruker HFX 270; NMR (100 MHz): Jeol JNM-MH 100, all chemical shifts being reported in δ values. The following abbreviations are used: s = singlet; d = doublet; t = triplet; bs = broad singlet; dd = double doublet; dt = double triplet; cm = complex multiplet.

Thin-layer chromatography (TLC) was performed on Macherey-Nagel sil G precoated plates using one of the following eluents: CH_2Cl_2:CH_3OH:H_2O (85:14:1) (solvent 1) and diethylether–Reichstein's mixture (Rothschild et al., 1973) (1:1) (solvent 2) (Reichstein's mixture: $CHCl_3$:CH_3OH:EtOAc 1:1:1). All TLC comparisons with authentic cardenolides were performed using these two solvent systems. In some cases a third one (Et_2O:EtOAc 1:1) (solvent 3) was also used. The cardenolides were visualized by spraying first with 2% *m*-dinitrobenzoic acid in CH_3OH, then with 5% methanolic KOH (Kedde reagent, Kedde, 1947; Lewbart et al., 1963). The cardenolides appear as purple spots after a few seconds.

The cardenolides of *Chrysolina coerulans* were extracted following the procedure of Reichstein (Rothschild et al., 1970). Purification was effected by silica gel column chromatography with increasing amounts of Reichstein's mixture in diethylether as eluent. For identification of the sugars, the cardiac glycosides were hydrolyzed by refluxing for 4 hr in a 0.5 N H_2SO_4 aqueous-methanolic (1:1) solution. The methanol was evaporated under reduced pressure, water was added, and the aqueous phase extracted with $CHCl_3$. Desalting of the aqueous phase on Zerolit DM-F and evaporation of the water yielded the sugar moiety which was silylated and analyzed by gas chromatography (GLC) on a 3% OV-17 column at 120° (Hewlett-Packard 402 instrument). In each case, xylose was identified by mixed injections with a silylated reference sample.

RESULTS

Distribution of Cardenolides within the Family

During a previous survey, cardenolides were detected in three genera belonging to the subfamily Chrysomelinae: *Chrysolina*, *Chrysochloa* and *Dlochrysa*. Five other genera of the Chrysomelinae, as well as two genera belonging to the Criocerinae, were found to be devoid of cardenolides (Pasteels and Daloze, 1977). We have now extended our study to members of two other subfamilies: Halticinae and Galerucinae. The genus *Diamphidia* was particularly interesting since it includes the well-known species from the Kalahari desert, used by the bushmen to prepare their arrow poison. It has even been claimed that the toxic compound involved is a saponin (Wigglesworth, 1968). Within the Chrysomelinae, our present survey includes not only new genera, but also other members of the genera *Chrysolina* and *Chrysochloa*. Indeed, since we had shown that some *Chrysolina* species do not secrete cardenolides (*C. hyperici*, *C. brunsvicensis* and *C. varians*, all living on *Hypericum*), it was tempting to accumulate data on insects living on the largest possible range of host plant species.

The results are given in Table I. Again, cardenolides were only detected in *Chrysolina* and *Chrysochloa* spp., whatever their food plants, with the

TABLE 1. DISTRIBUTION OF CARDENOLIDES IN *Chrysomelidae*[a]

Species	Material analyzed	Method	*N*	Food plant	Origin
Halticinae					
Haltica oleracea	Total extract	TLC + KR	0	Polyphagous	Belgium
Diamphidia nigroornata	Total extract of dried pupae and adults	TLC + KR	0	*Commiphora africana* (Burseraceae)[d]	South Africa
Galerucinae					
Galeruca tanaceti	Total extract	TLC + KR	0	*Compositae* sp.[b]	Switzerland
Chrysomelinae					
Chrysolinini					
Chrysolina banksi	Secretion	TLC + KR	9	*Ranunculus repens*[b] *Ranunculus acris*[c] (Ranunculaceae)	France (Brittany)
Chrysolina staphylaea	Secretion	TLC + KR	5	*Ranunculus repens*[b] *Ranunculus acris*[c] (Ranunculaceae)	France (Brittany)
Chrysolina diversipes	Secretion	TLC + KR	7	*Glechoma hederacea*[c] (Labiatae)	Belgium
Chrysolina haemoptera	Secretion	TLC + KR	8	*Plantago lanceolata*[c] (Plantaginaceae)	Belgium

Chrysolina didymata	Secretion	TLC + KR + MS	1	*Hypericum* sp.[b] (Hypericaceae)	France (Ardèche)
Chrysolina carnifex	Secretion	TLC + KR	0	*Artemisia*[b] (Compositae)	Spain (Costa Brava)
Chrysochloa tristis	Total extract	TLC + KR	3	*Adenostyles glabra*[b] (Compositae)	France (Alps)
Phaedonini					
Plagiodera versicolora	Total extract	TLC + KR	0	*Salix* sp.[c] (Salicaceae)	Belgium
Chrysomela tremulae	Secretion	TLC + KR	0	*Populus* sp.[c] (Salicaceae)	Belgium
Chrysomela 20-punctata	Secretion	TLC + KR	0	*Salix* sp.[c] (Salicaceae)	Belgium
Chrysomela discolor	Total extract	TLC + KR + MS	0	*Salix babylonica*[b] (Salicaceae)	South Africa
Mesoplatys cincta	Total extract	TLC + KR + MS	0	*Indigofera pulchra*[b] (Leguminosae)	Upper Volta
Phaedonia circumcincta	Total extract	TLC + KR	0	*Leguminosae*[b]	Ivory Coast
Timarchini					
Timarcha tenebricosa	Total extract	TLC + KR	0	*Galium* sp.[b] (Rubiaceae)	Belgium

[a] Methods of identification are: TLC, thin-layer chromatography; KR, Kedde reagent; MS, mass spectrometry. The number detected is *N*.
[b] Observed in nature.
[c] Fed in the laboratory.
[d] According to literature.

remarkable exception of *Chrysolina carnifex*. Kedde-positive spots observed in TLC were considered as a sufficient indication to conclude for the presence of cardenolides in those species for which the TLC pattern exhibits several positive compounds over a large spectrum of R_f values. Indeed such results are typical for the defensive secretion of species belonging to those genera, and for which the identification as cardenolides was confirmed by MS, IR, UV, and NMR spectroscopy.

The secretion of *Chrysolina didymata*, living on *Hypericum*, is interesting, since only one Kedde-positive compound was detected in TLC. MS and TLC comparisons with an authentic sample in three different solvent systems demonstrate that this compound is sarmentogenin (*2*), also found in the defensive secretion of *C. coerulans* (see below).

When only whole extracts of beetles are available, the detection of cardenolides is less straightforward. Some components of these complex mixtures can give Kedde-positive spots without being cardenolides. This was the case for *Chrysomela discolor* and *Mesoplatys cincta*, the extracts of which afforded two polar Kedde-positive spots in TLC. However, the color was not quite typical of cardenolides, being more red-brown and less purple. Mass and infrared spectra of the isolated compounds show that they are not cardenolides, but a mixture of as yet unidentified glycosides. Kedde-positive results with noncardenolide compounds were previously observed by us in extracts of plant material (*Mentha* × *villosa*).

Geographic Variations in the Pattern of Cardenolides

The rather complex patterns of cardenolides secreted by *Chrysolina polita* or *C. coerulans* were found to be characteristic of the beetle species and not of its food plant (Pasteels and Daloze, 1977).

In the course of the present study, we were surprised to find a slight but reproducible difference in the pattern of cardenolides detected in samples of *C. polita* collected in distant areas.

Two samples of *C. polita* were first compared. Adults of the first sample were taken from a laboratory brood and fed for four generations on *Mentha* × *villosa*. The specimens used to start the brood were collected in Greece (Horefton, Pilion) on *Lycopus europaeus* and *Mentha* sp. The second sample was collected in France (Brittany, near Pont-Aven) on *Mentha aquatica*. TLC patterns of both samples are given in Figure 1.

Two differences in TLC patterns are immediately apparent: spot 8 is always much more concentrated in the Breton population, and spot 10 is very faint and sometimes not detected in the Breton population.

Such differences could be due to several causes. The patterns of cardenolides produced maybe largely dependent on the physiological state of the insects and differences observed between small samples are to be expected.

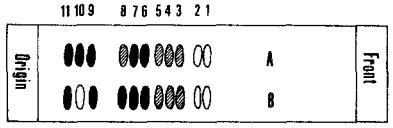


FIG. 1. TLC patterns (solvent 1) of cardiac glycosides produced by two different populations of *Chrysolina polita*. A. Population from Greece, and bred in the laboratory for four generations on *Mentha* X *villosa*. B. Population collected in France on *Mentha aquatica* O-very faint spot, barely detectable; ◍-low-intensity spot; ●-high- to very-high-intensity spot.

Indeed it is well known that a mixture of defensive compounds may show dramatic quantitative variations between different samples taken from apparently the same population (Jones et al., 1977). On the other hand, in some instances, the food plant could have an influence, which was overlooked in our previous experiments. Finally, the observed differences could be genetic: far-distant populations differing in details of the biosynthetic pathways of cardenolides.

Only this last hypothesis seems to hold for two reasons. First, we have never observed marked differences in the TLC patterns of the secretions of single beetles taken from the same population and from one generation to another. Second, *C. polita* from both the Greek and the Breton populations were bred from eggs on three different food plants, *M.* × *villosa*, *M. aquatica*, and *Lycopus europaeus*, their secretion collected separately, and compared by TLC. The pattern of cardenolides observed for each group was definitely characteristic of that of its parent population whatever the food plant was.

A sample of *C. polita* collected near Brussels on *M.* × *villosa* was found to produce a mixture of cardenolides similar to that of the Breton population.

Similar differences in the patterns of cardenolides produced by various populations of *Chrysolina herbacea* were also observed. Three samples were studied: one from Greece (Horefton, Pilion), one from the neighborhood of Brussels, and one from Marseille (France), all collected on several *Mentha* spp. The pattern of cardenolides secreted by the western European populations is markedly different from the one secreted by the Greek population (Figure 2).

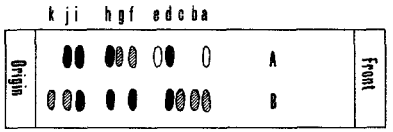


FIG. 2. TLC patterns of cardiac glycosides produced by different populations of *Chrysolina herbacea*. Same technique and conventions as in Figure 1. A. Population from France (Marseille) or Belgium (Brussels), B. Population from Greece.

Production of Cardenolides throughout the Life Cycle

Cardenolides have been detected in the eggs of *C. polita* and *C. coerulans*, but in very small quantity compared to the quantity observed in adults (Pasteels and Daloze, 1977). This prompted us to look for cardenolides in the larvae and pupae also. It should be noted that the larvae of the *Chrysomelini* do not possess the defensive glands of the adults, or the eversible defensive glands which have been described in the larvae of several *Phaedonini* and *Phyllodectini* (Hollande, 1909; Garb, 1915; Berti, 1968; Meinwald et al., 1977; Blum et al., 1978, and literature therein).

Methanolic extracts were prepared separately from 20 individuals of the following instars of *C. polita*: eggs; first, second, and fourth (last) larval instars; pupae; newly emerged adults; and 15-day-old adults. These extracts were compared by TLC, using Kedde reagent.

Cardenolides were detected in all extracts. However, even if our results cannot be considered to be fully quantitative, it is clear from the intensity of the observed spots that the quantity of cardenolides increases with age. The cardenolides found in the eggs, larvae, and pupae do not differ from those found in adults, but the complexity of the mixture produced seems also to increase with age. For example, the cardenolides 1–5 (Figure 1) were not detected in the eggs, larvae, and pupae. In these immatures only one compound was detected in the R_f region of compounds 6–8. Moreover, only one cardenolide was found in the eggs and larvae in the R_f region of spots 10 and 11 present in the adults and pupae. Such observations must be interpreted with caution and do not definitely demonstrate true qualitative differences between instars. Indeed, at least some of the apparently missing cardenolides in the eggs, larvae, and pupae could be present but in too small a quantity to be detected by TLC in the extracts of 20 specimens. Moreover, some of the cardenolides could be masked by the pigments present in the extracts of whole insects. This is most probably the case for cardenolides 1–4, which are not easily detected in the extracts of whole adults.

One Kedde-positive spot, migrating below cardenolide 11, was observed in the very polar region of all the extracts of whole *C. polita*. This compound is not present in the secretion of adults. However, since very polar non-cardenolide compounds were already found in some extracts of plants and beetles (see previous section), no definite conclusion should be drawn from this observation.

Cardenolides were also detected in the larvae of *C. herbacea* and *Chrysochloa tristis*.

Structures of Cardenolides from C. coerulans

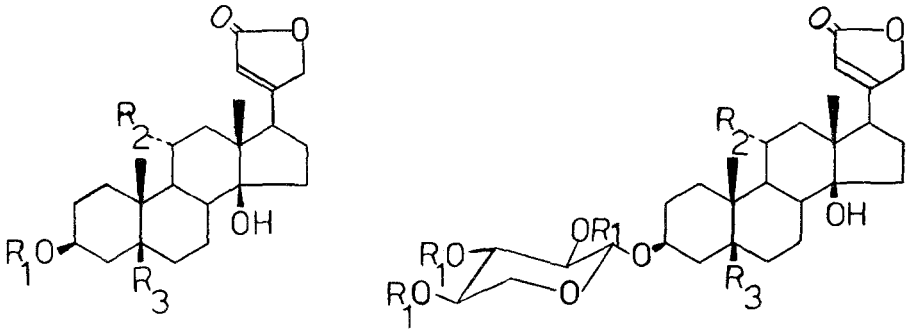
TLC of the defensive secretion of *C. coerulans* shows the presence of six main cardenolides. To obtain a sufficient amount of material, about 2000

whole beetles were finely ground and extracted according to published procedures (Rothschild et al., 1970) The cardenolides were concentrated in the $CHCl_3$ and $CHCl_3$-EtOH (3:2) extracts. Repetitive silica gel column chromatography afforded ten cardenolides, which will be referred to as compounds A, B, . . ., J, in order of increasing polarity.

The structure of six of these cardenolides (A, D, F, G, H, I) has been established, whereas the four other, only isolated in trace amounts, are still undetermined. In the following description, the numbers given for structures refer to Figure 3. We shall first deal with the structure determination of the two main compounds, G and H.

Compound G. This compound (15 mg) was identified as sarmentogenin-3β-xylopyranoside (5) on the basis of the following evidence:

G, $C_{28}H_{42}O_9$, amorphous, displays UV (217 nm, ϵ 14,000) and IR (υ_{OH} 3450, $\upsilon_{C=O}$ 1740, and $\upsilon_{C=C}$ 1630 cm^{-1}) spectra characteristic of a cardiac glycoside (Yamaguchi, 1970). Indeed the MS of G exhibits the typical fragmentation pattern (Brown et al., 1972) of a monohydroxydigitoxigenin derivative, with peaks at m/e 354, 339, 336, 321. The NMR spectrum (270 MHz, CD_3OD) of G completely confirms this assignment, showing signals



1 R_1= R_2 = H; R_3= OH

2 R_1= H; R_2= OH; R_3= H

3 R_1= H; R_2= R_3= OH

4 R_1= Ac; R_2= H; R_3= OH

5 R_1= H; R_2= OH; R_3= H

6 R_1= R_2= H; R_3= OH

7 R_1= H; R_2= R_3= OH

8 R_1= Ac; R_2= OAc; R_3= H

9 R_1= Ac; R_2= H; R_3= OH

10 R_1= Ac; R_2= OAc; R_3= OH

FIG. 3. Cardenolides produced by *Chrysolina coerulans* (1–3, 5–7) and derivatives there of.

characteristic (Tori and Aono, 1965) of a monohydroxylated digitoxigenin [0.9 (3H,s $^{18}CH_3$), 1.07 (3H,s $^{19}CH_3$), 2.90 (1H,t, J = 7 Hz, ^{17}C—H), 3.68 (1H,dt, J = 10 and 4.5 Hz, aglycone CH—OH), 4.00 (1H,bs ^{3}CH), 4.92 (2H,AB, J = 11 Hz, $^{21}CH_2$), 5.90 (1H,s ^{22}CH)] linked to a β-pentopyranose (Lemieux and Stevens, 1966; Durette and Horton, 1971) [3.17 (1H,t, J = 10 Hz, $^{5'a}CH$), 3.17 (1H,t, J = 7.5 Hz, $^{2'}CH$), 3.44 (1H,dt, J = 9 and 4.5 Hz, $^{4'}CH$), 3.80 (1H,dd, J = 10 and 4.5 Hz, $^{5'e}CH$), 4.24 (1H,d, J = 7.5 Hz, $^{1'}CH$)].

Acetylation of G (Ac_2O-pyridine, 100 hr) afforded the tetraacetate 8, $C_{36}H_{50}O_{13}$, whose high-resolution MS (see Table 2) fully supports the above conclusions (Blessington and Morton, 1970; Kochetkov and Chizhov, 1966). The NMR spectrum of 8 (100 MHz, $CDCl_3$) clearly shows the four $OCOCH_3$ groups [1.95 (3H,s), 2.00 (3H,s), 2.04 (6H,s)] and four C<u>H</u>OAc signals [4.87 to 5.30 (3H,cm, $^{2'}CH$, $^{3'}CH$ and $^{4'}CH$), 5.44 (1H,dt, J = 11 and 5.5 Hz, aglycone C<u>H</u>OAc)].

On 0.5 N H_2SO_4 hydrolysis, G afforded xylose together with a cardenolide aglycone (accompanied by dehydrated products (Fieser and Fieser, 1959) which was identical with sarmentogenin (2) (Katz, 1948) (MS and TLC in three different solvent systems).

The NMR spectra of G and 2 are very similar, except for the xylose signals in the spectrum of G. All these data unambiguously show that compound G is sarmentogenin-3β-xylopyranoside (5).

Compound H. This compound (8 mg), $C_{28}H_{42}O_9$, amorphous, is isomeric with G and shows similar spectral properties. Acid hydrolysis of H also afforded xylose as the only sugar. However, the secondary OH group present at C-11 in sarmentogenin (2) is lacking (no signal at 3.68 ppm) in the NMR of H, and replaced by a tertiary OH group as demonstrated by acetylation of H into the triacetate 9, $C_{34}H_{48}O_{12}$ (M 648), whose MS shows peaks characteristic of the aglycone moiety [*m/e* 373 (M^+ —OH—$C_{11}H_{14}O_7$), 355 (M^+ —OH—H_2O—$C_{11}H_{14}O_7$), 337 (M^+ —OH—$2H_2O$—$C_{11}H_{14}O_7$), 318 (see below)] and of triacetylxylose (Kochetkov and Chizhov, 1966) (*m/e* 259, 199,

TABLE 2. HIGH-RESOLUTION MS OF 8 (FIGURE 3)

Measured	Calculated	Attribution
673.3219	673.3222 for $C_{36}H_{49}O_{12}$	M^+ —OH
612.2900	612.2932 for $C_{34}H_{44}O_{10}$	M^+ —H_2O—CH_3COOH
457.2603	457.2588 for $C_{27}H_{37}O_6$	Diacetylsarmentogenin —OH
415.2466	415.2483 for $C_{25}H_{35}O_5$	Monoacetylsarmentogenin —OH
397.2374	397.2377 for $C_{25}H_{33}O_4$	Monoacetylsarmentogenin —OH—H_2O
337.2156	337.2166 for $C_{23}H_{29}O_2$	Monoacetylsarmentogenin —OH—H_2O—CH_3COOH
259.0826	259.0817 for $C_{11}H_{15}O_7$	Tri-*O*-acetylxylose —OH

157, 139, 99). The intense fragment at *m/e* 318 is considered (Brown et al., 1972) to be a diagnosis peak for periplogenin (1) (Speiser and Reichstein, 1947). Comparison of the NMR spectra of H and its triacetate with those reported (Höriger et al., 1970) for periplogenin (1) and its monoacetate (4) supports these assignments. Consequently H must be periplogenin-3β-xylopyranoside (6).

The four remaining compounds, although isolated in small amounts ($\simeq$2–5 mg), were also investigated.

Compound A. This formula is $C_{23}H_{34}O_5$; MS exhibits peaks at *m/e* 372, 354, 336, 318, 244, and 201, typical of a monohydroxydigitoxigenin (Brown et al., 1972). The peak at *m/e* 318 is particularly prominent, suggesting that A could be identical with periplogenin (1) (Speiser and Reichstein, 1947). This was confirmed by direct comparison with an authentic sample (MS, TLC in three different solvent systems).

Compound D. This compound, $C_{23}H_{34}O_5$ (MS), exhibits a mass spectrum nearly identical with that of sarmentogenin (2), except for some minor peaks attributable to impurities. The identity of D and sarmentogenin was confirmed by TLC comparison of both samples in three different solvent systems.

Compound F. This compound, $C_{23}H_{34}O_6$ (M^+ 406), exhibits fragmentation peaks at *m/e* 388, 370, 352, 334, and 199 in its mass spectrum. This suggests that F is a dihydroxydigitoxigenin derivative. Bearing in mind the structures of G and H (5 and 6 respectively), one can speculate that F is probably identical with the known compound bipindogenin (3) (Fechtig et al., 1960). This was indeed demonstrated by direct comparison (MS, IR, TLC in three different solvent systems) between F and authentic bipindogenin (3).

Compound I. The mass spectrum of this compound is very similar to that of F, showing peaks at *m/e* 388, 370, 352, 334, and 199. Moreover, on acetylation it yields a tetraacetate (10), $C_{36}H_{50}O_{14}$ (M^+ 706). Its mass spectrum displays, besides the peaks of triacetylxylose, characteristic fragmentation peaks at *m/e* 671, 670, 610, 455, 431, 413, 412, 394, 352, 335, and 334. These data, coupled to the fact that it yields xylose on acid hydrolysis, suggest that I is bipindogenin-3β-xylopyranoside (7).

The cardenolide content of one individual specimen of *C. coerulans* is estimated to be 20–30 μg.

DISCUSSION

The ability to secrete cardiac glycosides seems to be restricted to a narrow range of taxa within the chrysomelids, and this confirms our previous results (Pasteels and Daloze, 1977). Cardiac glycosides were found only in the secretion of *Chrysolina* and *Chrysochloa* species, and reported previously as

well in *Dlochrysa fastuosa*. These genera are closely related, belonging to the Chrysomelini within the subfamily Chrysomelinae. The family is, however, quite large, and our surveys have obviously covered only a small part of it.

There is little doubt that the cardenolides are synthesized by the beetles themselves and not sequestered from their food plants. These insects are narrowly oligophagous. However, when we consider all the species together, the total spectrum of food plants is quite large. It includes plants from no less than six families: Compositae, Labiatae, Scrofulariaceae (Pasteels and Daloze, 1977), Hypericaceae, Ranunculaceae, and Plantaginaceae. Cardenolides were reported in none of these plants eaten by the insects, and it is most unlikely that all of them (if any) do contain such compounds. Moreover, the adults of *Chrysolina polita* bred in the laboratory for four generations solely on the food plant *Mentha* × *villosa*, produce cardenolides detectable in the secretion of a single beetle, and it was demonstrated that this host plant does not contain such compounds (Pasteels and Daloze, 1977). Since insects cannot biosynthesize sterols, phytosterols are the most likely precursors of the chrysomelid cardenolides. Experiments with labeled compounds are planned to determine which sterols can be used by the insects for the synthesis of cardenolides.

The secretion of one cardenolide (sarmentogenin) by *Chrysolina didymata* was unexpected. This species is the only one which produces a single cardenolide. Moreover the three other species of *Chrysolina*, *C. polita*, *C. varians*, and *C. hyperici*, also feeding on *Hypericum*, do not secrete cardiac glycosides (Pasteels and Daloze, 1977). Such deficiency in their biosynthesis ability does not seem to be due to an absence of the necessary precursors in *Hypericum*, since *C. didymata* is able to perform it, but rather to be a secondary event in the evolution of the insects. Such evolution could be the consequence of a shift to a toxic food plant, and the ability to sequester hypericin as a cheaper means of defense (Pasteels, 1976). It was indeed reported by Rees (1969) that *C. brunsvicensis* stores hypericin. More investigations are needed, however, to understand fully the defensive strategies of the beetles feeding on *Hypericum*: they possess well-developed defensive glands producing an abundant secretion, the nature and activity of which remain unknown, but which does not contain hypericin.

In the course of the present survey, still another *Chrysolina* was also found to be devoid of cardenolides: *C. carnifex* feeding on *Artemisia* spp. Plants of the genus *Artemisia* produce bitter sesquiterpene lactones (Rodriguez, 1976). We do not know, however, if this species accumulates secondary plant substances.

The differences observed in the patterns of cardenolides secreted by several populations of *C. polita*, as well as of *C. herbacea*, collected in either France and Belgium, or Greece, are constant from one generation to the other, independent of the food plant eaten by the insects. Such constant

quantitative and possibly qualitative differences in the patterns of defensive compounds produced by far-distant populations of the same species were never reported to our knowledge, but are not really surprising. They just reflect some of the genetic differences which usually occur between populations. This demonstrates, however, that the patterns of cardenolides secreted are genetically determined and not dependent of the quality of the food.

The discovery that the larvae secrete cardenolides was unexpected. The larvae are devoid of defensive glands. The cardenolides are thus synthesized in other tissues. It remains to determine where the cardenolides are located in the larvae, and how they participate in the protection of the species. The main function of the glands in adults could be to excrete a highly concentrated solution of cardenolides, possibly synthesized elsewhere. The secretion would deter predators before they could harm the insects. Indeed the cardenolides are very bitter, and we have observed that birds quickly reject the beetles without swallowing them.

The three cardenolides aglycones identified so far from chrysomelid beetles [periplogenin (1), sarmentogenin (2), and bipindogenin (3)] have already been described from several plants belonging to the genus *Strophantus* (Tamm, 1956; Singh and Rastogi, 1970). Moreover (1) and (2) have also been isolated from toads, together with the well-known bufadienolides (Höriger et al., 1970; Fujii et al., 1976). However, xylosides of these three aglycones have not been described until now. Other cardiac glycosides produced by chrysomelid beetles are currently being investigated in our laboratories.

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SECONDARY ATTRACTION IN *Gnathotrichus retusus* AND CROSS-ATTRACTION OF *G. sulcatus* (COLEOPTERA:SCOLYTIDAE)[1]

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Abstract—Laboratory bioassays indicated that frass produced by *Gnathotrichus retusus* males and male gut extracts were attractive to both sexes of beetles. The frass became attractive to females within 2 days of the commencement of boring activity, and attractive frass was produced for at least 10 days. Attraction of frass rose sharply after pairing of males with females. However, this increase in attraction could be due to increased boring activity and pheromone production by males following establishment of the mutualistic fungus. In field trapping experiments, male-infested logs were always highly attractive, but in one experiment, female-infested logs and logs infested by both sexes were also attractive. Thus, females could be involved in secondary attraction. High cross-attraction of *G. sulcatus* to *G. retusus*-infested logs in field experiments, as well as a moderate but significant response by *G. retusus* to sulcatol in the laboratory, suggests that they share sulcatol as a common pheromone.

Key Words—Coleoptera:Scolytidae, *Gnathotrichus retusus, Gnathotrichus sulcatus,* aggregation phenomone, secondary attraction, cross-attraction.

INTRODUCTION

Pheromone-based secondary attraction (Borden et al., 1975) has been demonstrated in two of the three most important western North American ambrosia

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beetles, *Trypodendron lineatum* (Olivier) (Rudinsky and Daterman, 1964a,b; Chapman, 1966) and *Gnathotrichus sulcatus* LeConte (Cade, 1970; Borden and Stokkink, 1973). A tricyclic acetal, lineatin, has been identified as the aggregation pheromone in *T. lineatum* (MacConnell et al., 1977), and a 65:35 mixture of *S*-(+)- and *R*-(−)-6-methyl-5-hepten-2-ol, sulcatol, is the aggregation pheromone in *G. sulcatus* (Byrne et al., 1974; Borden et al., 1976). The utility of sulcatol in the management of *G. sulcatus* has been demonstrated in the forest (McLean and Borden, 1977a) and in a commercial sawmill (McLean and Borden, 1975, 1977b).

No research has been done on secondary attraction in *G. retusus* (LeConte), the other important ambrosia beetle in western North America (Johnson, 1958). Only when the chemical basis for secondary attraction in all three species is known will it be possible to institute truly comprehensive pheromone-based pest management programs for ambrosia beetles. Accordingly, our objective was to demonstrate whether or not secondary attraction occurred in *G. retusus*.

METHODS AND MATERIALS

G. retusus adults were collected as they emerged in a rearing chamber from logs and stumps taken from various lower mainland and Vancouver Island, British Columbia, localities. They were sexed and stored up to 4 weeks on moist, absorbent paper in glass jars at 0–4°C until used in experiments.

From April 1974 to April 1975, various baits were tested for attraction of both sexes of *G. retusus* in an open-arena, air-stream olfactometer identical to that used for *G. sulcatus* (Borden and Stokkink, 1973). Included among the baits were sawdust from western hemlock, *Tsuga heterophylla* (Raf.) Sarg., frass produced by males on days 1–3 after commencing boring activity on a hemlock log [females produce little to no frass; the term frass is used as by MacConnell et al. (1977) even though solid fecal material is produced only after beetles begin to feed on the mutualistic fungus about 1 week after attack], benzene extracts of guts excised from emerged and boring agults of both sexes, and benzene solutions of the *G. sulcatus* pheromone, sulcatol.

Two additional laboratory experiments were conducted in 1974. In October, 5 groups of 25 males were each allowed to bore into a western hemlock log under constant laboratory conditions. The frass was collected daily for 10 days in gelatin capsules affixed outside the galleries, weighed, stored at −40°C, and after the 10-day period was bioassayed with at least 60 beetles of each sex run in groups of 20. At 2, 4, 6, 8, and 10 days, 25 males were excised from one of the logs and the lengths of the galleries they had produced were measured. The excised males were held at −40°C for one month, after which the guts were dissected from them, extracted in benzene, and bioassayed at a concentration of 2 male equivalents to at least 40 insects of each sex.

In April, 3 groups of 25 newly emerged males were each allowed to attack a western hemlock log. Females were allowed to join the males on the 5th day. For 9 days, frass was collected daily ,weighed, and stored at −40° C. After the 9-day period, the frass from each day was bioassayed with both sexes. Each stimulus of approximately 100 mg was tested for attraction of at least 40 insects of each sex run in groups of 20. Control assays were run for the response to air and to fresh hemlock sawdust from the same tree as the log which the beetles had been allowed to attack.

Field experiments were conducted in 1975 at Pt. Roberts, Washington, and in 1976 at two southern Vancouver Island locations, one near Nanaimo, and the other 35 km further south on Yellow Pt. The following treatments were tested: blank control; unattacked *T. heterophylla* log 70 cm long × 20–25 cm in diameter; log infested with 50 male *G. retusus* approx. 48 hr before the experiment was set up in the field; log infested with 50 females; and log infested with 50 pairs (50 males and 50 females). Because of an apparently poor establishment of males, 50 additional males were added to the logs infested with paired insects in the first 1976 replicate. Each log was enclosed in a fine-mesh cloth bag, placed on a 30-cm-high platform, and surrounded by a cylindrical 6-mm mesh hardware cloth trap, 90 cm high × 34 cm in diameter, coated with Stikem Special. For each replicate, the log-trap setups were randomly placed at least 25 m apart in a linear sequence.

Two replicates were run at Pt. Roberts from May 14–25, 1975. Collections were made every 2 days throughout the experiment, but inclement weather after 2 days resulted in little beetle flight. Therefore, collections 2–5 were summed prior to statistical analysis. One replicate was run at each Vancouver Island location from May 19–26 and one from June 2–9, 1976. In each case the beetles were collected from the traps only once, at the end of the replicate. After their removal from the traps, the beetles were held in Shell solvent, and speciated and sexed in the laboratory. After the experiments, the logs were debarked, examined for attack success, and inspected for volunteer attack by natural populations of *G. retusus* or other species.

The data from laboratory experiments were analyzed by χ^2 tests. Field data were transformed to $\log_{10}(x + 1)$ prior to analysis of variance.

RESULTS

Male-produced frass and gut extracts from boring males 3 and 4 days after attack, respectively, were clearly the most attractive baits in laboratory tests (Table 1). There was a significant response by males to western hemlock sawdust, and surprisingly high responses by females to sulcatol, even though they were approximately half of the highest responses to male frass or gut extracts. There was no attraction to female guts or either benzene or air controls.

TABLE 1. RESPONSE OF *G. retusus* TO VARIOUS BAIT STIMULI IN 1974 AND 1975 BIOASSAYS IN A LABORATORY OLFACTOMETER

Stimulus	Amount or concentration	Male response			Female response			χ^2 (♂vs♀)[b]
		No. tested	No. responders	Response (%)[a]	No. tested	No. responders	Response (%)[a]	
Unbaited air control	—	60	0	0.0a	58	1	1.7a	NS
W. hemlock sawdust	approx. 40 mg	38	5	13.2b	38	2	5.3ab	NS
Male frass								
Day 1	approx. 300 mg				15	5	33.3cd	—
Day 1	approx. 40 mg	39	3	7.7ab	77	9	11.7b	NS
Day 2	approx. 40 mg	38	0	0.0a	59	9	15.3bc	**
Day 3	approx. 40 mg	38	3	7.9ab	56	23	41.1d	**
Days 1–3	approx. 25 mg	61	20	32.8c	60	34	56.7d	**
Benzene control	0.5 ml	79	2	2.5a	92	3	3.2a	NS
Female gut extract								
Emerged adults	2♀ equiv.	42	1	2.4a	39	0	0.0a	NS
In log 3 days	2♀ equiv.	41	0	0.0a	40	3	7.5ab	NS
Male gut extract								
Emerged adults	2♂ equiv.	60	4	6.7ab	60	7	11.7b	NS
In log 3 days	2♂ equiv.	97	15	15.5b	99	39	39.4c	**
In log 4 days	2♂ equiv.				75	34	45.3c	—
Sulcatol	0.2 μg				15	4	26.7bc	—
	0.46 μg	63	5	7.9ab	60	11	18.3b	*

[a] Percents within sawdust–frass group or gut extract–sulcatol group followed by same letter not significantly different, χ^2 test, $P < 0.05$.
[b] NS = not significant; * = significant, $P < 0.05$; ** = significant, $P < 0.01$.

For the first 3 days after beginning to bore into a log, frass production by males reached 3 mg/beetle, but declined thereafter to approximately 2 mg/beetle until the 9th day, when production sharply increased again to nearly 3 mg/beetle by day 10 (Figure 1). This sharp rise in production may coincide with establishment and growth of the mutualistic fungus and the beetles' increased feeding thereon. Measurement of the length of between 20 and 22 galleries from which beetles were excised on days 2, 4, 6, 8, and 10 disclosed mean boring rates/beetle/day of 3.53, 3.37, 1.24, 2.46, and 4.16 mm, respectively. After 10 days, one gallery was 73 mm long.

The response by males to male-produced frass over a 10-day period fluctuated between 10 and 35%, with no striking trends (Figure 2), except that the response from day 2 on was significantly greater (χ^2 test, $P < 0.05$) than the 6.4 and 6.6% responses to host sawdust and air controls, respectively. The response by females was not significantly different (χ^2 test, $P < 0.05$) only on day one from the 10.0 and 8.1% responses to host sawdust and air, respectively, or from the response of males. It rose sharply to a peak of 68.5% on day 3, and plateaued at a slightly lower level until day 7 when it declined to 33.2%, not significantly different from the male response (Figure 2). A sharp rise in response occurred to day 9 frass.

There was almost no response at all by females to benzene extracts of male guts from beetles excised from logs every 2 days. This lack of response may be due to the fact that the beetles were frozen and could have lost their attraction prior to being dissected and their gut extract bioassayed one month later. Alternatively, the guts may not have been macerated immediately in the solvent, allowing residual enzymatic activity to break down the pheromone. This experimental error was probably also the cause of failure of a similar experiment with guts in October 1975.

Frass production by males prior to being paired with females on the 5th day of attack (Figure 3) was similar to that in Figure 1. Following pairing of each male with a female, the frass production rose to a peak of 3.54 mg/pair on day 7. It is almost certain that even after males are paired, they continue to produce the majority of frass. Dissection of 30 galleries on day 9 of the experiment disclosed that the male was always at the head of the tunnel, a position which our observations indicate is also maintained by *G. sulcatus*.

Response of both sexes to male-produced frass during the first half of the experiment (Figure 4) was similar to the response to other unpaired male frass (Figure 2). After females were allowed to join the males, there was a marked increase in response by both sexes to the frass. This effect could be due to a female contribution to the secondary attraction, either through pheromone production themselves or by stimulating pheromone production by males. As the female assumes the function of clearing the gallery of frass, the male is free to bore continuously, and thereby could increase pheromone production over that of unpaired males. Alternatively, more active pheromone production by males could be in response to establishment of the mutualistic fungus.

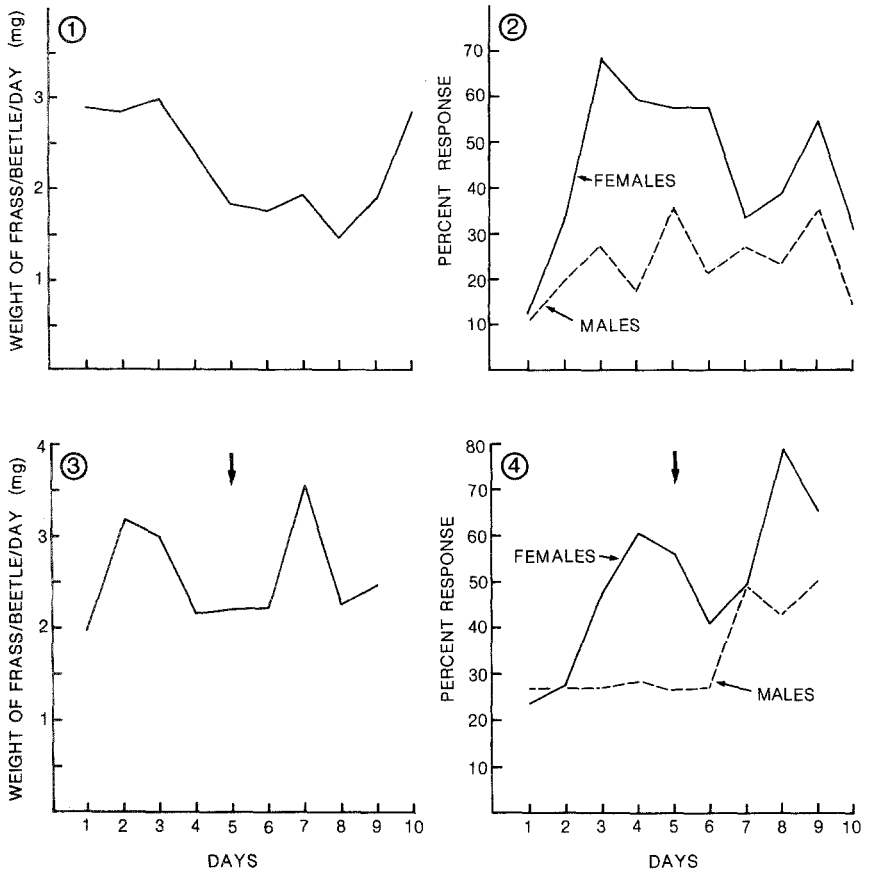

FIG. 1. Weight of frass produced by *G. retusus* males boring in western hemlock log, Fall 1974.

FIG. 2. Response of *G. retusus* in laboratory olfactometer to frass produced by males on days 1–10 after being allowed to bore into western hemlock log.

FIG. 3. Weight of frass produced by *G. retusus* boring in western hemlock log, Spring 1974. Males only for first 5 days. Arrow indicates pairing of each male with a female on day 5.

FIG. 4. Response of *G. retusus* in laboratory olfactometer to frass produced from western hemlock log infested by males (days 1–5) and both sexes in pairs (days 6–9). Arrow indicates pairing of each male with a female on day 5.

The 1975 field experiment at Pt. Roberts, Washington, clearly showed that *G. retusus* of both sexes responded most significantly to male-infested logs, and less actively to logs infested by females or both sexes (Table 2). ANOVA disclosed a significant treatment-by-time interaction, reflecting greatly reduced catches after the first 2-day collection period. In addition, there were approximately 4 times as many of the more locally abundant *G. sulcatus* caught as *G. retusus*, with the response to male-infested logs being significantly higher than to any other treatment (Table 2). The fairly large response by *G. sulcatus* to unattacked control logs is probably at least partially an artifact. There was a volunteer attack by 5 pairs and one male *G. sulcatus* on the log to which almost all the response occurred.

The 1975 results were partially corroborated by the 1976 results. The trend in the two replicates at Nanaimo agreed with the Pt. Roberts data. However, the combined data for the two locations disclosed that male-infested logs were clearly attractive to *G. retusus* of both sexes, but there was also attraction to female-infested logs and logs with both sexes (Table 2). The additional 50 males added to the paired insect treatments in the first replicate could account for some of the variability in the data. Of the 440 males and 435 females caught in response to this treatment 87.3 and 71.0%, respectively, were to the first replicate trap at Yellow Pt. Two other responses may be at least partially artifacts caused by inadvertent placing of treatments adjacent to *G. retusus*-infested Douglas-fir stumps, which could have created a strong overriding attraction. Of the 79 males and 87 females attracted to the unattacked log controls, 55.7 and 62.1%, respectively, were to one of the four replicates which was near a naturally infested stump. Similarly, of the 482 males attracted to the female-infested treatments, 65.8% were to the treatment near a naturally infested stump. However, only 46.9% of the 228 females captured were in response to this treatment.

In both 1976 locations there was strong cross-attraction of *G. sulcatus* to *G. retusus*-infested logs.

DISCUSSION

Our results demonstrate conclusively that a pheromone-based secondary attraction occurs in *G. retusus*, and as in *G. sulcatus* (Cade 1970, Borden and Stokkink 1973), male beetles are implicated as producers of the attractive agent. Positive responses to male frass (Table 1; Figures 2 and 4), gut extracts (Table 1), and male-attacked logs (Table 2) support this conclusion. The role of the female is less clear. No response occurred to a female gut extract stimulus (Table 1). However, the rise in attraction of frass following pairing of males with females (Figure 4), and the significant response to female-infested

TABLE 2. TOTAL NUMBERS OF *Gnathotrichus* SPP. CAPTURED ON STICKY TRAPS ENCLOSING *G. retusus* INFECTED LOGS

		Number of beetles captured[a]			
		G. retusus		*G. sulcatus*	
Location and Dates	Treatment	Males	Females	Males	Females
Pt. Roberts, Wash., May 14–24, 1975	No log	0a	0a	1a	1a
	Unattacked log	6a	4a	144b	98b
	Log + 50 ♂♂	150c	183c	576c	439c
	Log + 50 ♀♀	8a	15a	65b	56b
	Log + 50 prs.	27b	30b	193b	173b
Nanaimo and Yellow Pt., B.C., May 19–26 and June 2–9, 1976	No log	12a	6a	4a	3a
	Unattacked log	79a	87b	48a	19a
	Log + 50 ♂♂	343b	367b	156b	129b
	Log + 50 ♀♀	542b	284b	132b	68a
	Log + 50 prs	440b	435b	166b	118b

[a]Totals, within columns for each experiment, followed by same letter not significantly different, Newman-Keuls test, $P < 0.05$.

logs and paired beetles in one of two field experiments (Table 2), suggest that both sexes could be involved in secondary attraction of this species. Such a phenomenon definitely occurs in *Dendroctonus brevicomis* LeConte in which the female beetles produce the pheromone brevicomin (Silverstein et al., 1968) and the males produce frontalin (Kinzer et al., 1969).

The rise in response to frass produced by males or both sexes after about 1 week in the log (Figures 2 and 4) could be associated with the establishment of the mutualistic fungus or the activity of other microorganisms, rather than, e.g., increased activity in response to females (Figure 4). The fungus could stimulate increased pheromone production and/or release by the beetles, or could contribute directly to the attractive odor. A basidiomycete mycangial fungus of *Dendroctonus frontalis* Zimmermann is capable of producing sulcatol (Brand and Barras, 1977). However, we have been unable to obtain any response by *G. sulcatus* to cultures of fungus taken from its galleries (unpublished data). Alternatively, other microbial metabolites could contribute to secondary attraction. For example, Brand et al. (1977) found that certain yeast metabolites enhance the attraction of *D. frontalis* to near-threshold concentrations of known secondary attractants.

The cross-attraction of *G. sulcatus* to *G. retusus*-infested logs (Table 2) could reflect a response to host volatiles. In the laboratory, we have obtained consistent responses of 60% or higher by *G. sulcatus* to the odor of sawdust from insect-free, western hemlock sapwood exposed to air for 3 weeks. How-

ever, the moderate, but significant, attraction of *G. retusus* to sulcatol in the laboratory (Table 1), together with the striking attraction of *G. sulcatus* to *G. retusus*-infested logs in the field, suggests that the two species may share the pheromone sulcatol. We have no information on how species specificity in pheromone response is maintained. However, it could be based on the presence of an additional compound in *G. retusus* [there is no evidence for any other pheromone than sulcatol in *G. sulcatus* (Byrne et al., 1974)], or on the utilization of different enantiomers of the same pheromone, as speculated by Borden et al. (1976).

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EFFECT OF GROSS CARDIAC GLYCOSIDE CONTENT OF SEEDS OF COMMON MILKWEED, *Asclepias syriaca,* ON CARDIAC GLYCOSIDE UPTAKE BY THE MILKWEED BUG *Oncopeltus fasciatus*

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Abstract—Milkweed bugs, *Oncopeltus fasciatus*, were fed seeds of common milkweed, *Asclepias syriaca*, that contained differing concentrations of cardiac glycoside. Whole seeds had a mean cardiac glycoside concentration of 4.01 mg equivalents to digitoxin per g dry weight, and seed embryos had a mean concentration of 5.56 mg/g dry weight. Bugs fed these seeds concentrated cardiac glycoside: their mean concentration was 6.85 mg/g dry weight. Milkweed bugs fed seeds of lower cardiac glycoside content sequestered a greater percent of the available glycoside than bugs fed seeds of high glycoside content. The quantitative variation of cardiac glycoside content of the seeds of this single species did not significantly affect the growth of bugs. In a separate feeding preference experiment, bugs were offered seeds of both high (5.18 mg/g dry weight) and low (2.30 mg/g dry weight) cardiac glycoside content. The bugs showed no indication of selecting seeds of either high or low glycoside content.

Key Words—Warning coloration, cardiac glycosides, phytotoxin, milkweed bugs. *Oncopeltus fasciatus*, Hemiptera, Lygaeidae, *Asclepias syriaca.*

INTRODUCTION

The milkweed bug, *Oncopeltus fasciatus* (Dallas) (Hemiptera, Lygaeidae) usually feeds on milkweed plants, Asclepiadaceae (Feir, 1974). This plant family is characterized by a large number of species which contain cardiac glycosides, a group of secondary plant substances that affects heart function

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in warm-blooded vertebrates (Robinson, 1967). Milkweed plants are often toxic to mammal livestock feeding on them, resulting in considerable livestock mortality (Kingsbury, 1964). It is known that some insects, however, are relatively insensitive to large concentrations of cardenolides (Rothschild and Kellett, 1972). Furthermore, certain groups of insects utilize as their primary food source families or species of plants possessing the same secondary substance. Various species of lygaeid bugs (e.g., *Caenocoris nerii*) and danaine butterflies (including the monarch) feed mainly on plants of the family Asclepiadaceae (Feir, 1974; von Euw et al., 1971; Brower, 1969).

The cardenolides ingested by these insects appear to protect them from vertebrate predators. Many lines of evidence converge on this conclusion: (1) both groups of insects ingest and sequester the cardiac glycoside from the plants they eat, and both do not possess these substances if they feed on a plant lacking them (Brower et al., 1967; Scudder and Duffey, 1972); (2) bluejays fed monarch butterflies reared on toxic plants display an emetic response and will then refuse monarchs as food on sight alone (Brower, 1969); (3) both monarch butterflies and milkweed bugs are brightly colored. Speculation centers on the possible evolutionary advantage of warning coloration (Rothschild, 1972); (4) high concentrations of cardenolides occur in the ventral metathoracic scent glands and dorsolateral thoracic and abdominal spaces of the milkweed bug (Duffey and Scudder, 1974). The secretions from *O. fasciatus* and the scent glands of other hemipterans have pungent-smelling unsaturated aliphatic aldehydes (Games and Staddon, 1973). These chemicals may help a predator to identify a milkweed bug as unpalatable by providing a distinctive olfactory or gustatory cue.

The experiments presented in this paper exploited natural variation in the cardenolide content of wild collected milkweed, *Asclepias syriaca* L., seeds to investigate three questions: (1) Do milkweed bugs sequester in their bodies amounts of cardiac glycoside that are directly proportional to that of their food, or do they sequester varying proportions of cardiac glycoside from seeds having a high or low concentration of cardiac glycoside? In the latter case, milkweed bugs might have a higher minimum concentration of glycoside per bug, making an emetic response from a vertebrate predator more likely. (2) Does the concentration of cardiac glycoside in *A. syriaca* seeds affect the growth of the milkweed bug? (3) Do milkweed bugs selectively feed on seeds of either high or low glycoside content? This might result in a change in the amount of cardiac glycoside in their bodies.

METHODS AND MATERIALS

Preparation of Seeds and Bugs. Seed pods were collected from 70 *Asclepias syriaca* plants in October 1972, on Ellwell Island in the Connecticut

River in Northampton, Massachusetts. Seeds from each were put in a separate glassine envelope with holes punched in it, were dried for 16 hr at 60°C in a forced-draft oven, and then stored in desiccators over anhydrous $CaSO_4$ until used.

To determine the cardiac glycoside concentration of whole seeds from each seed pod, a sample of 20–25 seeds was randomly selected out of each envelope and ground to a fine powder with a mortar and pestle. The gross concentration of cardiac glycoside equivalents to digitoxin was determined in 0.1 g amounts of this powder in 5.0 ml of ethanol, utilizing the method of Brower et al. (1972), as modified in Brower and Moffitt (1974), and expressed as mg/g dry powder (Brower et al., 1975). Seeds fed to the bugs were from the same seed pods and were premoistened by putting them on a screen above water in a jar for 24 hr prior to feeding.

The stock of milkweed bugs (*Oncopeltus fasciatus*) studied was obtained from the University of Connecticut. The feeding experiments were run in 236-ml clear plastic containers provided with a vial half filled with water, with a cotton wick at the end for a water source. All the feeding experiments were run at room temperature in the lab, exposed to natural light from a north-facing window between February 16 and March 31, 1973. The order in which the experiments were run was randomized.

Effect of Glycoside Concentration in Food on Sequestering of Glycoside and Growth of Oncopeltus fasciatus. For each replicate, ten first instar nymphs, all weighing less than 1 mg each (wet weight), were placed with 0.4 g (dry weight) of *A. syriaca* seeds, all from one pod, whose cardiac glycoside concentration was determined previously by means of a seed subsample. Concentrations of glycoside in 0.1 g (dry weight) subsamples of whole seed powder from the 40 different pods used for the 40 respective replicates ranged from 1.71 to 7.85 mg glycoside/g dry weight (Table 1). Within-pod variation was also measured in two separate pods: in one, the coefficient of variation of cardiac glycoside content for three 0.1-g subsamples of whole seed powder was 8.15% (mean = 1.86 mg/g); in another, it was 8.20% (mean = 4.45 mg/g). The among-pod variation ranged over nearly an order of magnitude, whereas within-pod variation was effectively negligible.

However, milkweed bugs eat primarily the cotyledons and do not digest the seed coat (Beck et al., 1958). Therefore, for each of these experimental replicates, a 0.04-g sample of randomly selected seed embryos, including the cotyledons, hereafter referred to simply as "seed embryos," were separated from seed coats and also assayed for cardiac glycoside content. One sample for each experimental replicate was put in a 2-ml volumetric flask, resulting in the same volumetric ratio as above. Every other day, dead bugs and molted skins were removed, dried, weighed, and discarded. Bugs fed for 24 days, at which time some were adults. Bugs remaining after 24 days of feeding were

sacrificed by freezing. The bugs from each replicate were then prepared for extraction by drying at 60°C for 16 hr, weighed, and ground to a fine powder with a mortar and pestle. This powder was heated at 60° C for an additional 6–10 hr prior to extraction. A sample of 0.04 g of the bug powder from each experiment was weighed. Smaller samples were made of seed embryos and bugs than of whole seeds for each replicate because of the smaller amount of material available. Cardiac glycoside concentrations were then determined in the same manner as with the seed embryos. The amount of seed that bugs ingested was determined for 20 randomly selected replicates. Forceps were used to clean frass off the seeds upon which the bugs had fed; then the seeds were dried and weighed.

Selected samples of bugs, seed embryos, and whole seeds were reextracted for cardiac glycoside using the same procedure, to determine if the procedure extracted all the cardiac glycoside present. The resulting absorbancy values (about 10% of the original ones) were added to the originals. As a correction, 10% was added to the absorbances of the other replicates. These corrected values were converted to mg of glycoside (digitoxin equivalents) per g dry weight of whole seeds, seed embryos, or bugs. Two-tailed paired *t* tests and linear regression analyses (Steel and Torrie, 1960) were used to test the effect of cardenolide concentration of seeds on the growth and cardenolide concentration of the milkweed bugs.

Feeding Preference of Oncopeltus fasciatus *for Seeds of High vs. Low Cardenolide Concentrations.* This experiment was conducted to determine if bugs preferentially fed on seeds of either high or low cardiac glycoside content. Twenty-three containers were each provided with 0.2 g (dry weight) of seeds from pods known to have a high mean glycoside concentration (between 3.70 and 6.25 mg/g dry weight), and 0.2 g of seeds from pods of low mean glycoside concentration (1.56–2.68 mg/g dry weight). Glycoside concentrations of randomly selected seeds from individual seed pods were previously determined. The two groups of seeds in each container were marked with pink or blue dots, color being randomly assigned to each seed pod, so they could be distinguished and weighed separately at the end of the experiment. Five first instar nymphs (about 1.5 mg wet weight each) were placed in each container and allowed to feed for 6–15 days. At the end of each experiment, seeds were cleaned of frass as completely as possible with forceps. Bugs and seeds were then dried at 60°C for 16 hr, and final dry weights were obtained.

To reduce variability in the data arising from greater total ingestion in some replicates, the amount of both high and low glycoside seeds of each replicate was divided by the total bug dry weight biomass gain for that replicate. These data were then analyzed with a paired-sample *t* test (Steel and Torrie, 1960) that considered the loss in weight (ingestion) of high and low glycoside seeds in each replicate as a pair.

RESULTS

Ingestion Experiment. Milkweed bugs had significantly higher concentrations of cardiac glycoside (mean concentration 6.85 mg glycoside/g dry weight) than did either the whole seeds (4.01) or seed embryos that they ate (5.56) Table 1) (two-tailed paired t test; $t = 10.38$, $df = 38$, $P < 0.001$ for whole seeds compared with bugs and $t = 5.23$, $df = 39$, $P < 0.001$ for seed embryos compared with bugs).

The cardiac glycoside concentration in milkweed bugs was affected significantly by the concentration both in whole seeds and seed embryos, as shown by the regression coefficients differing significantly from zero ($0.02 < P < 0.05$ and $P < 0.001$, respectively; Table 2). The coefficient of determination, r^2, between seed embryo cardenolide concentration and bug cardenolide concentration (0.3243) was greater than that relating whole seed and bug cardenolide concentration (0.1197). This was expected because the bugs feed primarily on the seed embryos.

Bugs fed on seeds of low cardenolide concentration sequestered significantly greater proportions of cardenolide than did bugs eating seeds of higher cardenolide concentration. Regression coefficients for the least-squares plots of (1) whole seed and bug cardenolide concentration, and (2) seed embryo and bug cardenolide concentration were compared to hypothetical regression coefficients that would have been obtained had the bugs sequestered a constant proportion of the cardenolide available in their food. In each case, the regression coefficient was significantly different from that expected ($P < 0.001$ in each case; Figures 1 and 2).

TABLE 1. SUMMARY OF CARDENOLIDE CONCENTRATIONS (mg EQUIVALENTS OF DIGITOXIN PER g DRY WT MATERIAL) AND DRY WEIGHTS (mg) OF WHOLE SEEDS, SEED EMBRYOS, AND BUGS FED THOSE SEEDS

	Range	Mean	SD	*N*
Cardenolide concentration				
Whole seeds	1.71–7.85	4.012	1.464	39
Seed embryos	1.88–9.05	5.557	1.811	40
Bugs	4.11–11.15	6.847	1.489	40
Dry weights				
mean wt individual seeds	3.24–13.24	6.178	1.905	32
mean wt individual seed embryos	1.07–3.52	2.105	0.607	32
growth of bugs (total for each replicate)	22.5–146.1	111.1	30.4	38
Number of live bugs (total at end of each replicate)	2–10	6.894	1.842	38

TABLE 2. SUMMARY OF LINEAR REGRESSION ANALYSES ($Y = a + bX$) OF CARDENOLIDE CONCENTRATION (mg EQUIVALENTS OF DIGITOXIN PER g DRY WT MATERIAL) AND DRY WEIGHTS (mg) OF WHOLE SEEDS, SEED EMBRYOS, AND BUGS

	N	b	a	Standard error of estimate	$P(b = 0)$
Cardenolide concentration of whole seeds compared with:					
Cardenolide concentration of bugs	39	0.343	5.407	1.342	$0.02 < p < 0.05$
Growth in dry wt of bugs	37	1.048	107.108	30.293	$p > 0.50$
Dry wt seed ingested by bugs	19	2.023	187.065	47.997	$p > 0.50$
Cardenolide concentration of seed embryos compared with:					
Cardenolide concentration of bugs	40	0.468	4.245	1.209	$p < 0.001$
Growth in dry wt of bugs	38	−1.738	120.931	29.822	$p > 0.50$
Dry wt seed ingested by bugs	20	0.768	191.745	46.923	$p > 0.50$
Average dry wt of individual seeds compared with:					
Cardenolide concentration of of bugs	32	0.080	6.295	1.350	$p > 0.50$
Growth in dry wt of bugs	30	2.849	95.289	29.869	$0.30 < p < 0.40$
Dry wt seed ingested by bugs	15	20.064	78.471	36.613	$0.01 < p < 0.02$
Average dry wt of individual seed embryos compared with:					
Cardenolide concentration of bugs	32	0.195	6.378	1.353	$p > 0.50$
Growth in dry wt of bugs	30	8.596	94.756	29.910	$0.30 < p < 0.40$
Dry wt seed ingested by bugs	15	39.134	119.305	39.732	$0.02 < p < 0.05$
Dry wt seed ingested compared with:					
Cardenolide concentration of bugs	20	−0.001	7.105	1.326	$p > 0.50$
Growth in dry wt of bugs	19	0.593	−7.352	11.914	$p < 0.001$

The growth of milkweed bugs and the amount of seed they ingested were not significantly affected by the size of individual whole seeds or seed embryos, nor by the cardenolide content of their food (Table 2). There was no significant correlation observed between whole seed or seed embryo size and cardenolide concentration of whole seeds or seed embyros (Table 3).

Feeding Preference Experiment. Milkweed bugs did not eat more seeds of high glycoside content than of low glycoside content (Table 4; paired t test: $t = 0.8496$, $df = 22$, $0.40 < P < 0.50$).

DISCUSSION

Isman et al. (1977a) found that cardiac glycoside content of milkweed bugs collected in California on different *Asclepias* species, or fed their respective seeds, reflected the concentration of the seeds of the respective plant species. My finding extends theirs in showing that the cardenolide content of milkweed bugs feeding on one species of milkweed is affected by the variation in cardenolide concentration within that species. Ward (1972) found a unimodal variation in cardiac glycoside concentration among seeds of different *Asclepias syriaca* plants which resembles the unimodal distribution of cardiac glycoside concentration found in a sample of wild monarch butterflies in Massachusetts (Brower et al., 1972). Since most of the milkweed growing in Massachusetts is *A. syriaca* (Woodson, 1954; personal observation), one may hypothesize that the cardiac glycoside content of a

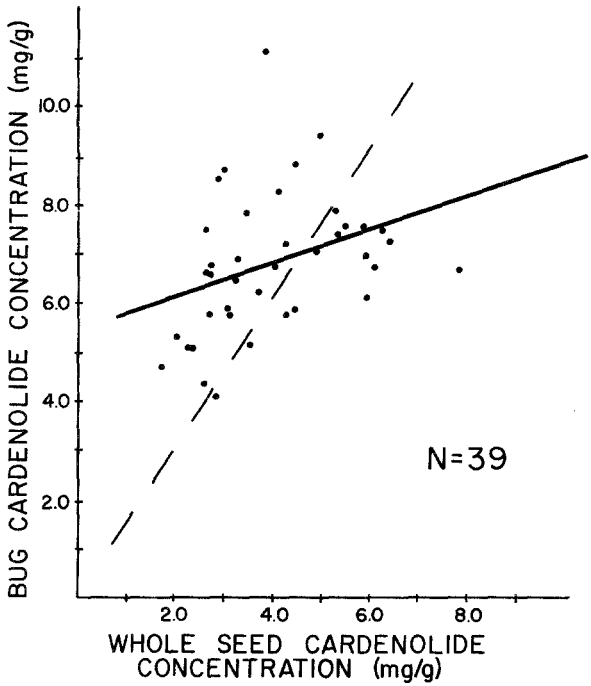

FIG. 1. Regression of bug cardenolide concentration on whole seed cardenolide concentration, expressed in mg equivalents of digitoxin per g dry wt material. The solid line is the least-squares line of best fit ($Y = 0.343X + 5.407$). The broken line represents the hypothesis that bugs sequester a constant proportion of available cardenolide from whole seeds ($Y = 1.536X$; regression coefficient, $b = \Sigma XY/\Sigma X^2$; Steel and Torrie, 1960, p. 179).

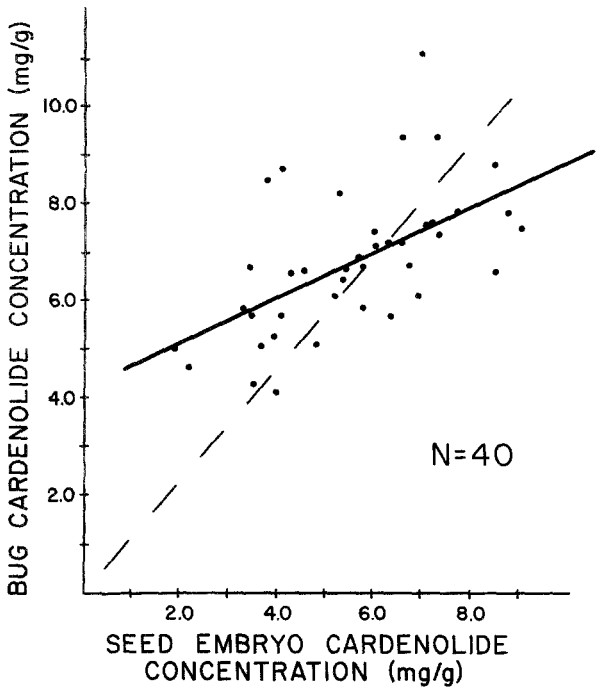

FIG. 2. Regression of bug cardenolide concentration on seed embryo cardenolide concentration, expressed in mg equivalents of digitoxin per g dry wt material. The solid line is the least-squares line of best fit ($Y = 0.468X + 4.245$). The broken line represents the hypothesis that bugs sequester a constant proportion of available cardenolide from seed embryos ($Y = 1.160X$; regression coefficient $b = \Sigma XY/\Sigma X^2$).

particular monarch butterfly is dependent on that of the plant it ate, as this research has shown to be the case with milkweed bugs. The cardiac glycoside concentration of Massachusetts *A. syriaca* seeds in this study (1.71–7.85 mg digitoxin equivalents/g dry weight) is similar to *A. syriaca* seed concentrations (1.34–9.03) found by Roeske et al. (1976), and higher than that which they found in *A. syriaca* leaves (0.06–2.64). The emetic potency of monarch butterflies fed on two different species of milkweeds was correlated with the cardiac glycoside content of those milkweeds (Roeske et al., 1976). Brower et al. (1967) found that cabbage-reared monarch butterfly larvae lacked emetic properties and were palatable when fed to captive bird predators. It is therefore reasonable to assume that the larvae do not manufacture cardiac glycosides. Similarly, Duffey and Scudder (1972) found that milkweed bugs fed sunflower seeds, which do not contain cardenolides, also did not sequester cardenolides.

Milkweed bugs concentrate cardiac glycoside from their food, indicating preferential storage of cardiac glycoside. Lygaeid bugs are more efficient at

TABLE 3. SUMMARY OF LINEAR CORRELATION ANALYSES OF CARDENOLIDE CONCENTRATION (mg EQUIVALENTS OF DIGITOXIN PER g DRY WT MATERIAL) AND DRY WEIGHTS (mg) OF WHOLE SEEDS, SEED EMBRYOS, AND BUGS

	N	Correlation coefficient (r)	$P(r = 0)$
Cardenolide concentration of whole seeds compared with:			
Average dry wt individual seeds	32	0.087	$P > 0.50$
Average dry wt individual seed embryos	32	0.053	$P > 0.50$
Cardenolide concentration of seed embryos compared with:			
Average dry wt individual seeds	32	0.215	$0.20 < P < 0.50$
Average dry wt individual seed embryos	32	0.142	$0.20 < P < 0.50$
Cardenolide concentration of bugs compared with:			
Growth in dry wt bugs	38	−0.075	$P > 0.50$

storage than are cerambycid beetles, whose storage of cardiac glycoside is minimal (Isman et al., 1977b). Duffey (1976) found that *O. fasciatus* that were reared on *A. syriaca* were palatable to toads, while those fed *A. curassavica* were unpalatable. On the other hand, Feir and Suen (1971) found *A. syriaca*-reared milkweed bugs to be unacceptable to common fence lizards. Brower and Moffitt (1974) found that Massachusetts monarch butterflies weighing 188 mg (dry weight) and containing 0.403–0.480 mg of glycoside contained one ED_{50} unit for the average bluejay predator. Milkweed bugs were much smaller (mean dry weight 16 mg) than were these monarch butterflies.

TABLE 4. VARIATION IN CARDENOLIDE CONCENTRATION (mg EQUIVALENTS OF DIGITOXIN PER g DRY WT MATERIAL) BETWEEN SEEDS OF HIGH AND LOW CARDENOLIDE CONCENTRATION, DRY WEIGHT (mg) EATEN OF EACH, AND GROWTH (mg) OF BUGS IN 23 REPLICATES OF FEEDING PREFERENCE EXPERIMENT

	Variation	High cardenolide seeds		Low cardenolide seeds
Cardenolide concentration	Mean	5.178		2.302
	SD	0.765		0.439
Dry wt seed ingested	Mean	32.6		27.2
	SD	22.5		16.8
Dry wt growth of bugs	Mean		118.9	
	SD		63.5	
Dry wt seed ingested / Dry wt growth of bugs	Mean	0.267		0.230
	SD	0.133		0.096

However, milkweed bugs fed *A. syriaca* may still cause emesis in some vertebrate predators for two reasons. First, their cardiac glycoside concentration (6.85 mg/g dry wt of bug) is higher than that of Massachusetts monarch butterflies (2.049 mg/g dry weight; Brower and Moffitt, 1974) that eat primarily *A. syriaca* (Urquhart, 1960). Second, milkweed bugs display gregarious behavior, which increases their feeding efficiency on *A. syriaca* (Ralph, 1976). This probably also increases the likelihood that some vertebrate predator species will find and eat several milkweed bugs at once and ingest enough glycoside to experience an emetic response.

Values obtained in this study for cardiac glycoside content of milkweed bugs were comparable to those obtained by Isman et al. (1977a), who found that *O. fasciatus* fed seeds of different California *Asclepias* species had cardenolide contents ranging from 0.034 to 0.281 mg of cardenolide per insect. Isman (1979) found that wild lygaeid bugs collected on *Asclepias curassavica* in Costa Rica had cardenolide contents ranging from 0.203 to 0.231 mg/insect.

Milkweed bugs fed *A. syriaca* seeds of different cardenolide concentrations did not store a constant proportion of that cardenolide in their bodies. Instead, those bugs eating seeds of low cardenolide concentration stored a greater percentage of that cardenolide than did bugs fed seeds of high cardenolide concentration. This differential retention may result in a higher percentage of the bug population (or groups of bugs) being emetic to vertebrate predators. Brower et al. (1970) state that increases in the proportion of emetic prey lead to decreases in predator pressure and enhancement of survival. This idea could be reasonably extended to aggregates of milkweed bugs as well as individuals, because of their gregarious behavior.

The growth rate of milkweed bugs and the amount of food they ingested were not measurably affected by differing intraspecific concentrations of cardiac glycoside in their food. This is consistent with Isman's (1977) finding that the growth of *O. fasciatus* was not significantly affected by variation in cardiac glycoside concentration among the different species of milkweed included in their diets. In contrast, Brower and Glazier (1975) and Brower and Moffitt (1974) found that female monarch butterflies with very high concentrations of cardenolides tended to weigh less than those with lower concentrations, and that butterflies with lower concentrations also appeared to be more successful migrants. Brower and Glazier (1975) cite several experiments in which insects showed little sensitivity to high concentrations of the secondary substances in their host plants. Perhaps the toxicity of a host plant secondary substance for insects feeding on that plant is low but is synergistically affected by high stress (e.g., migration). In this way, the secondary substance might appreciably affect the survival of the insect in nature.

The present feeding preference experiment indicated that milkweed bugs do not feed preferentially on seeds of either high or low cardenolide concentration when seeds of differing concentrations are offered to them. Likewise, milkweed bugs showed no feeding preference for any particular part of the *A. syriaca* plant, even though these parts differed in cardenolide content (Feir and Suen, 1971). These data indicate that milkweed bugs physiologically or physically regulate cardenolide rather than preferentially eat individual seeds that have a higher cardenolide concentration than other seeds in the same seed pod. This also implies that milkweed bugs do not select *A. syriaca* plants of a certain cardenolide concentration in the wild. It does not, however, exclude the possibility that bugs may prefer one species of milkweed to another due to qualitative differences in cardiac glycosides or quantitative variation greater than that encountered in these experiments.

The experiments outlined in this paper indicate that milkweed bugs increase the gross content of cardiac glycoside in their bodies in at least two ways: (1) bugs concentrate cardiac glycoside from their food in their tissues, and (2) bugs that eat seeds containing relatively little cardiac glycoside physiologically or physically, rather than behaviorally, sequester a greater proportion of that glycoside in their tissues.

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AN IMPROVED PREPARATION OF SOME INSECT SEX ATTRACTANTS: SYNTHESIS AND SEPARATION OF GEOMETRICAL ISOMERS BY FORMATION OF UREA COMPLEXES[1,2]

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Abstract—Wittig condensations of aldehydes or ketones with phosphonium salts are frequently used methods for the syntheses of straight-chain or branched alkenes. Suitable choice of reaction conditions may provide the desired geometrical isomer as reaction product. However, we have found that mixtures of geometrical isomers can be conveniently separated on a large scale by the relatively inexpensive method of urea inclusion complex formation. The recovery of both isomers from the separation procedure is almost quantitative. Urea inclusion complexes are formed preferentially with *E* isomers. Formation of the inclusion complex is affected by the skeletal structure and by the nature of the terminal functional group. By applying this method of separation at a convenient stage, several insect pheromones were prepared without the necessity of a stereoselective step. The technique was used for syntheses of (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal (components of the *Heliothis virescens* pheromone) and for the synthesis of candidate attractants possessing a trisubstituted double bond.

Key Words—Insect sex attractants, synthesis, isomer separation, urea complexes.

INTRODUCTION

Many lepidopteran pheromones are derivatives of long-chain olefins, with a terminal aldehyde, ester, or alcohol functional group. Insect responses to

[1]This work was conducted under a Cooperative Agreement between the University of Maryland and the Agricultural Research Service.
[2]Mention of a commercial product in this paper does not constitute an endorsement of this product by the USDA.

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these compounds are often critically dependent upon the presence of the correct geometrical isomer or a precise blend of geometrical isomers. A prerequisite for the practical use of these attractant pheromones or related compounds in pest management programs, either for trapping or for mating disruption, will be the availability of relatively large quantities of the pure geometric isomers at reasonable cost. Since stereoselective synthetic methods rarely yield the desired isomers in a sufficiently pure state, methods for the separation of geometrical isomers that can be adapted to large-scale operation must be devised. We have found that formation of urea inclusion complexes can be a useful method for the preparative-scale separation of individual geometrical isomers from their mixtures.

The addition of an appropriate compound with a linear or nearly linear skeletal structure to a methanolic solution of urea results in the formation of a crystalline complex having the "guest" molecule held in the channel of the helical lattice of hydrogen-bonded urea molecules. Compounds unable to fit in this channel remain in solution and are separated by filtration. The complex is then destroyed by the addition of water to recover the original substance. Butenandt et al. (1962) made use of this process at several points in the synthesis of the various isomers of bombykol [(*E*,*Z*)-8, 10-hexadecadien-1-ol], the first identified sex attractant pheromone, but the process has not received much attention in pheromone work since that time.

We have examined some of the structural requirements for the formation of such urea complexes and have shown how the technique can be applied to specific pheromone syntheses.

METHODS AND MATERIALS

The geometrical purity of the products was determined by vapor-phase chromatography (GLC) on a Hewlett-Packard model 5840 gas chromatograph. Separations were achieved using a SP 1000 wall-coated open tubular (WCOT) glass capillary column (60 m × 0.25 mm ID) operated at 195°. The nonenoate esters were analyzed on a Varian model 2740 gas chromatograph on a glass column (75 cm × 2 mm ID) packed with a graphitized carbon black (0.2% SP-1000 on 60/80 mesh Carbopak A; Supelco, Inc., Bellefonte, Pennsylvania) using a flame-ionization detector. A Waters model 201 high-pressure liquid chromatograph was used to analyze (*Z*)-9-tetradecenal. This was fitted with Whatman Partisil ODS 11 reverse-phase column (25 cm × 4.6 cm ID) with an efficiency of 28,100 plates per meter, and methanol/water was used as the solvent. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian T-60 spectrometer. Infrared (IR) spectra were recorded on a Perkin-Elmer model 457 A grating spectrometer using neat film. Mass spectra (MS) were run on a Finnigan 1015 GC-MS system.

Intermediates were prepared by standard methods. Chromatography, spectra (IR, NMR, and MS), and elemental analyses were used for characterization and confirmation of purity of each compound.

(11-*Methoxy*-11-*oxoundecyl*)*triphenylphosphonium Bromide.* A mixture of 436 g methyl 11-bromoundecanoate, 400 g triphenylphosphine, and 1.0 liter of dried acetonitrile was heated under reflux in a 3-liter flask for 8 hr while the flask was swept with nitrogen. The solvent was removed under reduced pressure to give an oil. The oil was triturated twice with 500-ml portions of ether to yield 737 g (87%) of the solid salt, which was dried in an oven at 75°C overnight and used without further purification. Other phosphonium salts were prepared similarly. These salts were used to prepare unsaturated esters and an unsaturated nitrile by the Wittig reaction, using the method of Sonnet (1969) with slight modification.

Methyl 12-*Methyl*-11-*pentadecenoate.* Dry methanol (200 ml) was treated with 12.5 g of sodium in a 3-liter 3-necked flask equipped with a mechanical stirrer, distillation head, gas inlet tube, and a mineral oil trap. A nitrogen sweep was continually maintained. After all the sodium had reacted, methanol was removed by distillation and 200 ml of dry benzene was added and distilled off, followed by a second portion of benzene. After all the benzene had been removed, 300 ml of dry dimethylformamide was added and the mixture was allowed to cool to room temperature. (11-Methoxy-11-oxoundecyl)triphenylphosphonium bromide (300 g) was added and the red solution was stirred for 1 hr. 2-Pentanone (46.7 g) was added dropwise from a dropping funnel, and the solution was stirred at room temperature for 16 hr. Water (1500 ml) was added, the upper organic layer was separated, and the aqueous layer was extracted twice with 200 ml portions of petroleum ether. The organic layers were combined, washed with 100 ml of water, dried ($MgSO_4$), and evaporated under reduced pressure to give 101 g of an oil. The product distilled at 149°C (0.03 mm) to give 81.4 g (55%); the *E*/*Z* ratio was 42/58.

Separation of E and Z Isomers: Methyl (*Z*)-12-*Methyl*-11-*pentadecenoate.* The mixed esters were treated with 135 g of urea dissolved in 850 ml of methanol and allowed to stand for 3 hr. The crystalline complex was removed by filtration and washed with ether. The filtrate and washings were concentrated to dryness, and the urea residue was extracted with two 200-ml portions of petroleum ether. Filtration and evaporation of the petroleum ether extract yielded 60.0 g. The complexation process was repeated two more times to give a product with a *Z*/*E* ratio of 94.4/5.6 (GLC analysis of the corresponding aldehyde). The yield of *Z* isomer was 48 g.

Methyl (*E*)-12-*Methyl*-11-*pentadecenoate.* The collected solid and the urea residue remaining after the separation of the *Z* isomer were combined and treated with 500 ml of water to decompose the complex. The resulting upper organic phase was removed, and the lower aqueous phase was extracted

with two 200-ml portions of petroleum ether. The combined organic phase was dried and evaporated under reduced pressure to yield 28.8 g *E* isomer. GLC analysis of the corresponding aldehyde showed that the *E*/*Z* ratio was 95.6/4.4.

(*Z*)-12-*Methyl*-11-*pentadecenal.* Methyl (*Z*)-12-methyl-11-pentadecenoate (30 g) was reduced to (*Z*)-12-methyl-11-pentadecen-1-ol (25 g, 93% yield) with lithium aluminum hydride in dry tetrahydrofuran. Chromium trioxide/pyridine oxidation (Ratcliffe and Rodehorst, 1970) converted the unsaturated alcohol to the aldehyde (18.2 g, 74%). GLC analysis showed that the overall purity was 96.5%, with *Z*/*E* ratio 94.4/5.6. The retention time was identical to that of an authentic sample.

(*E*)-12-*Methyl*-11-*pentadecenal.* In a similar fashion, lithium aluminum hydride reduction of methyl (*E*)-12-methyl-11-pentadecenoate (21.8 g) and oxidation of the resulting (*E*)-12-methyl-11-pentadecen-1-ol yielded 15.5 g (79%) of the aldehyde (overall purity by GLC, 94.7%, with *E*/Z ratio 95.6/4.4). The retention time was identical to that of an authentic sample.

The sample failed to complex with urea, indicating that urea separation of the isomeric aldehydes would fail.

Methyl 11-*Hexadecenoate.* Pentanal (35.4 g) and (11-methoxy-11-oxoundecyl)triphenylphosphonium bromide (223 g) reacted at 10°C to yield 36 g (33%) of the isomeric mixture (bp, 135–140°C at 0.06 mm Hg). Since both isomers formed urea complexes, separation by treatment with urea was not possible.

11-*Hexadecen*-1-*ol.* Reduction of methyl 11-hexadecenoate (56.8 g) with lithium aluminum hydride in dry pyridine yielded 50.8 g (100%) of the isomeric mixture. Both isomers formed complexes when treated with excess urea, so again the mixture could not be separated by complexation with urea.

(*Z*)-11-*Hexadecenal.* Chromium trioxide/pyridine oxidation of 11-hexadecen-1-ol (38 g) yielded 33.7 g (88%) of the crude aldehyde. The product was treated with 119 g urea in 600 ml methanol. The solution was allowed to stand several hours, and then the crystals were collected by filtration and washed with ether. The filtrate and washings were evaporated to dryness. Petroleum ether extraction of the urea residue followed by evaporation of solvent yielded 15.5 g of a noncomplexing fraction. After chromatography on silica gel with petroleum ether–diethyl ether (90:10), the yield was 10.3 g. GLC showed that the overall purity was 98.1%. No *E* isomer was detectable by HPLC. GLC analysis showed that *Z*/*E* ratio was 95.5/4.5.

The fraction recovered from the urea complex (14.4 g) was a mixture of *E* and *Z* isomers; *E*/*Z* ratio was approx. 50/50.

Methyl 9-*Tetradecenoate.* The reaction of pentanal (77.7 g) and (9-methoxy-9-oxononyl)triphenylphosphonium bromide (475 g) yielded 59.7 g (28%) of a mixture of isomers, boiling at 155–165°C at 0.75 mm Hg. Liquid chromatography showed the ratio of isomers to be 92:8 *Z*/*E*. Both isomers formed complexes and could not be separated by treatment with urea.

(*Z*)-9-*Tetradecen*-1-*ol.* Methyl 9-tetradecenoate (36.0 g) treated with 5.0 g lithium aluminum hydride yielded 31.2 g (98%) of an oil, containing the two isomers. The GLC retention time was identical to that of an authentic sample.

Urea (10.0 g) dissolved in 100 ml methanol was added to 27.5 g of this mixture of isomers. After the mixture had stood at room temperature for 16 hr, the crystals were removed by filtration, the solvent was evaporated, and the residue was extracted with two 200-ml portions of petroleum ether. Evaporation of solvent yielded 23.2 g (*Z*)-9-tetradecen-1-ol (ratio of isomers shown to be 97.6:2.4 *Z*/*E* by HPLC). GLC analysis of the acetate showed that the *Z*/*E ratio was* 94.6:5.4.

(*Z*)-9-*Tetradecenal.* Oxidation of 5.0 g (*Z*)-9-tetradecen-1-ol with chromium trioxide yielded 3.0 g aldehyde (61%; overall purity by GLC, 97%). GLC analysis showed that the *Z*/*E* ratio was 94.6:5.4. Subsequent treatment with urea for four cycles gave a *Z*/*E* ratio of 99:1.0. No further purification was possible.

(*Z*)-9-*Tetradecen*-1-*ol Formate.* Acetic anhydride (100 ml) was cooled to 0°C and treated dropwise with formic acid (50 ml). The solution was heated to 50°C for 15 min; then cooled to 0°C (Fieser and Fieser, 1967).

(*Z*)-9-Tetradecen-1-ol (51 g) in dry pyridine (150 ml) was treated with the freshly prepared formic acetic anhydride to give 57.9 g ester (100%). No impurities were detected by GLC.

Methyl (*Z*)-4-*Nonenoate.* (4-Methoxy-4-oxobutyl)triphenylphosphonium bromide (762 g from methyl 4-bromobutanoate) and pentanal (148 g) reacted to yield 152 g (52%) of a mixture of *Z* and *E* isomers, bp, 103–105°C at 21 mm Hg. The *Z*/*E* ratio was 95:5.

A portion of the ester (75 g) was treated with 25 g of urea (two times), followed by one treatment with 5.0 g of urea, to give 64.9 g of noncomplexing *Z* isomer *Z*/*E*, 98.4:1.6). The complexing fraction (1.3 g) had a *Z*/*E* ratio of 23:77.

9-*Tetradecenenitrile.* (8-Cyanooctyl)triphenylphosphonium bromide (79.9 g from 9-bromononanenitrile) reacted with 14.4 g of pentanal to yield 20.9 g (60%) of crude product.

The mixture of isomers was treated with 60 g urea in 300 ml of methanol to give 16.8 g of noncomplexing *Z* isomer. Thin-layer chromatography on a $AgNO_3$-impregnated plate showed no *E* isomer.

RESULTS AND DISCUSSION

The formation of urea inclusion complexes has found application in a number of areas, such as the fractionation of fatty acids or the separation of alkanes from naphthenes. The subject of urea complexes was reviewed briefly by Fieser and Fieser (1967). An interesting application is described by Fujiwara et al. (1976); a mixture of the urea complexes of (*Z*,*E*)-9,11-

tetradecadien-1-ol acetate and (*Z*,*E*)-9,12-tetradecadien-1-ol acetate was used to obtain prolonged pheromonal activity against a cutworm, *Spodoptera litura* (F.).

The steric requirements for complex formation are very rigid, both as to cross-sectional dimensions and chain length. A straight-chain hydrocarbon with seven or more carbon atoms will form a complex, but the presence of branching, such as a methyl group somewhere along the chain, makes the compound too bulky to be accommodated in the urea channel and prevents complex formation. The slight kink in a hydrocarbon chain resulting from a double bond having the *E* configuration is usually not sufficient to prevent complex formation, but compounds with the *Z* configuration frequently are not able to form complexes.

A polar terminal functional group, which can form hydrogen bonds with the urea molecules, has considerable effect on the dimensions of the urea channel. The carbomethoxy group is relatively bulky, with a cross-sectional diameter of 5.1 Å (Radell and Connolly, 1960), compared with 2.86 Å, the cross-sectional diameter of a normal alkane. The channel of a complex formed with a methyl ester is therefore larger and can accommodate unsaturated chains in either the *E* or the *Z* configuration. Thus, the methyl esters of (*E*)- and (*Z*)-11-hexadecenoate both formed complexes and could not be separated. However, the additional bulk of a methyl group on the chain was sufficient to prevent complex formation with methyl (*Z*)-12-methyl-11-pentadecenoate, while the *E* isomer readily formed the inclusion complex and separation of the two isomers was possible.

With a smaller terminal functional group, such as a $-CH_2OH$ group (cross-sectional diameter, 2.8 Å), the reduced diameter of the urea channel may improve selectivity sufficiently to permit a separation which is not possible with the esters. The methyl 9-tetradecenoates could not be separated, but the *E* isomer of 9-tetradecen-1-ol could be removed by complex formation, and the excluded (*Z*)-9-tetradecen-1-ol contained only 2.4% *E* isomer when treated with a limited amount of urea. In this case selectivity was marginal, and with 11-hexadecen-1-ol satisfactory separation was not achieved when an excess amount of urea was used.

Terminal groups such as $-CHO$ and $-CN$, with still smaller effective diameters, bring about a further decrease in the size of the urea channel and a consequent increase in selectivity. With 11-hexadecenal, the channel is small enough so that essentially pure (*Z*)-11-hexadecenal could be obtained in the noncomplexing fraction. In this case, the complex contained a mixture of the *E* and *Z* isomers in about equal amounts. Satisfactory separation of (*Z*)-9-tetradecenitrile was also obtained. With 12-methyl-11-pentadecenal, the methyl substituent prevented complex formation with either isomer.

To obtain selectivity, a balance between cross-sectional diameter and length of the straight-chain portion of the molecule is needed. This is

illustrated by the 4-nonenoate esters. The *E* isomer has a sufficiently long linear portion to form a complex, and thus separation can be achieved when limited amounts of urea are used. The corresponding 4-nonenols do not complex and cannot be separated.

The separations that were achieved by means of the urea complex technique are summarized in Table 1.

A number of the compounds prepared in this study have insect pheromonal activity. These include (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal, the components of the sex attractant pheromone of the tobacco budworm, *Heliothis virescens* (F.) (Roelofs et al., 1974; Tumlinson et al., 1975); (*Z*)-9-tetradecen-1-ol formate, an effective mating disruptant for *H. virescens* and *H. zea* (Boddie) (Mitchell et al., 1975); and (*Z*)-11-hexadecen-1-ol, which with its acetate make up the major components of the sex attractant pheromone of the clover cutworm moth, *Scotogramma trifolii* (Rottenberg) (Underhill et al., 1976).

This method thus shows promise for the preparation of many pheromonal components possessing a linear or nearly linear carbon skeleton. As we have illustrated, separation of geometric isomers can often be conveniently accomplished by the formation of a urea complex as a result of the judicious selection of a terminal functional group. The inclusion of a urea complex separation step in the synthetic scheme eliminates the necessity for some time-consuming chromatographic procedures, and the technique is readily applicable to large-scale preparations. Since in many cases complex forma-

TABLE 1. SEPARATION OF GEOMETRIC ISOMERS OF $RR_1C{=}CH(CH_2)_nX$ BY UREA COMPLEX FORMATION

R	R_1	*n*	X	Separation[a]
C_3H_7	CH_3	9	CO_2CH_3	+
C_3H_7	CH_3	9	CHO	−
C_4H_9	H	9	CO_2CH_3	−
C_4H_9	H	9	CN	+
C_4H_9	H	9	CH_2OH	−
C_4H_9	H	9	CHO	+
C_4H_9	H	7	CO_2CH_3	−
C_4H_9	H	7	CH_2OH	+
C_4H_9	H	2	CO_2CH_3	+
$C_3H_7CH{=}CH$[b]	H	8	CH_2OH	+
$C_3H_7C{\equiv}C$[b]	H	8	$CO_2C_2H_5$	+
$C_3H_7C{\equiv}C$[b]	H	8	CH_2OH	+

[a] + indicates that at least one isomer could be obtained in essentially pure form; − indicates that no separation was achieved.
[b] From Butenandt et al. (1962).

tion with the *E* isomer is preferred, separations and useful enrichments of isomers can be achieved by carefully controlling the amount of urea used.

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SPECIALIZATION OF OLFACTORY CELLS TO INSECT- AND HOST-PRODUCED VOLATILES IN THE BARK BEETLE *Ips pini* (SAY)[1]

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Abstract—Electrophysiological recordings in *Ips pini* were made from single olfactory cells stimulated by six concentrations of eleven compounds—either pheromones or host constituents. The receptor cells were grouped according to their differential responses to these biologically significant compounds. Cells specialized to either ipsdienol or ipsenol (pheromones for several *Ips* species) appeared to be relatively uniform in their sensitivity to all compounds tested. Another group of cells specialized to *cis*- and *trans*-verbenol were more variable in their sensitivities to the substances tested. The activation of separate groups of cells by the different pheromones and host compounds shows (by exclusion) that behaviorally expressed synergistic as well as inhibitory action mainly is due to an interaction in the central nervous system rather than an interaction of the compounds on the receptors. All "ipsdienol cells" responded to both enantiomers of ipsdienol. Although some differences between responses to the enantiomers by individual cells were observed, these did not form a consistent pattern, and no net differences could be found between the eastern and western populations of *I. pini*.

Key Words—Bark beetle, *Ips pini*, Coleoptera, Scolytidae, pheromone, enantiomer, olfaction, single cell, electrophysiology.

INTRODUCTION

Pheromones of the bark beetle genus *Ips* are structurally similar to host compounds which have been implicated as their precursors. For example, *trans*-

[1]Coleoptera: Scolytidae.

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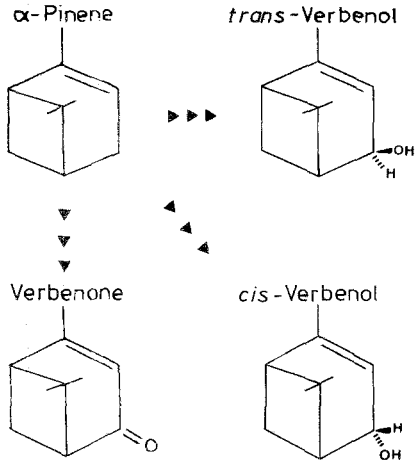

FIG. 1. Structure of the host terpene, α-pinene, and the three insect-produced compounds *trans*-verbenol, *cis*-verbenol, and verbenone, indicating α-pinene to be a precursor for the three others.

and *cis*-verbenol have been shown to be manufactured from α-pinene (Figure 1) (Hughes, 1973; Brand et al., 1975) while ipsenol and ipsdienol released by the insects are produced from myrcene obtained in the host (Figure 2) (Hughes, 1974).

As aggregating attractants, various combinations of ipsenol, ipsdienol, *trans*- and *cis*-verbenol and host odorants act synergistically for some *Ips* species and antagonistically for others. *I. paraconfusus* Lanier responds to a mixture of ipsenol, ipsdienol, and *cis*-verbenol (Silverstein et al., 1966) while *I. pini* (Say) is attracted to ipsdienol but attraction is inhibited by the addition of ipsenol (Birch and Wood, 1975; Birch and Light, 1977). *I. calligraphus* (Germar) requires a combination of *cis*-verbenol, ipsdienol, and host terpenes for maximum response (Renwick and Vité, 1972).

Enantiomeric configuration may also be important; Vité et al. (1976) showed that *I. grandicollis* (Eichhoff) responds to the (+) enantiomer of ipsenol and, in the attractant of *I. pini* (Lanier et al., 1972), geographic variability could be explained by the eastern and western populations keying up on opposite enantiomers of ipsdienol (Lanier et al., unpublished).

The present work investigates whether or not there are separate olfactory cells for the various insect- and host-produced volatiles and attempts to ascertain if behavioral differences of the eastern and western strains of *I. pini* may be explained by differences in the olfactory cells.

METHODS AND MATERIALS

Materials. Ips pini used in these tests were from laboratory colonies originating in Tully, New York; Worley, Idaho; and Mt. Shasta, California. The beetles were 2–10 days postemergence and only females were tested.

Preparation and Recording. Stereoscanning micrographs of the antennal club of *Ips pini* (Angst and Lanier, 1979) showed sensilla trichodia and basiconica similar to those implicated to have olfactory function in other bark beetle species (Borden and Wood, 1966; Payne et al., 1973; Dickens and Payne, 1978). In the present study electrophysiological recordings were made from single olfactory cells by inserting a tungsten microelectrode into the base of sensilla as described by Boeckh (1962). However, because of the high hair density on the club, it was difficult to resolve individual hairs. The microelectrode was, therefore, inserted at a certain angle into the area of olfactory organs (cf. Mustaparta, 1975). The indifferent electrode was placed in the mouth. A scheme of the recording arrangement is shown in Figure 3. Methods for mounting beetles were similar to those of Payne (1970) and Angst and Lanier (1979).

Stimulation. A cartridge technique was used for stimulation (Kaissling and Priesner, 1970). Different concentrations of the various compounds dissolved in hexane were deposited on strips of filter paper. The solvent was allowed to evaporate and the paper placed in a 12-cm glass tube (cartridge). The

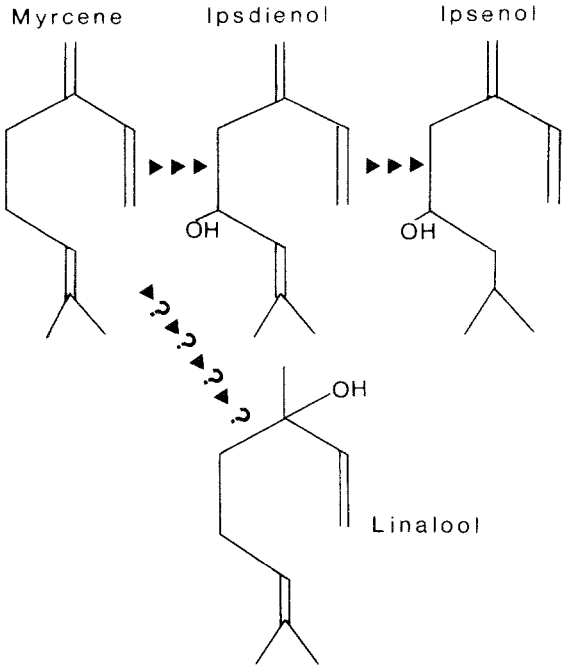

FIG. 2. Structure of the host terpene, myrcene, and the insect-produced compounds ipsdienol, ipsenol, and linalool, indicating myrcene to be used as a precursor in the production of the pheromones.

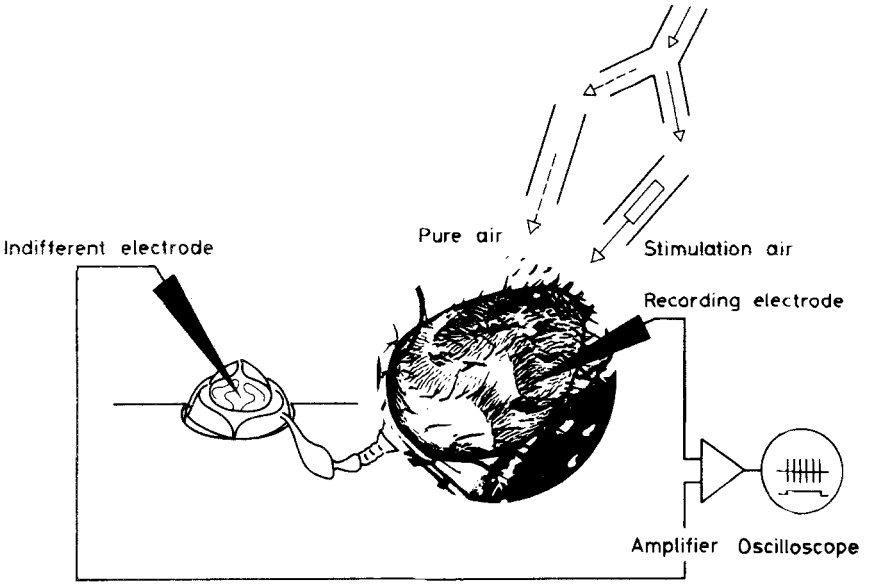

FIG. 3. Recording and stimulation arrangement for single olfactory cell recordings in *Ips pini*, showing the position of the electrodes. The input resistance of the preamplifier was 10^9MΩ and the noise level 5 μVpp (low pass 10 kHz, high pass 100 Hz, 20 dB/DEK).

receptor cells were then stimulated by blowing a purified air stream through the cartridge over the antennae. Each compound was used at six serial concentration levels ranging from 0.005 to 500 μg of the pure compounds applied on each strip of paper. The compounds were purified and concentrations controlled by gas–liquid chromatography.

Substances. Seven of the eleven compounds tested represent the major volatiles from the frass (boring dust which includes fecal pellets and host fragments) of *I. pini*. These were the (+) and (−) enantiomers of ipsdienol, *trans*-verbenol, verbenone, camphor, linalool, and 1-octanol (Stewart, 1975). The remaining four compounds are ipsenol, *cis*-verbenol, and the two host-terpenes, α-pinene and myrcene.

RESULTS

Recordings were made from 95 single olfactory cells. Usually, spikes of one amplitude were obtained, but a few recordings showing two distinctly different spike amplitudes indicated that the olfactory sensilla could be in-

nervated either by one or two cells; two simultaneously recorded cells were usually activated by different compounds.

The responses of the cells remained constant for several hours, and most of them were tested against all eleven compounds at six concentrations. For many cells the test series were repeated two or three times. When the sensitivity of the cell changed, it was in the same degree to all substances. The spontaneous activity of the cell was consistently low (0–5 imp/sec) with spike amplitudes varying between 0.03 and 0.1 mV. The responses (Figure 5b and c) showed the usual pattern of an initial high discharge followed by decay to a stationary level until the stimulation was terminated (Kaissling, 1971). The effect of a compound on a receptor cell is expressed as the number of spikes during 500 msec of the stimulation period from the time the first spike occurred.

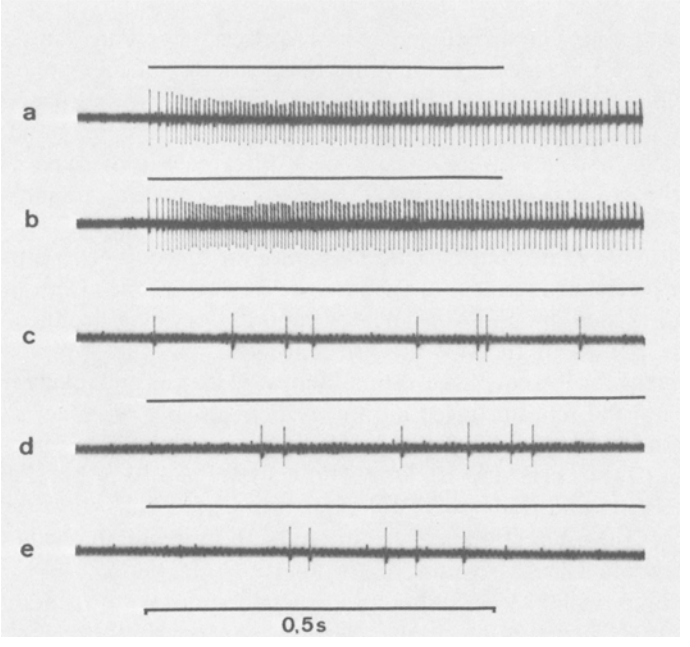

FIG. 4. Responses of a single olfactory cell of *Ips pini* to different compounds, showing selective activation by the enantiomers of ipsdienol, while other compounds were not effective. From above: responses to the vapor of 50 μg (−)-ipsdienol (a), 50 μg (+)-ipsdienol (b), 500 μg ipsenol (c), 500 μg *trans*-verbenol (d), 500 μg α-pinene (e). The stimulation period is indicated by the black bar above each trace.

All of the cells responded selectively and are therefore grouped below according to their reaction to the compounds tested (Table 1). Figures 4 and 5 demonstrate the selective responses of two cells to ipsdienol and *trans-/cis*-verbenol, respectively.

Responses to Ipsdienol. This is the principal aggregation pheromone in *I. pini* and is produced as a mixture of the (+) and (−) enantiomers in the eastern (New York) population, whereas beetles of the western (Idaho and California) populations produce only the (−) variant (Stewart, 1975). Therefore we compared the responses of the ipsdienol receptors from different strains of *I. pini* to the pure (+) and (−) enantiomers and to the racemic mixture. Of the 95 recordings made, 45 were from receptor cells specialized to ipsdienol. All of these 45 cells responded strongly to both enantiomers and the racemic mixture, but they only responded weakly, if at all, to the highest concentrations of the other compounds (Table 1, Group A, cell 1–8). Thus, the highest concentrations of linalool and ipsenol activated some ipsdienol cells, with linalool evoking a stronger response than ipsenol (cf. Table 1, Group A, cell 4–8).

The enantiomers of ipsdienol appeared to affect the receptor cells of beetles from eastern and western populations to the same extent. Figure 6 shows one example of dose–response curves for both enantiomers and the racemic mixture. Only slight differences between these three curves were obtained for any of the cells, and there were no consistent differences in the ranges of the curves for the two populations. However, within both populations individual cells varied slightly in response to the enantiomers.[5]

Responses to Other Bark Beetle Pheromones. A group of 15 cells were found to be specialized to the other genus-specific pheromone of *Ips*, ipsenol, even though this compound has not been reported as being produced by *I. pini.* The responses of the two highest concentrations were typically very strong, whereas the lower concentrations activated the cells relatively weakly (Figure 7) and the cell illustrated did not respond to any other compound. However, some of these cells responded weakly to the highest concentrations of ipsdienol (Table 1, Group B). Only two cells seemed to respond almost equally to ipsenol and ipsdienol (Table 1, Group C, cell 7). A more detailed description of the interaction between ipsenol and ipsdienol in *I. pini* at the receptor cells was given previously (Mustaparta et al., 1977).

Recordings from 23 cells showed highest responses to an additional group of three structurally similar beetle-produced compounds—*trans*-verbenol, *cis*-verbenol, and verbenone (Figure 1). *trans*-Verbenol and verbenone are released by *I. pini* (Stewart, 1975), and all three compounds have been shown to influence the aggregation of several bark beetle species (review by Bordon, 1974). Some of these 23 cells responded markedly stronger

[5]Recent recordings of single cells using another stimulation technique revealed consistent differences in the responses of ipsdienol cells to the (+) and (−) enantiomers.

TABLE 1. RESPONSES OF SINGLE OLFACTORY CELLS TO DIFFERENT TEST COMPOUNDS AT HIGHEST INTENSITY TESTED (CARTRIDGE LOAD OF 500 μg PURE COMPOUND)[a]

Test compounds	Cell responses									
Group A[b]	1	2	3	4	5	6	7	8	9	
Ipsdienol +	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Ipsdienol −	+++	+++	+++	+++	+++	+++	+++	+++	+++	
Ipsenol	0	0	0	0	0	0	+	+	+	
trans-Verbenol	0	0	0	0	0	0	0	0	+	
cis-Verbenol	0	0	0	0	−	−	0	0	0	
Verbenone	0	0	0	0	0	0	0	0	0	
Linalool	0	0	0	+	+	+	+	++	+	
Camphor	0	0	0	0	0	0	0	0	0	
1-Octanol	0	0	0	0	0	0	0	0	0	
α-Pinene	0	0	0	0	0	0	0	0	0	
Myrcene	0	0	0	0	0	0	0	0	+	
Group B[c]	1	2	3	4	5	6	7	8	9	10
Ipsdienol ±	0	0	+	+	+	0	0	+	+	0
ipsenol	+++	+++	+++	+++	+++	0	0	0	0	0
trans-Verbenol	0	0	0	0	0	+++	+++	++	++	+
cis-Verbenol	0	0	0	0	0	+++	++	+++	+	+
Verbenone	0	0	0	0	0	+	+	+	+++	+++
Linalool	0	0	0	0	0	0	0	0	0	0
Camphor	0	0	0	0	0	0	0	0	0	0
Myrcene	0	0	0	0	0	0	0	0	0	+
α-Pinene	0	0	0	0	0	0	0	0	0	0
1-Octanol	0	0	0	0	0	0	0	0	0	0
Group C[d]	1	2	3	4	5	6	7	8		
Ipsdienol ±	0	0	0	0	0	0	+++	+++		
Ipsenol	0	0	0	+	0	0	+++	0		
trans-Verbenol	0	0	+	+	0	0	0	+++		
cis-Verbenol	0	0	−	−	0	0	0	+		
Verbenone	0	0	0	0	0	0	0	+		
Linalool	++	++	0	0	0	0	0	0		
Camphor	0	0	+++	+++	0	0	0	0		
1-Octanol	0	0	0	0	0	0	0	0		
α-Pinene	0	0	0	0	0	0	0	0		
Myrcene	0	0	0	0	++	++	0	0		

[a] Representatives from the different response groups are listed, showing the distinction between them and the relatively uniform responses within the group. +++: the highest response, 50–90 imp/0.5 sec; ++: medium response, 30–50 imp/0.5 sec; +: low response, 10–30 imp/0.5 sec; 0: no response, 0–10 imp/0.5 sec; −: not tested.

[b] Representatives of ipsdienol cells, 1–9.

[c] Representatives of ipsenol cells, 1–5, and of verbenol cells, 6–10.

[d] Two linalool cells, 1–2, two camphor cells, 3–4, two myrcene cells, 5–6, and two nontypical cells, 7–8 (cf. text).

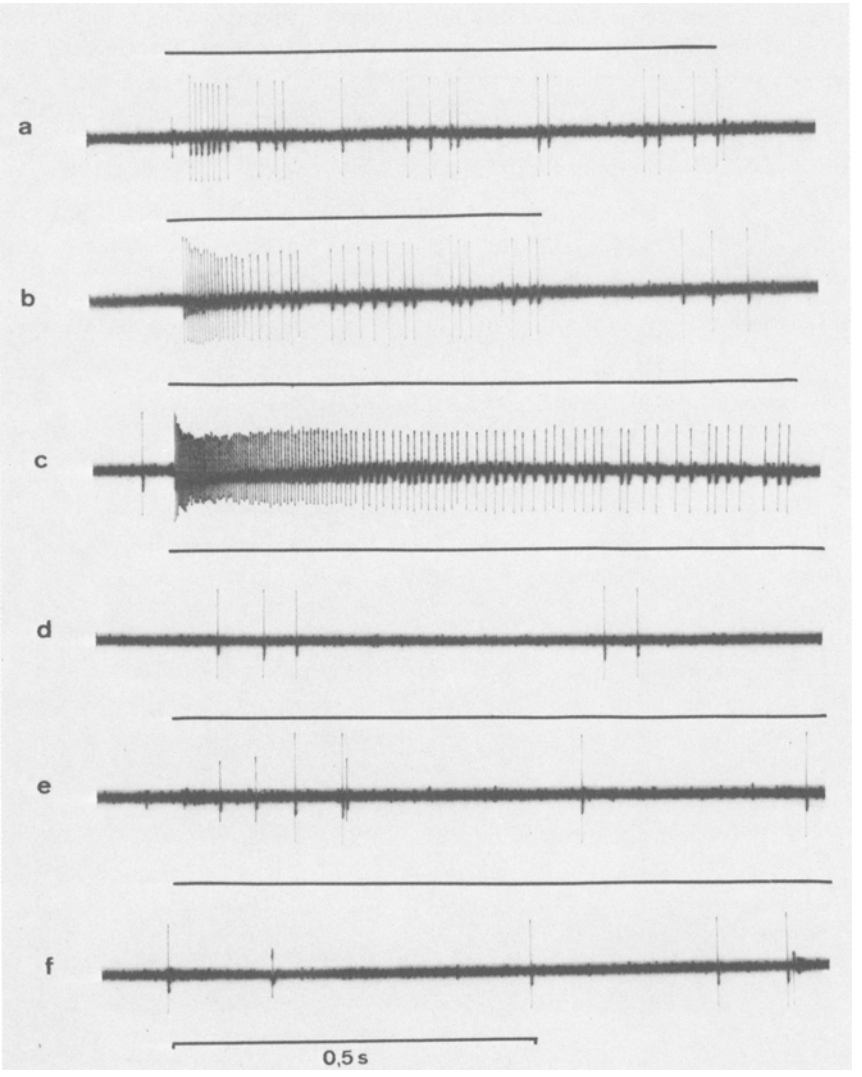

FIG. 5. Responses of a single olfactory cell of *Ips pini* to different compounds, showing selectivity for *cis*- and *trans*-verbenol (a, b, and c) while other compounds (d, e, and f) had no effect even at the highest concentrations. From above: Response to the vapor of 5 μg *cis*-verbenol (a), 5 μg *trans*-verbenol (b), 500 μg *trans*-verbenol (c), 500 μg α-pinene (d), 500 μg ipsdienol (e), 500 μg ipsenol (f). The stimulation period is indicated by a black bar above each trace.

to one or another of these compounds while others seemed to be almost equally activated by two or all three (Table 1b). The threshold concentrations for the different cells varied (0.05–0.5 μg), but none of the cells had thresholds as low as those of the cells specialized to ipsdienol (0.005 μg). The effects of other compounds tested were at best weak responses at the highest concentrations. However, the cells of this group responded more often to various compounds than did the ipsdienol and ipsenol cells. Dose–response curves for one of these cells are shown in Figure 8. Only three cells responded almost equally to ipsdienol and the verbenols (Table 1c, cell 8).

A few cells responded selectively to camphor and linalool (Table 1c)—compounds that are found in significant amounts in the frass of *I. pini*. Linalool is insect-produced (Young et al., 1973) while the origin of camphor is uncertain. The three camphor cells showed a characteristic strong and long-

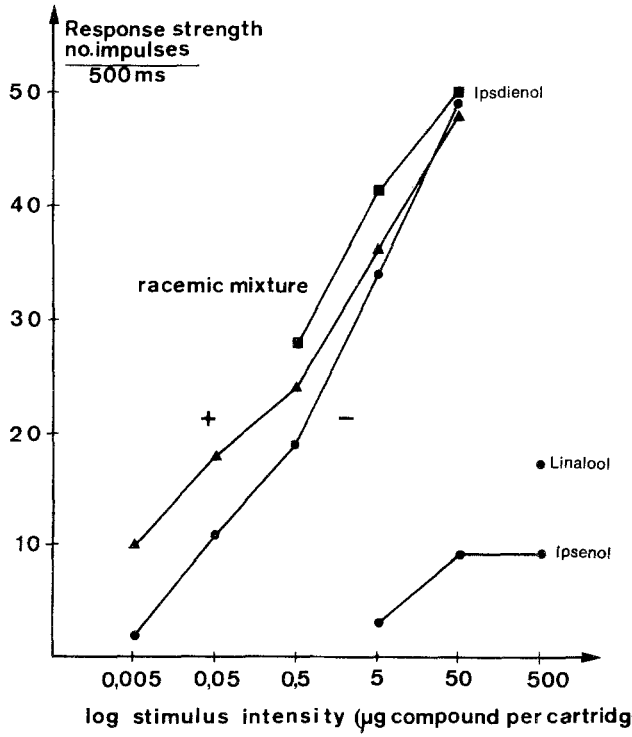

FIG. 6. Dose–response curves for a single olfactory cell in *Ips pini* (N.Y.), specialized to ipsedienol. The response curves for both enantiomers and the racemic mixture are shown as are its responses to linalool and ipsenol. The responses are given as the number of spikes during the first 500 msec after stimulation starts and are plotted for six intensities. The stimulus intensities are the cartridge loads (0.005–500 μg) of the pure compound.

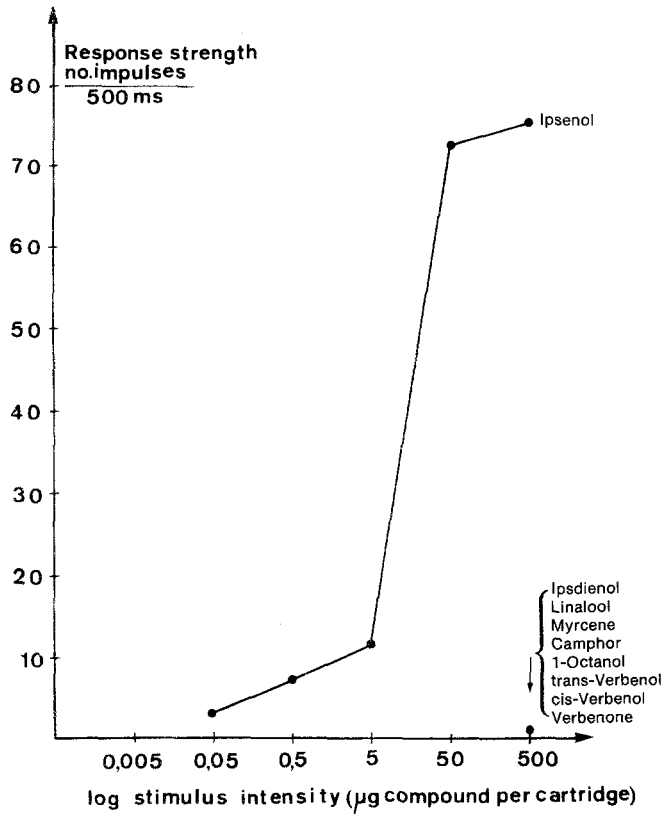

FIG. 7. Dose–response curve for a single olfactory cell in *Ips pini*, specialized to ipsenol. The responses are plotted for five different intensities originating from different cartridge loads (0.05–500 µg) of the pure compound. The insensitivity to the other compounds is indicated by no response to their highest concentrations.

lasting response to camphor while other substances had weak effect. However, only the two highest concentrations of camphor were tested on these cells. The linalool cells were only moderately activated by linalool (about 30–40 imp/500 msec as maximum response), and the threshold concentrations of these cells were relatively high, between 0.5 µg and 5.0 µg (Figure 9). No cells were found to be significantly activated by 1-octanol which also is present in the frass.

Responses to Host Compounds. Six cells were found to be exclusively activated by myrcene, which is an important host compound and has been indicated to be a precursor for ipsenol, ipsdienol (Hughes, 1974), and possibly linalool. These cells responded only moderately (20–40 imp/500 msec) to myrcene, and their threshold concentrations were relatively high (0.5–5.0 µg)

(Figure 10). Very few of the 95 recorded cells responded at all to α-pinene at the concentration tested, and none responded to it selectively, even though this compound is important behaviorally in several other bark beetle species (Borden, 1974).

DISCUSSION

The olfactory cells in *Ips pini* can be placed in separate groups according to their selective responses to only one or a very few compounds which are known pheromones or prominent components of the insect- or host-produced volatiles. We recognize that the eleven compounds tested do not represent the full repertoire of odorants to which *I. pini* receptors respond, but it seems

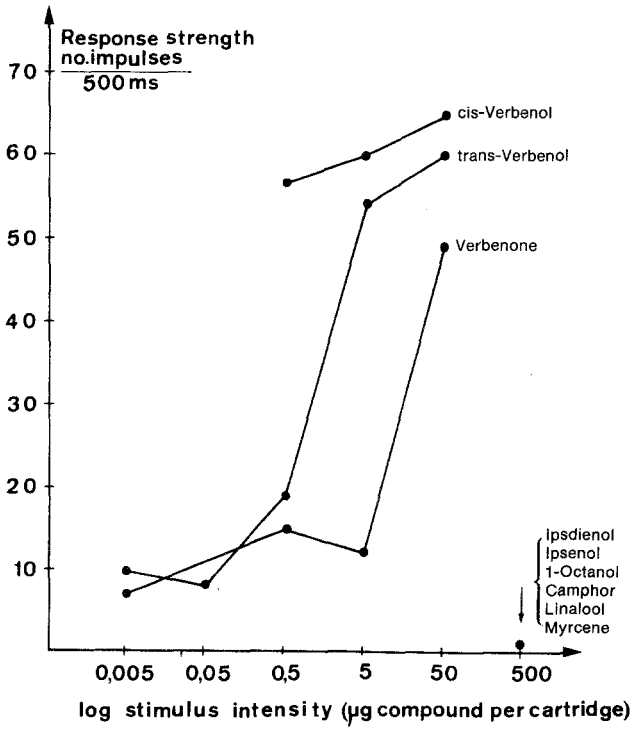

FIG. 8. Dose–response curves for a single olfactory cell of *Ips pini*, specialized to *cis*-verbenol, *trans*-verbenol, and verbenone. The responses are plotted for six different intensities of *trans*-verbenol and verbenone and three intensities of *cis*-verbenol. The insensitivity to the other compounds is indicated by no response to their highest concentration. The stimulus intensities are the cartridge loads (0.005–500 µg) of the pure compounds.

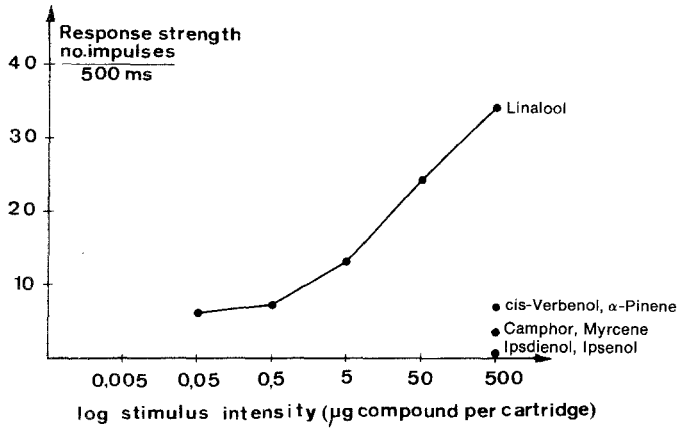

FIG. 9. Dose–response curve of a single olfactory cell in *Ips pini*, specialized to linalool. The responses are plotted for five intensities of the cartridge load (0.05–500 μg). The low response to the highest intensity of other compounds is indicated.

probable that the group we selected includes chemicals to which this insect is most perceptive.

The uniform sensitivity of some cells to various compounds indicates that they belong to one functional group. Thus, the ipsdienol cells which responded weakly to linalool and ipsenol showed consistently stronger responses to linalool than to ipsenol, while those ipsenol cells which responded to another substance all reacted to ipsdienol as the second most effective compound. These results indicate that the ipsdienol and ipsenol receptor cells are

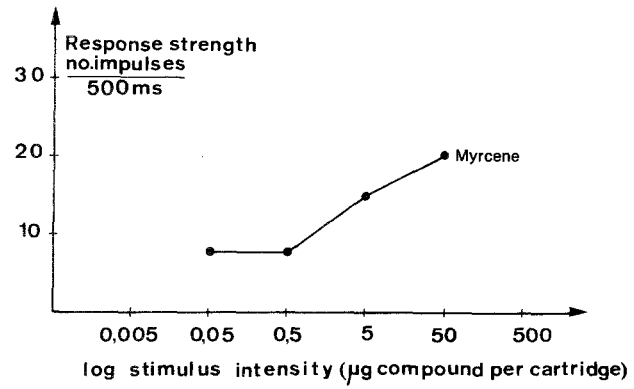

FIG. 10. Dose–response curve of a single olfactory cell in *Ips pini* responding only to myrcene. The responses are plotted for four intensities, originating from different cartridge loads (0.05–50 μg) of the pure compound.

relatively uniform, each having one acceptor type—for ipsdienol and ipsenol, respectively.

The group of cells specialized to *cis*-verbenol, *trans*-verbenol, and verbenone seem less uniform. The individual variation in responses to *trans*-verbenol, *cis*-verbenol, and verbenone indicates that at least three acceptor types are involved in the interaction between these cells and their compounds.

There seems to be minimal interaction between pheromones and host odors at the receptor cells of *I. pini.* Cells specialized for pheromones responded minimally, if at all, to host terpenes; and cells which responded selectively to host compounds were not activated by insect-produced compounds. Thus, the olfactory cells of *I. pini* seem to be specialized to receive separate parts of the odor bouqet produced by the insect and its host. Preliminary recordings from *I. paraconfusus* have shown the same type of receptor cells as *I. pini* (Mustaparta et al., unpublished). This indicates that the synergistic effect of ipsenol, ipsdienol, *trans*-/*cis*-verbenol, and host compounds, as demonstrated for *I. paraconfusus*, *I. latidens* (Le Conte) (Silverstein et al., 1966), and *I. calligraphus* (Germar) (Renwich and Vite, 1972) results mainly from central nervous integration of signals from separate cells, rather than from interaction of compounds on individual olfactory receptors. Similarly, the inhibition of the aggregation of *I. pini* by addition of ipsenol to male-infested logs is apparently the result of central integration rather than blocking of ipsedienol receptors (Mustaparta et al., 1977). In contrast, pheromones and host tree terpenes (either attractants or inhibitors) have been shown to stimulate the same receptor cells in the southern pine beetle *Dendroctonus frontalis* Zimm (Dickens and Payne, 1977, 1978).

We observed response thresholds of the ipsdienol cells to be 100–1000 times lower than thresholds of other cells to their most activating compounds. Angst and Lanier (1979) found a similar high sensitivity to ipsdienol by recording electroantennograms in *I. pini.* This, together with the high proportion of the ipsdienol cells (45 of 95 recorded), seems consistent with field tests showing that ipsdienol is the principal aggregating pheromone of *I. pini* (Lanier and Birch, personal communication).

It was surprising to find that the response of ipsdienol receptor cells did not parallel the differential production of the (+) and (−) enantiomers of the eastern (New York) and western (California and Idaho) populations. Thus, the strong preferential responses of *I. pini* for the pheromone of the same population (Lanier et al., 1972) cannot simply be explained by population differences in receptor cells. However, recent field tests (Lanier, unpublished) showed that *I. pini* from New York were strongly attracted to racemic ipsdienol but only slightly attracted to either enantiomer. Thus, the small differences in the dose–response curves for the enantiomers that we observed may result in a behavioral discrimination, similar to that described by

Vareschi (1971). Discrimination of pheromone enantiomers seem to be of importance for many insect species (reviewed by Elliott et al., 1979).

In conclusion, the olfactory cells of *I. pini* can be grouped according to their responses to various substances. The cells responded highly selectively to one or a very few compounds and overlap of response substances occurred only at the highest concentrations. These results indicate that the synergistic as well as the inhibitory interaction of these compounds upon the aggregation if *Ips* species are generally due to a central nervous integration of signals from separate groups of receptor cells rather than an interaction of compounds on the receptors.

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PHEROMONE OF THE MALE FLESH FLY, *Sarcophaga bullata*

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Abstract—Hexanal, isolated from a whole animal extract of the flesh fly, *Sarcophaga bullata*, attracts over 65% of the females tested with no apparent effect on males.

Key Words—Diptera, Sarcophagidae, flesh fly, *Sarcophaga bullata*, pheromone, hexanal.

INTRODUCTION

Flesh flies of the family Sarcophagidae (Diptera) are common insect pests in the United States. Adult female *Sarcophaga bullata* (Parker) are larviparous and are attracted to meat or flesh where they discharge their first instar larvae. This species is a potential vector of disease (especially dysentery); in warm climates flesh flies may be responsible for intestinal myiasis (Watson, 1942; Linduska and Lindquist, 1952) and for the invasion of wounds or ulcers (Dove, 1937).

Although the male flesh fly initiates the mating strike, we have reported (Girard et al., 1975) that the males have a sex attractant which attracts females over long distances. We report the isolation and identification of this sex pheromone.

METHODS AND MATERIALS

The *S. bullata* pupae were purchased from the Carolina Biological Supply Company (Burlington, North Carolina). Male and female pupae were

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segregated for bioassay studies. Adult females were 2–3 days old when tested. Our Y-tube olfactometer (Monteith, 1956) and bioassay techniques have been described before (Girard et al., 1975). The pupae were placed in cages at 30° C and 100% humidity with a daily regime of 8 hr light, 16 hr dark.

One kilogram of *S. bullata* pupae were allowed to emerge in cages at 29° C. After emergence, the flies were reared for three days at 29° C on a diet of sugar, water, and condensed milk. Two days after emergence, the insects were anesthetized with carbon dioxide and ground in a Waring blender with nanograde ethyl acetate. An adequate amount of anhydrous sodium sulfate was added to the mixture to absorb any water present in the homogenate. The extract was passed through a Millipore filter. The extract was then evaporated to dryness under vacuum, and the residue was stored in hexane.

The hexane extracts were chromatographed on Sephadex LH-20; fractions were eluted with benzene–methanol, 1:1. When 188 g of the extract was subjected to this gel permeation chromatography, seven fractions were isolated. Only two fractions (6 and 7) were active. Fraction 6, which was most active, weighed only 0.24 g. This fraction was chromatographed with a Varian 1440 gas chromatograph on a 6-ft × 1/8-in. glass, 3% OV-17 on Gas Chrom-Q column. The injection was made at 50° C, and the oven was programed at 4°/min to 250° C.

Active components isolated from the GLC were then analyzed by mass spectroscopy. A Varian Mat III GC-MS was used with a 6-ft, 3% OV-17 on gas Chrom Q column.

RESULTS AND DISCUSSION

As we reported before (Girard et al., 1975), the response of 25 *S. bullata* virgin females toward live virgin males was observed in the bioassay chamber; this experiment was replicated 20 times. An average of 18.2 females went into the arm of the Y tube with the live males; 3.3 entered the control arm, with only humidified air flowing; and 1.0 made no response. These experiments demonstrate that over 70% of the virgin females responded to the virgin males.

Assays were then performed to demonstrate that the females were responding to the males because of an attractant rather than other stimuli. The last two abdominal segments of 25 male abdomens were excised and crushed on filter paper. The responses of 25 S. *bullata* virgin females to the abdomens were observed in the same assay chamber; this observation was also repeated 20 times. An average of 18.4 were attracted to the filter paper; 2.3 were attracted to the control; and 2.3 made no response. These experiments indicate that the female responds because of a chemical attractant rather than other stimuli.

The attractant was extracted from a kilogram of *S. bullata* with ethyl acetate. The extract was then subjected to gel permeation chromatography.

Of the seven fractions isolated only two, 6 and 7, were active. Since fraction 6 was the most active, we concentrated our efforts on this fraction. Fraction 6, which was very polar, accounted for only 1.2% of the total extract and was 82% as attractive as live males.

Fraction 6 was then chromatographed by GLC on a 6-ft × 1/8-in. ID glass, 3% OV-17 column into six fractions. Figure 1 shows a typical chromatogram and how fractions A1–F1 were collected. Each fraction was bioassayed, but only fraction A1 showed good activity. Fraction D1, however, showed low activity (~20%). Forty-five injections of a methylene chloride solution of fraction 6 were made and fraction A1 was collected in glass capillary tubes cooled by liquid nitrogen.

A small amount (~10%) of fraction A1, in methylene chloride, was injected into a Varian Mat III GC-MS at 40°. After the solvent front had passed through the column, the separator was opened and the GC oven programed at 2°/min to 150°C. Only a tailing methylene chloride peak was observed. A larger injection of this solution was made and again only methylene chloride was observed. The active material was eluting with the methylene chloride peak, and it had passed through the GC before the separator was opened.

In order to observe the volatile pheromone peaks, the sample tubes were washed with a high-boiling-point solvent, dichlorobenzene. A 5-μl injection of this solution contained 25% of our A1 fraction. Figure 2 shows the resulting chromatogram.

Peak I is methylene chloride, which is present in our sample tubes and is trapped by our liquid nitrogen trap from the GC. Peak II is a trace of methanol. Peak III, which is a shoulder on Peak IV, gives a mass spectrum which can be seen in Figure 3. This compound was identified as hexanal. Because of the small quantity present, the parent ion at m/e 100 was not

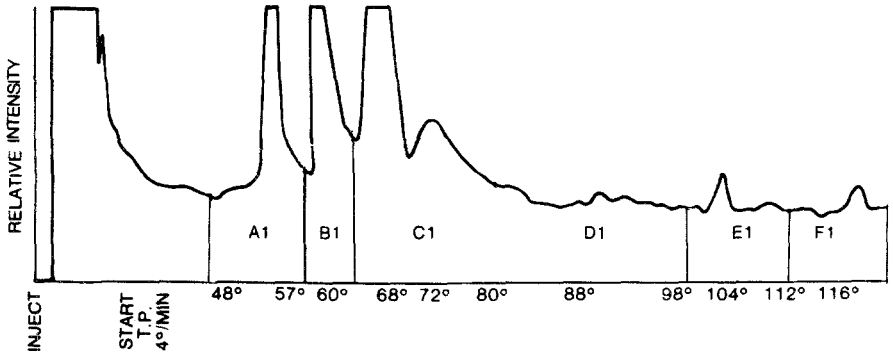

FIG. 1. Gas chromatogram of fraction 6 in methylene chloride Varian 1440GC, OV-17 6-ft, 40 cc He/min.

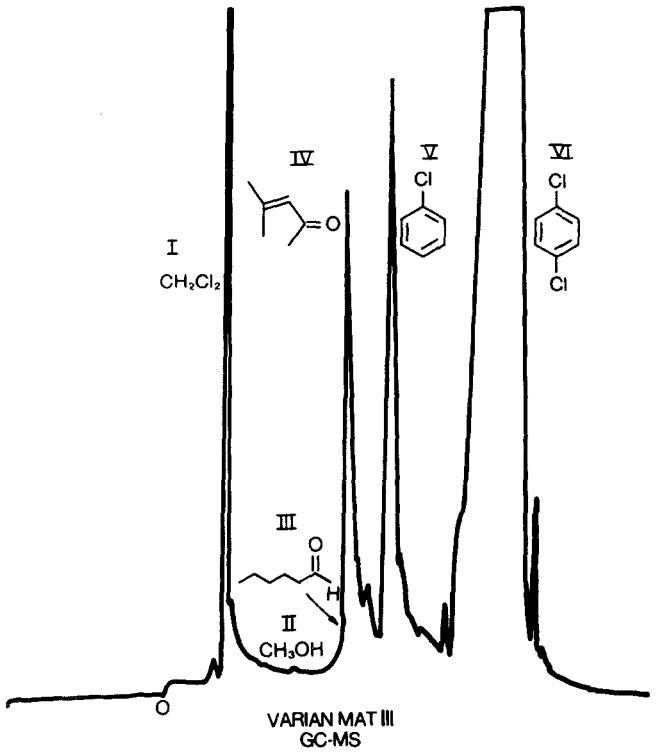

FIG. 2. Gas chromatogram of pheromone fraction A in dichlorobenzene.

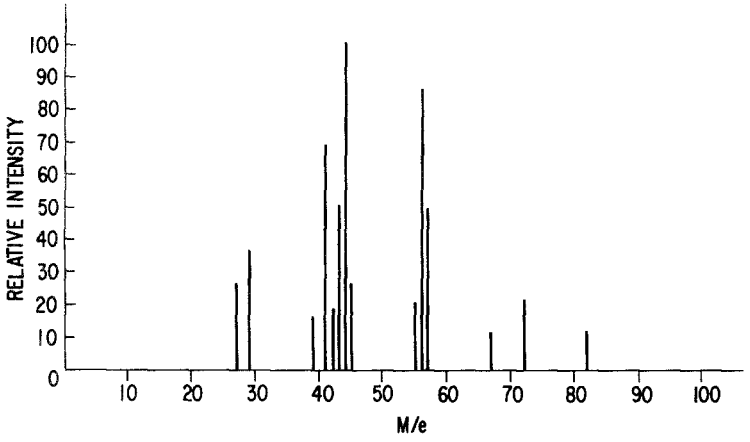

FIG. 3. Mass spectrum of peak III, pheromone fraction A.

observed. Major fragments at m/e 82 ($-H_2O$), 72 ($-C_2H_4$), 56 ($-C_2H_4O$), and 44 ($-C_4H_8$) gave supporting evidence for hexanal (Liedtke and Djerassi, 1969). An authentic sample of hexanal gave an identical retention time and fragmentation pattern. Peak IV gave a mass spectrum which can be seen in Figure 4. This compound was identified as mesityl oxide (4-methyl-3-penten-2-one). It gives a parent peak at m/e 98 and major fragments at 83 ($-CH_3$), 55 ($-CH_3$, $-CO$), and 43 (C_4H_7). An authentic sample of mesityl oxide gave an identical retention time and fragmentation pattern. Peak V was identified as monochlorobenzene, an impurity in the dichlorobenzene. Peak VI was identified as the dichlorobenzene solvent.

Authentic samples of mesityl oxide and hexanal were assayed in the 10 to 100-μg range. Table 1 lists the results of the bioassays with 50 μg in methylene chloride. Mesityl oxide showed no apparent activity throughout the entire concentration range. Hexanal, on the other hand, showed considerable activity throughout this concentration range with a maximum response in the 50 to 100 μg range. Our results indicate that at least 68% of the females tested were attracted to hexanal, whereas males show no apparent attraction.

It appears that hexanal is a male sex attractant for the female flesh fly *S. bullata*. The D1 area of our GC trace (86° C), when reinvestigated, appears to coincide with the retention time for hexanoic acid. Hexanoic acid itself shows no activity when tested alone. Several bioassays with hexanoic acid/hexanal in the ratio of 1:10 show good activity for the mixture with 65–70% of the females being trapped. Higher concentrations of hexanoic acid decrease the bioassay response. Hexanoic acid–hexanal synergism could not be clearly demonstrated.

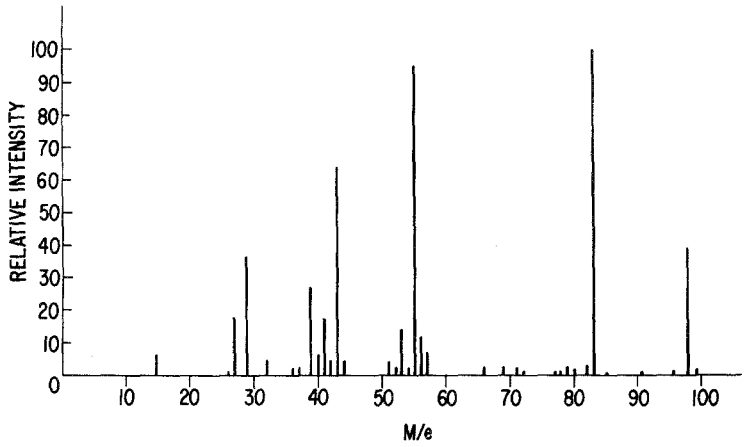

FIG. 4. Mass spectrum of peak IV, pheromone fraction A.

TABLE 1. BIOASSAY RESULTS

Mesityl oxide		
(45 females)		~50μg
Positive	20	
Negative	23	
No response	2	
Hexanal		
(75 females)		~50μg
Postive	51	
Negative	13	
No response	11	
44 Males		~50μg
Positive	15	
Negative	11	
No response	18	

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ELECTROANTENNOGRAM RESPONSES OF TWO POPULATIONS OF *Ips pini* (COLEOPTERA: SCOLYTIDAE) TO INSECT-PRODUCED AND HOST TREE COMPOUNDS[1]

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Abstract—Electroantennograms in two populations of *Ips pini* (Idaho and New York) were very similar, even though previous work documented behavioral specificity and revealed that the populations produce different ratios of the enantiomers of the aggregating pheromone ipsdienol. The insect- and host-produced compounds tested evoked characteristic response curves in both populations. EAG amplitude was highest to ipsdienol and the aggregation inhibitor ipsenol. Among the other compounds tested, responses were generally higher to the beetle-produced odorants (linalool, verbenone, *trans*-verbenol) than to host terpenes (1-octanol, α-pinene). The antennal club and its sensory receptors are described and illustrated by scanning electron micrographs.

Key Words—Coleoptera, Scolytidae, *Ips pini*, pheromones, population specificity, electrophysiology, electroantennogram.

INTRODUCTION

The pine engraver, *Ips pini* (Say), occurs throughout most pine and spruce forests of North America (Lanier, 1972). Owing to considerable morphological variations, populations of this species from widely separated localities have been known under seven different names (Hopping, 1964; Lanier, 1972). The most recent synonymy was that of the western *I. oregonis*

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(Eichhoff) with the eastern *I. pini* (Say) by Hopping (1964). Controlled breeding experiments, analysis of chromosomal and morphological detail, and demonstration of cross-attractiveness to phermones confirmed this synonymy (Lanier, 1972). However, in another experiment in which pheromone-laden frass from eastern (*pini*) and western (*oregonis*) beetles was presented simultaneously, western females showed a significant preference for western frass (Lanier, 1972). Field experiments supported the supposition of geographic variation in the pheromone bouquets of *I. pini* from Idaho, California, and New York (Lanier et al., 1972; Piston and Lanier, 1974).

If a female beetle can distinguish between odors of males of its own and a different population, we can conclude that the males must produce distinct pheromone bouquets. This difference may be qualitative, quantitative, or both. Young et al. (1973) and Stewart (1975) analyzed volatiles collected from Idaho and New York males boring into red pine (*Pinus resinosa* Ait.) logs. The only qualitative difference found was in the proportion of the two enantiomers of the pheromone ipsdienol. *Ips pini* males from Idaho produce the (−)-enantiomer only, whereas males from New York produce a mixture of 65% (+)- and 35% (−)-ipsdienol. Recent laboratory bioassays and a field test in New York (Lanier, unpublished data) indicate that these enantiomeric differences can account for the observed preferences of *I. pini* for pheromones produced by the same population. In addition to this qualitative difference, GLC analysis of volatiles extracted on Porapak Q® and solvent extracts of the abdomina of males revealed a quantitative difference; Idaho males produced twice as much ipsdienol as New York males (Claesson, personal communication[3]).

It can be hypothesized that females distinguish between populations by the different composition of the enantiomers or by the amount of ipsdienol produced. The first case would mean that the two enantiomers must be perceived differently by olfactory receptors; if the second case (quantity) were decisive, receptors of the two *Ips* populations may have different thresholds for response to ipsdienol. We recorded electroantennograms (Schneider, 1957) to find possible differences in olfactory reception by females of the two populations. We were especially interested in whether the two populations of *I. pini* differentiate between the (+) and (−) enantiomers and whether they varied in their responses to various concentrations of ipsdienol.

METHODS AND MATERIALS

Materials. The *Ips pini* used were taken from colonies originating from red pine at Heiberg Experimental Forest near Tully, New York, and from

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ponderosa pine, *Pinus ponderosa* Laws., at Worley, Idaho. The beetles were reared on red pine logs; females were held at approx. 3° C up to 15 days before they were used for EAG recordings.

Substances applied for stimulation were obtained from Chemical Samples Co. or were synthesized. All samples were cleaned by GLC (purity 99%). The optical purity of (+)- and (−)-ipsdienol was 93%.

Preparation. Techniques for preparing beetles were modified from those described by Payne (1970) and Payne et al. (1970). A block of Plexiglass (3 × 3 × 2 cm) with a hole 1.7 mm diam. that narrowed to 1.5 mm at the upper end was used to mount the beetles. On its surface was fastened a metal sheet containing a hole of 2 mm matching the opening of the block. The beetles were inserted into the lower end of the hold and pushed with a wire towards the narrow end where they were held firmly in place. Only the head and about one-third of the thorax protruded. The head was immobilized by pressing it into the thorax with the metal sheet on which an antenna was held in place by pressing it gently onto sticky tape (Figure 1). The beetle holder was mounted on a magnet to facilitate its positioning on a metal substrate.

Recordings. Electroantennograms were recorded with Ag–AgCl glass electrodes filled with 3 M KCl solution (Schneider, 1957). The tips of the electrodes were approximately 5–10 μm diam. Micromanipulators were used to insert the recording electrode into the club of the antenna and the indifferent electrode into the mouth of the beetle (Figure 1).

Stimulation. The stimuli were given as puffs of odorant into a stream of compressed purified air continuously blowing over the antenna at 0.15 liters/min (Roelofs and Comeau, 1971). Each puff, 1 ml of ordorant-containing air, was delivered from a glass syringe through a 4-ml pipet

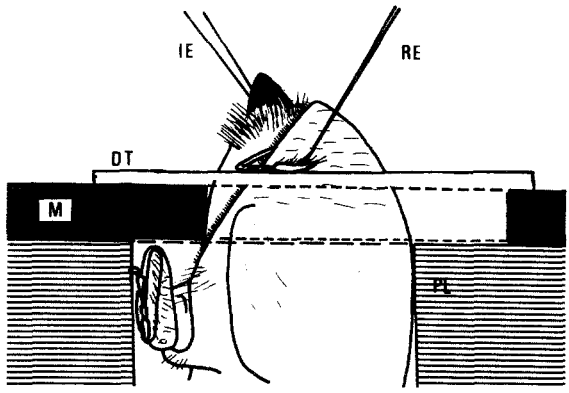

FIG. 1. *Ips pini* fixed for recording electroantennograms, longitudinal view. DT = double sticky surface; IE = indifferent electrode; M = metal sheet; PL = plexiglass; RE = recording electrode.

containing filter paper treated with a given compound and concentration. The compounds were dissolved in hexane to produce stock solutions of 5, 50, and 500 μg/ml which were placed on the filter paper in 1- to 1000-μl aliquots to make a series of ordorant concentrations ranging from 0.005 to 500 μg of each compound. After the solvent evaporated, pipets were closed with Teflon stoppers and stored in a freezer. To prevent contamination (after each stimulation), the syringe was cleaned with air from the cylinder and the contaminated air was drawn off by a vacuum. A stimulus with pure hexane served as a control and the EAG obtained was subtracted from the other responses. EAGs were normalized as a percent of the response to 500 μg of racemic ipsdienol (Payne, 1975). To allow complete recovery of the sensory cells the stimuli were given in intervals of 1–7 min, depending on the amplitude obtained. The same samples were used to compare individuals of the two populations. Each beetle was stimulated once with each compound and concentration. Means and standard deviations were computed for responses of 10 beetles per population for each stimulus concentration.

RESULTS

Antenna and Olfactory Receptors. Scanning electron micrographs show that in *I. pini*, as in *I. paraconfusus* (Borden and Wood, 1966), two bands of short sensilla are located on the face of each antennal club (Figure 2). These sensilla basiconica are pointed and approximately 1 μm thick and 8 μm long (Figure 3). Each band contains approx. 200 sensilla and there are approx. 90 sensilla on the tip of the club. A second type of s. basiconica (1 μm thick and 25 μm long with rounded tips), called sensilla trichodea by Borden and Wood (1966), are found along the edge and the tip of each antennal club. Toothed sensillae, probably mechanoreceptory s. chaetica (Schneider, 1964), are located in the same places as the trichodea but in lower numbers (15 in total). Additional s. chaetica occur on the scape, the funicle, and on the back of the club.

Electrophysiology. Electroantennogram (EAG) responses to stimulation by given compounds were obtained as slow negative potentials which reached a peak within 0.5 sec, declined immediately afterward, and reached the resting potential after about 20 sec (Figure 4). Amplitudes of EAGs varied between 0 and 5 mV, depending on the concentration and the quality of the stimulation. No notable differences in the shapes of the EAGs for different compounds were observed. We detected no significant differences (Duncan's multiple range test) between the New York and Idaho populations of *I. pini*, either in response thresholds or in the relative response to the various compounds.

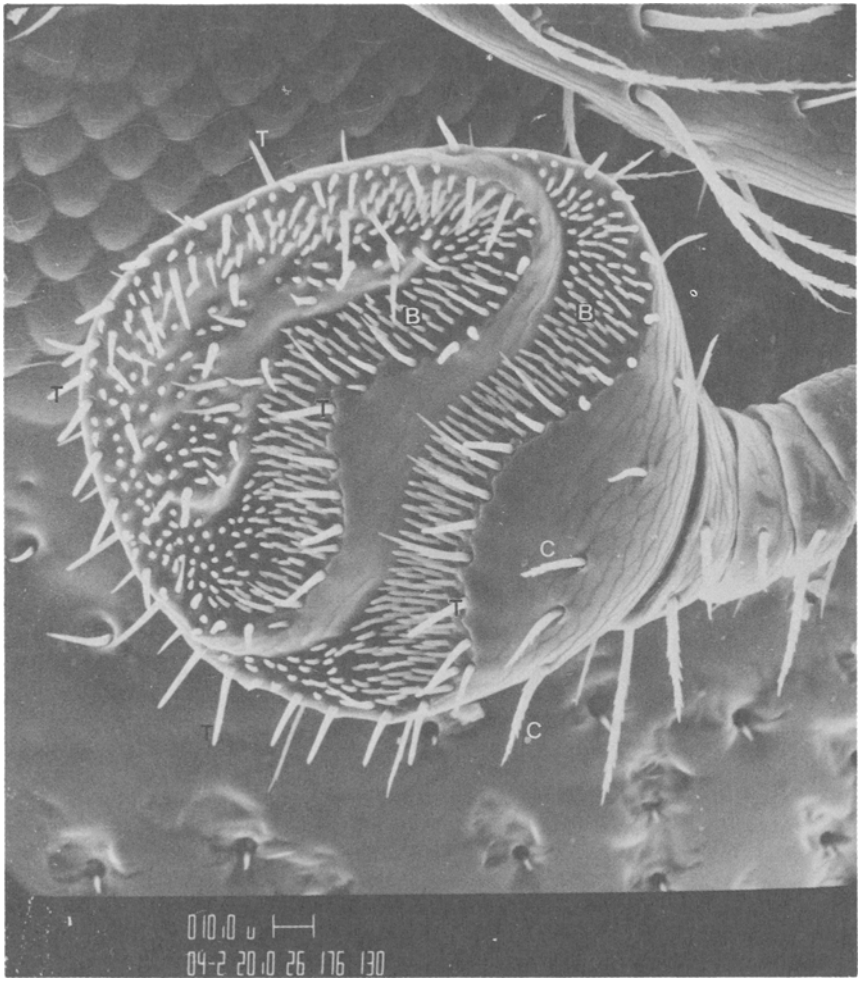

FIG. 2. Scanning electron micrograph of front surface of antennal club of *Ips pini*, Idaho, female (B = bands with sensilla basiconica; T = s. trichodea; C = s. chaetica).

Especially significant was a lack of difference in their responses to (+)- and (−)-ipsdienol (Figures 5 and 6).

The threshold concentrations for response were ipsdienol (0.01 μg), ipsenol (0.05 μg), verbenone (0.1 μg), *trans*-verbenol (0.5 μg), linalool (5 μg), 1-octanol (10 μg) and (+)-α-pinene (10 μg). Thus, the threshold concentrations for ipsenol and (+)-α-pinene exceed the threshold for ipsdienol by factors of 5 and 1000, respectively.

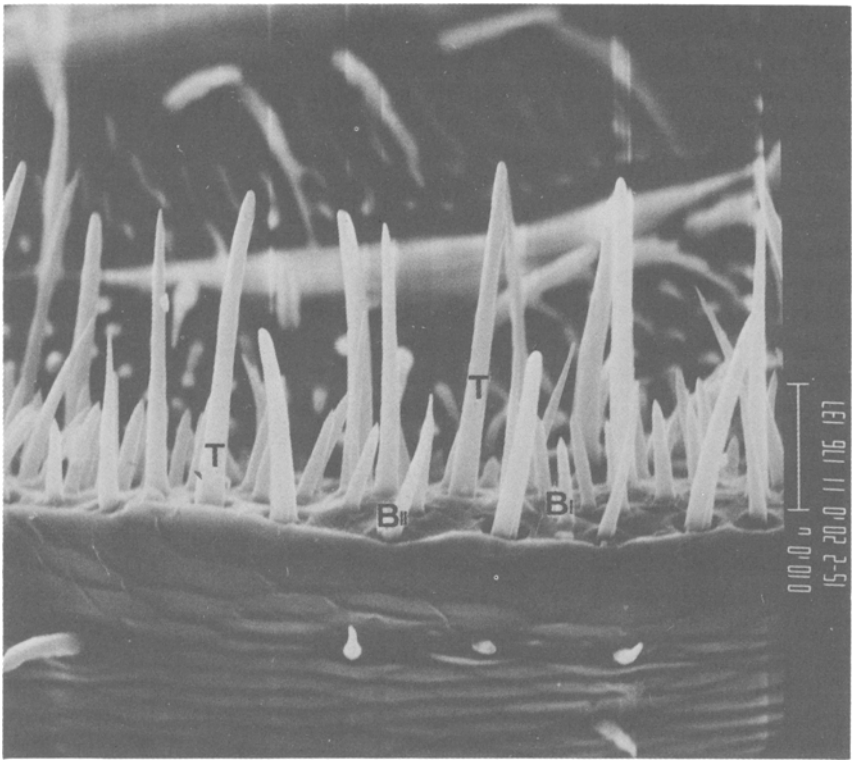

FIG. 3. Scanning electron micrograph of sensilla on the antennal club of female *Ips pini*, Idaho, viewed from the tip (T = sensilla trichodea; BI = s. basiconica type I; BII = s. basiconica type II).

DISCUSSION

Because EAGs from *I. pini* from Idaho and New York populations did not differ, we conclude that the two populations do not have major differences in their receptor systems, even though they produce enantiomers of ipsdienol in different ratios (Stewart, 1975) and respond preferentially to pheromone produced by males of the same population (Lanier et al., 1972).

Since EAG amplitude is considered to be directly related to the number of receptors responding (Boeckh et al., 1965), we can hypothesize either that individual receptor cells do not distinguish between (+)- and (−)-ipsdienol or that varying responses by different receptor cells are compensatory. Studies of single cells have indicated that the latter alternative is probably true (Mustaparta et al., 1979; Mustaparta, personal communication). Specificity for enantiomers was observed in electrophysiological studies of the gypsy

moth (Yamada et al., 1976), the honey bee and the migratory locust (Kafka et al., 1973), and the southern pine beetle (Dickens and Payne, 1977). It has also been found in behavioral studies of several other insects (Riley et al., 1974; Borden et al., 1976; Hedden et al., 1976; Vité et al., 1976; Wood et al., 1976).

Response curves to different compounds showed conspicuous differences in threshold concentration, amplitude of response, and saturation level. As expected, ipsdienol evoked the greatest response at all concentrations; insects usually show the greatest response to their own pheromones (Priesner, 1968; Roelofs and Comeau, 1971; Payne, 1970, 1975). The second greatest response was provoked by ipsenol, an inhibitor for *I. pini* (Birch and Wood, 1975), but part of the aggregating pheromone for certain other *Ips* species. Verbenone, *trans*-verbenol, linalool, and (+)-camphor stimulated intermediate responses somewhat greater than the low responses to 1-octanol and (+)-α-pinene. The latter two compounds probably are of little significance to *I. pini*.

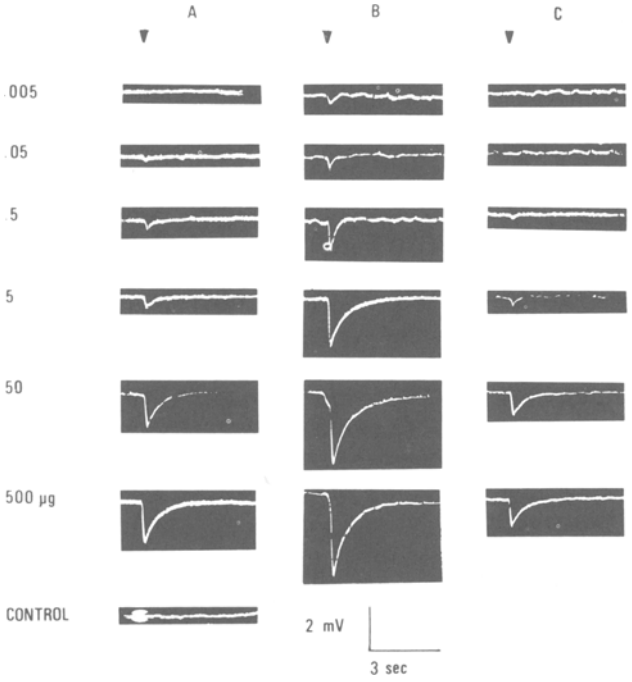

FIG. 4. EAGs of an Idaho *Ips pini* female to (A) linalool, (B) ipsdienol [racemic mixture] and (C) (+)-α-pinene. Δ = Start of the stimulations. Maximum stimulus duration is approx. 0.5 sec.

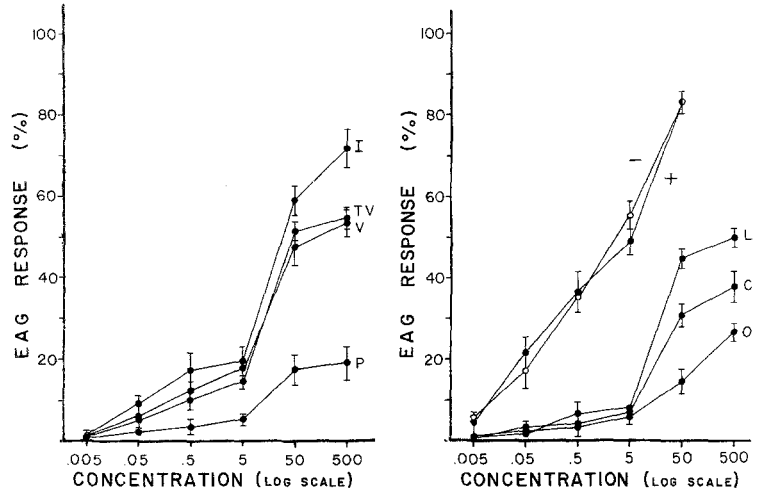

FIG. 5. EAGs of New York *Ips pini* females to various compounds. Left: ipsenol (I), *trans*-verbenol (TV), verbenone (V), and (+)-α-pinene (P). Right: (+)- and (−)-ipsdienol (+ and −), linalool (L), (+)-camphor (C), and 1-octanol (O). Vertical bars indicate standard errors for the points plotted. Due to a limited supply (+)- and (−)-ipsdienol were not tested at the 500 μg concentration.

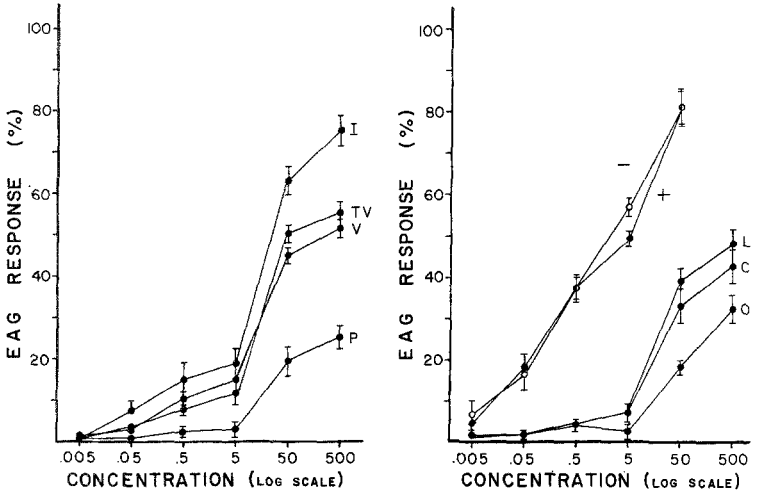

FIG. 6. EAGs of Idaho *Ips pini* females to various compounds. Left: ipsenol (I), *trans*-verbenol (TV), verbenone (V), and (+)-α-pinene (P). Right: (+)- and (−)-ipsdienol (+ and −), linalool (L), (+)-camphor (C), and 1-octanol (O). Vertical bars indicate standard errors for the points plotted. Due to a limited supply (+)- and (−)-ipsdienol were not tested at the 500 μg concentration.

In addition to giving high amplitude EAG responses, compounds of known importance to *I. pini* had the lowest thresholds and highest saturation levels. The low threshold for ipsdienol would enable *I. pini* to perceive the pheromone at long distances from its source and the high saturation level for this compound would enable the insect to perceive a considerable concentration gradient as it approached the odorant source.

Acknowledgments—Several members of the College of Environmental Science and Forestry provided essential support for this work. Mr. Andrew Eggers aided in setting up the electronic equipment. Dr. R.M. Silverstein and his staff provided the purified compounds tested, and Dr. W.A. Côté and his staff made the scanning electronmicrographs. We also are grateful to Drs. T.L. Payne and J.C. Dickens (Department of Entomology, Texas A&M University), for their advice on EAG techniques, and Drs. A. Claesson and H. Mustaparta, ESF postdoctoral fellows, for their assistance. We appreciate the constructive reviews of the manuscript by Drs. T.L. Payne and D.C. Allen of the College of Environmental Science and Forestry.

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THE OPHIOPHAGE DEFENSIVE RESPONSE IN CROTALINE SNAKES: EXTENSION TO NEW TAXA

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Abstract—A total of 21 new taxa of New World pit vipers (Serpentes: Crotalinae) responded by elevating the middle portion of the body in a defensive posture (body bridge) when exposed to the skin substances of certain colubrid snakes (Colubridae). Newborn snakes from two of the three species tested gave the response. Several new species of colubrid snakes also are documented as capable of eliciting a response, and it is suggested that the term *ophiophage defensive response* be used to denote body bridging and associated defensive behaviors instead of the restrictive "kingsnake defense posture." Most of the snakes which elicit the response in crotaline snakes are known to feed on lizards and/or snakes. There is no apparent correlation between the stimulus snakes' ability to elicit a response in the crotaline snakes and sympatry with the crotaline snakes.

Key Words—Snake behavior, chemoreception, predator–prey, kairomone, Colubridae, Crotalinae, ophiophage, defensive response.

INTRODUCTION

Klauber (1927) and Cowles (1938) provided the first reports of an unusual defensive posturing in rattlesnakes (genus *Crotalus*) to the presence of kingsnakes (*Lampropeltis getulus*), notorious ophiophagous snakes. This response, originally referred to as the "kingsnake defense posture" by Cowles (1938), is characterized by the elevation of the middle portion of the body to form a bend (body bridge), which directs body blows toward an advancing kingsnake, a decided contrast to the vertical coiling position assumed by rattlesnakes to other types of perturbations. By presenting rattlesnakes with sticks smeared with cloacal sac contents or rubbed against the dorsal and ventral surfaces of stimulus snakes, Bogert (1941) showed that chemical cues from

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the dorsal skin of *Lampropeltis getulus* are necessary and sufficient to elicit a response. *Masticophis flagellum piceus* (= *Coluber f. frenatum*) and *Pseustes sulfureus* also elicited a response, but a number of boid, crotaline, and non-ophiophagous colubrid snakes did not. Rattlesnakes also respond by body bridging to the scent of the spotted skunk, *Spilogale putorious*, (Cowles, 1938). 1-Butanethiol, once believed to be the primary malodorous constituent of the scent of the common skunk, *Mephitis mephitis*, (Aldrich, 1896) evoked a response only when accompanied by tactile stimulation (Cowles and Phelan, 1958). However, a recent reanalysis of the scent of *M. mephitis* by Andersen and Bernstein (1975) failed to indicate the presence of 1-butanethiol; this may account for the partial response with the snakes.

The species of New World pit vipers which respond to *Lampropeltis* species by body bridging and other defensive behaviors has been extended, through several reports, to include members of the genera *Agkistrodon* (Neill, 1947; Carpenter and Gillingham, 1975) and *Sistrurus* (Inger, cited in Burghardt, 1970; Carpenter and Gillingham, 1975) in addition to a number of species and subspecies within the genus *Crotalus* (Meade, 1940; Bogert, 1941; Klauber, 1956; Carpenter and Gillingham, 1975). Carpenter and Gillingham (1975) have tabulated the species known to respond and have suggested that body bridging may be a behavioral characteristic of the subfamily Crotalinae. They were unsuccessful in eliciting a response from a variety of colubrid snakes when placed in encounters with kingsnakes.

This is a preliminary report from our survey of body bridging in crotaline snakes in response to the skin substances of ophiophagous or possibly ophiophagous snakes.

METHODS AND MATERIALS

The testing procedure entailed the presentation of cotton balls attached with clips on the end of 1-m wooden rods to individual snakes. The experimental ball was moistened with methanol, a solvent found to be suitable for collecting skin substances from the various species that elicit the defensive posture, and rubbed against the dorsal surface of a stimulus snakes. A fresh ball was presented to a crotaline snake by placing it a few cm from the snout. If no response appeared after the first minute, the snout or anterior trunk of the snake, depending upon the position the snake was in at the time of testing, was lightly tapped and observations continued for another minute. A control ball, moistened only with methanol, was presented to each subject in an identical fashion. For approximately half the snakes tested, the presentation of the experimental preceded that of the control ball; the remaining snakes were tested with the control ball first. On a subsequent day, the order of control and experimental presentations was reversed in a counterbalanced design.

Zoos and private collections were the source of the majority of specimens tested in this study. Observations were conducted either in the snakes' home cages, where water bowls and shelters were removed prior to testing, or in large plastic or metal cans. All of the substrates used for observations of the snakes' behavior were flat surfaces on which any postural responses could readily be discerned. Body bridging, the elevation of the trunk of the snake, was used as the sole criterion for a response. The methanol used as a solvent to collect the skin substances, and present on both control and experimental balls, probably constituted an aversive stimulus, as did the visual effects of the presentations and the tactile stimulation involved in the snakes touching or being touched by the cotton balls. Thus, under the conditions encountered in this study, escape, head-hiding, and other behaviors which may be associated with body bridging, could not reliably be recorded as a positive response.

Approximate total length was recorded for each snake tested together with information on the individuals' history and sex where available.

Skin extracts were taken from whichever snakes (of those known or suspected to elicit a response) were available at each facility visited. Extracts from the suspected stimulus snakes were tested first with a known responder.

RESULTS

We report here only those previously undocumented taxa of crotaline snakes that exhibited a body-bridging response in one or more individuals. Because of the relatively small sample size for many of the taxa tested, the lack of a response should not be taken as conclusive evidence that a response does not occur in a particular species or subspecies (see Discussion). For each taxon, the number of individuals tested, the number observed to respond, and their approximate total lengths are shown in Table 1.

In addition to the adult and juvenile specimens listed in Table 1, we tested three litters of crotaline snakes with the skin substances of ophiophagous snakes. Since we were primarily concerned with the reaction of these snakes in their first exposure to ophiophage chemicals, the testing of litters involved the presentation of only one series of control and experimental balls such that half received the experimental ball first and half received the control ball first.

One litter of four captive-born cantils, *Agkistrodon bilineatus bilineatus*, each 43 days old and approximately 15 cm total length at the time of testing, were tested with the skin substances from *Lampropeltis getulus bolbrooki*. None of these snakes exhibited body bridging during the test sessions. Of a litter of eleven captive-born western diamondback rattlesnakes, *Crotalus atrox*, each 21 days old and about 22 cm total length, three exhibited body bridging when tested with an extract from *Lampropeltis getulus niger*. One litter of five northern copperheads, *Agkistrodon contortrix mokasen*, each ap-

TABLE 1. PREVIOUSLY UNREPORTED TAXA EXHIBITING BODY BRIDGING

Test taxa (Crotalinae)	Snakes tested		Snakes responded		Stimulus taxa (Colubridae)
	Number	Total length (cm)	Number	Total length (cm)	
Agkistrodon bilineatus bilineatus	4	60–75	2	60–75	*Lampropeltis g. niger*[a]
					L. g. splendida
A. contortrix contortrix	2	45–60	1	60	*Lampropeltis g. holbrooki*
A. c. mokasen	6	60–90	4	60	*Lampropeltis g. holbrooki*
					L. g. floridana[a]
					L. g. niger
					Drymarchon corais couperi
A. c. pictigaster	3	45–60	1	60	*Lampropeltis g. floridana*[a]
Bothrops alternatus	1	30	1	30	*Lampropeltis mexicana alterna*[a]
B. asper	1	120	1	120	*Lampropeltis getulus holbrooki*[a]
B. melanurus	2	30–35	1	30	*Lampropeltis getulus holbrooki*[a]
B. neuwiedi	4	55–90	1	90	*Masticophis taeniatus ornatus*[a]
B. undulatus	4	30–60	2	45–60	*Coluber c. constrictor*[a]
					Drymarchon corais couperi[a]
					Lampropeltis getulus holbrooki[a]

Crotalus adamanteus	4	60–90	2	60–90	*Lampropeltis triangulum sinaloae*[a] *Drymarchon corais couperi*
C. enyo enyo[b]	1	40	1	40	*Lampropeltis getulus holbrooki*[a]
C. molossus molossus	1	80	1	80	*Lampropeltis getulus holbrooki*[a]
C. m. nigrescens	3	60	2	60	*Lampropeltis triangulum sinaloae* *Coluber c. constrictor*[a]
C. polystictus	1	30	1	30	*Lampropeltis triangulum nelsoni*
C. pricei pricei	1	30	1	30	*Lampropeltis triangulum niger*[a]
C. ruber lucasensis[b]	2	30–90	1	30	*Lampropeltis mexicana alterna*[a] *L. getulus holbrooki*[a]
C. scutulatus salvini	4	60–70	1	60	*Drymarchon corais couperi*[a]
C. stejnegeri	1	40	1	40	*Lampropeltis triangulum nelsoni* *L. getulus holbrooki*[a]
C. tigris	3	60–75	1	60	*Coluber c. constrictor*[a]
C. viridis concolor	1	60	1	60	*Lampropeltis getulus niger*[a]
C. willardi willardi	2	45–60	1	45	*Masticophis taeniatus ornatus*[a]

[a]Sympatry between the test snake and this stimulus snake is unlikely.
[b]Bogert (1941) also found that *Crotalus enyo* and *C. ruber* exhibited a response; however, no subspecies were indicated.

proximately 15 cm total length, was found under a rock with an adult female in Cumberland County, Tennessee, during August 1977. It is unlikely that these snakes had had previous experience with any ophiophagous snakes, since the young generally do not disperse until several to many days after birth (Fitch, 1960). When tested with the skin extract from *Lampropeltis getulus holbrooki* 16 days after their capture, two out of five individuals responded by body bridging.

DISCUSSION

Previous studies of body bridging in crotaline snakes have concentrated on responses to subspecies of *Lampropeltis getulus*. Inger (unpublished) and Carpenter and Gillingham (1975) also found *Lampropeltis calligaster calligaster* to be effective. Thus, the term "kingsnake defense posture" has been used to refer to this behavior. Bogert (1941), however, stated that *Masticophis flagellum piceus* and *Pseustes sulfureus* also possess skin substances capable of eliciting the response. Aside from a few new subspecies of *Lampropeltis getulus*, we have observed that the dorsal skin of *Drymarchon corais couperi* (independently discovered earlier by Marchisin, personal communication), *Coluber constrictor constrictor*, *Masticophis taeniatus ornatus*, *Lampropeltis mexicana alterna*, *L. triangulum nelsoni*, and *L. t. sinaloae*, mostly known snake feeders, possess similar properties, and undoubtedly other such species will be discovered. Hence, we propose the term *ophiophage defensive response* to denote the body bridging and associated defensive behaviors of crotaline snakes and, should similar antipredator responses occur in other groups, of other snake taxa as well.

Actual body bridging has been used to establish the occurrence of a response in crotaline snakes to ophiophagous snakes; we also conservatively used trunk elevation as an indication of a response in this survey. From other reports on the ophiophage defensive response and our own observations, however, it is clear that recognition of predatory snakes through chemical cues may be manifest by other behaviors such as escape, head-hiding, and thrashing. Thus, body bridging represents just one of a constellation of recalcitrant reactions to ophiophagous snakes. A more complete inventory of responses and precise quantitative analysis, perhaps derived from actual encounters between snakes, would be useful. This would best be done with a common species available for repeated testing in controlled settings, conditions that did not prevail in the present study.

Carpenter and Gillingham (1975) have suggested that body bridging may be a behavioral characteristic of the subfamily Crotalinae. Body bridging as a defensive maneuver also has been reported in the elapid snakes, *Vermicella annulata* (Bustard, 1969) and *Denisonia maculata* (Johnson, 1970) in response to human molestation, and it is possible that they respond similarly to other perturbations. Given the limitations imposed by an elongate, limbless

Bauplan, it is not surprising to find some convergence in ophidian defensive postures and displays (Greene, 1977). We regard both the behavioral response, postural or otherwise, and its release by chemical stimuli as the unique features of the ophiophage defensive response. This is underscored by the fact that the scent of the spotted skunk elicits a response (Cowles, 1938), although the adaptive significance of the defensive response in this context remains to be investigated beyond Cowles' inconclusive observations on rattlesnake–skunk interactions. Nevertheless, the increasing number of crotaline snakes shown to respond to ophiophage skin substances lends tentative support to Carpenter and Gillingham's suggestion that the response may be a behavioral characteristic of the subfamily Crotalinae. It would thus be interesting to ascertain whether the strictly arboreal varieties, such as some *Bothrops* species, exhibit responses and, if so, in what form. Body bridging, at least, would seem to be an inappropriate defensive behavior for a species occurring among branches since purchase could easily be lost. If body bridging turns out to be ubiquitous in all crotaline species except for arboreal ones, the response could still be viewed as an ancestral crotaline response secondarily lost in species living where the response is no longer adaptive.

More fundamental is assessment of whether this response has been acquired convergently, due perhaps to some crotaline-peculiar chemosensory capacity, or has been generated in a common ancestor. This will depend upon the extension of our knowledge to new taxa, a consideration of the ecologies of the various species, and, ideally, the establishment of neuroanatomical and muscular correlates of this behavior.

A lack of responsiveness in crotalines to ophiophagous snakes, primarily *Lampropeltis* species, has been noted by others and probably accounts for some of the discrepancies which have appeared in the literature as to which species react. Bogert (1941) observed the attenuation of a response in individuals that had been in captivity and repeatedly exposed to the odor of predatory snakes. Carpenter and Gillingham (1975) stated that some of the snakes in their study that had been in captivity for several months exhibited no or weak responses to encounters with kingsnakes. It is unclear whether the lack of response or its decrease can be attributed to captive conditions per se or to repeated testing with ophiophagous snakes or their skin substances. In this regard, we have observed very dramatic responses with some crotalines that had been in captivity for several years.

The size or age of the snake may be another determinant of responsiveness to ophiophage cues. Bogert (1941) stated that large specimens of *Crotalus atrox* failed to react to kingsnakes, whereas juvenile and young adults responded without exception. Carpenter and Gillingham (1975) noted that individuals of *Agkistrodon contortrix laticinctus* that were larger than the kingsnakes in their study did not exhibit body bridging, although no measurements are given for either crotalines or kingsnakes. It is possible that larger

specimens are either less responsive or require more in the way of visual or tactile stimulation, in addition to chemical stimulation, to potentiate a response.

Our preliminary tests with litters of pit vipers point to behavioral polymorphism as one basis for the apparent variations in responsiveness. The relatively low ratio of snakes responding to the ophiophage skin substances may, in part, be attributed to our use of body bridging as the response criterion. As pointed out above, recognition of predatory snakes may be manifest in other forms. Carpenter and Gillingham (1975), for example, describe body flips of small *Agkistrodon c. laticinctus* in response to kingsnakes. From our data on neonates, however, it is clear that the response is not dependent upon previous experience with predatory snakes, an obvious advantage since the first encounter with an ophiophage could be the last, especially for smaller individuals.

We were successful in eliciting body bridging responses in a geographically diverse assemblage of New World crotaline snakes, including several species of the genus *Bothrops* (previously unreported), using a few species of ophiophagous snakes. In some cases the stimulus snakes used could be sympatric with the crotalines tested, but in many cases they would not be. In one case, *Crotalus stejnegeri*, a rare species known from a restricted area in southern Mexico, exhibited body bridging to the skin extract of *Lampropeltis g. holbrooki*, a subspecies indigenous to central United States. Other examples of response to allopatric stimulus snakes can be seen in Table 1. Bogert (1941) also found that several species of *Crotalus* from the western United States responded to *Lampropeltis g. getulus* from the eastern United States. Thus it appears that sympatry with the crotaline snakes is not essential when considering which snakes are capable of eliciting a defensive response. This suggests that (1) the crotaline chemoreceptors possess low specificity for the chemicals from the various predators, (2) the chemicals from the predators are the same or similar substances, or (3) different chemicals and chemoreceptors elicit the same response.

A similar picture has emerged from a few other kairomone systems involving the recognition of predators by actual or potential prey animals. Reed (1969) observed a fright response in several North American fish of the families Cyrinidae, Poecilidae, and Cyprinodontidae to chemicals from predatory fish, including two South American cichlids, *Astronotus ocellatus* and *Cichlasoma severum*. Müller-Schwarze (1972) tested the avoidance responses of naive black-tailed deer (*Odocoileus hemionus*) from the United States to droppings from the African lion (*Panthera leo*), the Bengal tiger (*Panthera tigris tigris*), the snow leopard (*Panthera unica*), the mountain lion (*Felis concolor*), and the coyote (*Canis latrans*). Only the mountain lion and coyote occur sympatrically with the deer. Although the odors of the mountain lion and coyote elicited the most consistent avoidance response, the odors from the snow leopard, African lion, mountain lion, and coyote did not differ in their

effect. The Florida apple snail, *Pomacea paludosa*, exhibits a burial response to some fresh-water turtle predators sympatric with it in addition to several turtles which are not, including a terrestrial turtle. *Gopherus polyphemus* (Synder and Snyder, 1971). Lastly, several marine gastropods (Clark, 1958; Yarnall, 1964; Ansell, 1969; Weldon, unpublished) and at least one anemone (Ward, 1965) exibit flight responses to chemicals emanating from predatory asteroids and/or gastropods that are and are not sympatric with them. Yarnall (1964) has suggested that the chemicals responsible for the elicitation of the flight responses are related to some common feature of the physiology of the predators, and indeed this appears to be true of other systems in which actual or potential prey recognize predators through chemical cues.

Mauzey et al. (1968) have hypothesized that the substances from predaceous asteroids that elicit escape responses in other echinoderms may have a dietary origin. This is a possibility with the crotaline–colubrid interaction, as most of the snakes that elicit the defensive reponse are known to be snake and/or lizard feeders (see Wright and Wright, 1957, for the diets of *Coluber c. constrictor*, *Drymarchon c. couperi*, *Lampropeltis c. calligaster*, *L. getulus* ssp., *Masticophis f. piceus*, *M. t. ornatus*; Mole, 1924 for *Pseustes sulfureus*; and Gehlbach and Baker, 1962, for *L. mexicana alterna. Lampropeltis t. sinaloae* has been reported as feeding on reptile eggs [Zweifel and Norris, 1955], and we know of no information on the diet of *L. t. nelsoni*).

We feel that these observations call for a broader consideration of the ophiophage defensive response by focusing not only on the species exhibiting a response, but also on the species eliciting it. Are the substances from the various predatory snakes which elicit a response the same or similar substances? Are they related to the diet of the snake or produced de novo? These are some of the questions that we hope to consider in future investigations.

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Note Added in Proof. Chiszar et al. (1978) have found that rattlesnakes (*Crotalus viridis*) and water moccasins (*Agkistrodon piscivorus*) exhibit less tongue flicking when placed into a cage previously occupied by a kingsnake (*Lampropeltis getulus*) than in a cage previously occupied by a hognose snake (*Heterodon nasicus*) or in an open-field situation. The disruption of exploration by the odor of kingsnakes as measured by tongue flicking was more dramatic in water moccasins

than in rattlesnakes. This is said to correlate with the extent to which the species overlap in nature, although the particular subspecies of *Lampropeltis* used in their study ranges from central Texas to central Mexico, and the subspecies of *Agkistrodon* from Virginia to central Alabama (Conant, 1975). No body bridging was observed in the pit vipers during the sessions in which they were exposed to kingsnake odor.

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COMPONENTS OF THE SEX PHEROMONE OF THE FEMALE SPOTTED STALK BORER, *Chilo partellus* (Swinhoe) (LEPIDOPTERA: PYRALIDAE): IDENTIFICATION AND PRELIMINARY FIELD TRIALS

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Abstract—Female *Chilo partellus* (Swinhoe) abdominal tip extracts were examined by gas–liquid chromatography (GLC) combined with simultaneous electroantennographic (EAG) recording from the male moth. Two olfactory stimulants were detected and identified as (*Z*)-11-hexadecenal (I) and (*Z*)-11-hexadecen-1-ol (II) by their GLC behavior, microchemical reactions, and comparison with synthetic materials. Both compounds were detected in volatiles emitted by the "calling" female moth. Synthetic (*Z*)-9-tetradecenyl formate, a structural analog of aldehyde (I), also elicited a significant EAG response from the male moth. Field trials carried out in India using synthetic (I) and (II) as bait in water traps showed that compound (I) was highly attractive to male *C. partellus*; compound (II) was not attractive, and its addition to (I) significantly reduced trap catches.

Key Words—Sex pheromone, sex attractant, Lepidoptera, Pyralidae, *Chilo partellus*, spotted stalk borer, electroantennography, (*Z*)-11-hexadecenal, (*Z*)-11-hexadecen-1-ol, (*Z*)-9-tetradecenyl formate.

INTRODUCTION

The spotted stalk borer moth, *Chilo partellus* (Swinhoe), is a pest of major economic importance in India and East Africa, the larvae attacking maize, sorghum, sugar cane, and rice. An investigation of the female sex pheromone was undertaken to provide a synthetic attractant for population

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monitoring, and we report here the identification of the pheromone components and preliminary field testing of the synthetic compounds carried out in India. This is part of a continuing program of work on the sex pheromones of *Chilo* pest species which, it is hoped, will also provide information on the role of pheromones in premating reproductive isolation in this genus.

METHODS AND MATERIALS

Insect Material and Preparation of Extracts. A laboratory culture of *C. partellus* based on insect material of Indian origin was maintained at the Centre for Overseas Pest Research, London. Larvae were reared for the first 10 days on maize (approx. 6 weeks old) and from then on until pupation on an artificial wheat germ diet similar to that of Chatterjee et al. (1968). The culture was maintained on a 14-hr light:10-hr dark cycle with photophase temperatures of 26–28° C and scotophase temperatures of 20–22° C. Pupae for pheromone work were kept at 24–26° C under constant light, and adult moths were sexed on emergence. On the first day after emergence, females were placed in the dark for 2 hr. After freezing for 10 min at −20° C, the abdominal tips (terminal 3–4 segments) were clipped and extracted by soaking in purified dichloromethane for 15 min at room temperature. The extracts were filtered through glass wool and concentrated to approximately 1 tip equivalent per microliter by rotary evaporation without heating. Later work indicated that the yield of pheromone could be increased by placing the females in the dark for 5–6 hr before clipping.

Entrainment of Pheromone. The composition of the pheromone actually emitted by a single female moth was examined using charcoal air filters similar to those described by Grob and Zürcher (1976) to trap the airborne volatiles. The moth was contained in a glass chamber (12 × 4 cm) through which air was passed at 2 liters/min (equivalent to 0.02 m/sec), after purification by passage through active carbon and humidification by bubbling through distilled water. Moths were used within 24 hr of emergence, and placed for 1 hr in the entrainment apparatus under full light. The lights were then switched off and observations made with a small safe-light at hourly intervals. The air filter was changed every 2 hr, and absorbed materials were extracted with four 10-μl portions of carbon disulfide. Aliquots of this extract were examined by GLC and by GLC linked to EAG recording.

GLC and EAG Analyses. GLC instrumentation and conditions were similar to those described previously (Nesbitt et al., 1975b). In an attempt to prevent degradation of one of the components of the pheromone during analysis, a glass SCOT Carbowax 20M column (50 m × 0.5 mm ID) was used instead of the original stainless-steel column. The isomeric composition of synthetic compounds was determined by analysis on a packed OV 275

column (10 m × 2 mm ID glass column, packed with 20% OV 275 on 100–120 mesh Gas-Chrom RZ).

GLC analysis combined with simultaneous recording of male moth EAG responses to the column effluent was carried out as described by Moorhouse et al. (1969). The "puffing" method for measuring EAG responses to test compounds by blowing them directly over the insect's antenna was carried out as described previously (Nesbitt et al., 1977).

Purification of Tip Extracts. After initial GLC–EAG work had established that female tip extracts contained two olfactory stimulants for the male moth—an aldehyde (I) and an alcohol (II)—these were purified by a method involving conversion of (I) into its bisulfite adduct, prior to various microchemical reactions.

Solvent was removed from female tip extract by rotary evaporation at room temperature, and saturated aqueous sodium metabisulfite solution was added to the residue. After 30 min at room temperature, extraction of the reaction mixture with dichloromethane gave (II) contaminated with only traces of (I). Pure (II) was obtained by collection of the appropriate peaks from Carbowax 20M and Apiezon L GLC columns as described previously (Nesbitt et al., 1975a). Component (I) (95% pure by GLC analysis) was recovered by addition of sodium carbonate to the aqueous phase remaining after extraction of (II), followed by reextraction with dichloromethane.

Microozonolysis. Component (I) was ozonolyzed in carbon disulfide by a method based on that of Beroza and Bierl (1967). The ozonides were reductively cleaved with triphenylphosphine, and the reaction product was examined by GLC on the following packed columns: (A) 10% SP-216-PS on 100–120 mesh Supelcoport; (B) 9% Silar-5CP on 80–100 mesh Chromosorb W; (C) 2.5% Apiezon L on 80–100 mesh Chromosorb G. Aliphatic dialdehydes used as GLC reference compounds were prepared by reductive ozonolysis of the corresponding cycloalkenes or monounsaturated aldehydes.

Oxidation. The alcohol component (II) was oxidized by a method based on that of Corey and Suggs (1975). Following purification as described above, (II) was treated with a freshly prepared, saturated solution of pyridinium chlorochromate in dichloromethane. After 30 min at room temperature, the reaction mixture was examined by GLC–EAG on SCOT Carbowax 20M and Apiezon L columns.

Synthesis of the Pheromone Components and Related Compounds. The *Z* and *E* isomers of 11-hexadecen-1-ol and 11-hexadecenal were prepared from 1,10-decanediol and 1-hexyne as described previously (Nesbitt et al., 1975b), and (*Z*)-9-tetradecenyl formate was prepared by an analogous acetylenic route (Beevor et al., 1977). For all these compounds the *Z* isomers contained 1–2% of the corresponding *E* isomer, and the *E* isomers contained less than 1% of the *Z* isomer.

Field Attractancy Tests. The traps used for field tests were square galvanized metal pans (60 × 60 × 7.5 cm deep) containing water and a trace of

detergent, fitted with lids and metal legs 0.5 m high (Campion et al., 1974; Marks, 1976). Synthetic test compounds, combined with an equal weight of 2,6-di-*tert*-butyl-*p*-cresol (BHT) as antioxidant, were dispensed from sealed polyethylene vials (36 × 16 mm with 1.5-mm-thick walls) suspended from the underside of the trap lids about 5 cm above the surface of the water. At an early stage in the work it was observed that the aldehyde (I) and alcohol (II) reacted together even in dilute hexane solution at −20° C, and these two compounds were subsequently dispensed from separate vials. The corresponding acetal, a possible interaction product, could not be detected by thin-layer chromatographic comparison with an authentic sample.

Virgin female moths used in traps were aged 20 hr or less and were renewed nightly.

The traps were set out 30 m apart between the plant rows in a 6-hectare field of almost-mature sorghum. The experimental design for the first test described was a randomized complete block of three replicates run over 15 nights; replicates were separated by 100 m. The data obtained was analyzed using a $\log(x + 1)$ transformation.

RESULTS

When female abdominal tip extract was examined by GLC–EAG using packed columns of varying polarity, two compounds were detected which elicited strong responses from the male moth antennal preparation. The major component (I) had the lower retention temperature on all columns and was present in amounts of approx. 20 ng/tip equivalent; the amount of the minor component (II) was approx. 3 ng/tip equivalent. From previous experience, these compounds were assumed to be sex pheromone components, and their GLC retention temperatures on five packed columns are given in Table 1.

The GLC behavior of (I) was recognized as being that of a C_{16}, monounsaturated, straight-chain aldehyde by its similarity to that of the major component of the female sex pheromone of *Chilo suppressalis*, (*Z*)-11-hexadecenal (Nesbitt et al., 1975b). Since lepidopterous species of the same genus often have pheromone components in common, (*Z*)-11-hexadecenal was examined under the same conditions used for the *C. partellus* female tip extract. This compound was found to chromatograph in the same way as component (I) on all five columns (Table 1), and it elicited a comparable EAG response from the male moth (responses to 1 ng of material presented through the GLC–EAG link: component (I), 0.60 mV; (*Z*)-11-hexadecenal, 0.54 mV). As expected for an aldehyde, component (I) was almost completely removed from tip extracts by treatment with saturated aqueous sodium metabisulfite solution, and this was used to separate and purify the two pheromone components.

TABLE 1. GLC DATA FOR *Chilo partellus* FEMALE TIP EXTRACT AND SYNTHETIC STANDARDS ON PACKED COLUMNS

	Retention temperatures (°C)				
	C[a]	D	E	F	G
Female tip extract					
Component (I)	208.9	181.9	180.6	147.0	162.5
Component (II)	218.9	205.0	189.5	165.6	165.4
Synthetic standards					
Tetradecyl acetate	208.8	175.1	182.0	140.6	159.2
Hexadecyl acetate	232.5	194.7	202.8	156.2	174.9
(*Z*)-11-Hexadecenal	208.8	182.3	180.7	146.9	162.4
(*Z*)-11-Hexadecen-1-ol	218.8	204.8	189.5	166.2	164.8

[a]Stationary phases: (C) Apiezon L; (D) Carbowax 20M; (E) Silicone gum SE 30; (F) EGSS-X; (G) Fluorosilicone oil QF1.

The GLC behavior of component (II) relative to that of hexadecyl acetate, particularly its high retention temperature on Carbowax 20M, suggested it was a C_{16}, monounsaturated, straight-chain alcohol. The compounds making up multicomponent pheromone systems have generally been found to possess related chemical structures. Hence (*Z*)-11-hexadecen-1-ol, which has the same ω distance and double-bond configuration as the major component (I), was examined by GLC–EAG under the conditions used for the tip extract. As shown in Table 1, its chromatographic behavior was very similar to that of component (II), and the synthetic compound also elicited an EAG response from male *C. partellus* at the nanogram level (responses to 1 ng of material: component (II), 0.40 mV; (*Z*)-11-hexadecen-1-ol, 0.41 mV).

The 11- position of the double bond in component (I) was confirmed by reductive ozonolysis. This gave a product which cochromatographed with synthetic 1,11-undecanedial on the three columns used [retention temperatures: column (A), 163.9°C; (B), 184.8°C; (C), 155.7°C). The configuration of the double bond was determined by comparison of the retention data for (I) on SCOT columns with that for synthetic (*Z*)- and (*E*)-11-hexadecenal (Table 2). In addition, component (I) was shown to cochromatograph with the *Z* isomer on the three stationary phases used. No GLC peaks and no EAG responses were observed at the retention temperatures for the *E* isomer during linked GLC–EAG analyses of female tip extract.

Insufficient amounts of component (II) were available for microozonolysis to confirm the double bond position, and chromatography on both metal and glass SCOT columns resulted in loss of this component (standard unsaturated alcohols were also totally destroyed when chromatographed on

TABLE 2. GLC DATA FOR *Chilo partellus* FEMALE TIP EXTRACT AND SYNTHETIC STANDARDS ON SCOT COLUMNS

	Retention temperatures (°C)		
	H[a]	J	K
Female tip extract			
Component (I)	152.8	188.1	175.4
Synthetic standards			
Dodecyl acetate	143.2	176.9	164.4
Tetradecyl acetate	150.7	184.7	174.8
(*Z*)-11-Hexadecenal	152.8	188.1	175.3
(*E*)-11-Hexadecenal	152.7	187.8	175.9

[a]Stationary phases: (H) DEGS; (J) Carbowax 20M; (K) Apiezon L.

these columns at low loadings). However, it was possible to oxidize purified (II) with pyridinium chlorochromate and analyze the product by GLC–EAG. A compound was obtained which cochromatographed with (*Z*)-11-hexadecenal on the three SCOT columns and had similar EAG activity.

Two compounds with the same GLC behavior and EAG activity as components (I) and (II) were obtained by collection of airborne volatiles from a virgin female moth. They were collected in amounts corresponding to 5–40 ng/hr of (I) and less than 1–3 ng/hr of (II) during the period 5–9 hr after lights-off. Yields were highest on those occasions when the female moth was seen to adopt a classical "calling" position.

EAG responses to (*Z*)-9-tetradecenyl formate, a compound in which the α-methylene group of (*Z*)-11-hexadecenal is replaced by oxygen, were lower than those elicited by this aldehyde, but comparable in magnitude to responses to (*Z*)-11-hexadecen-1-ol. The average responses to 2 ng of test compound in six replicate "puff" tests were: (*Z*)-11-hexadecenal, 1.29 mV; (*Z*)-11-hexadecen-1-ol, 0.82 mV; (*Z*)-9-tetradecenyl formate, 0.88 mV.

Field testing of (*Z*)-11-hexadecenal and (*Z*)-11-hexadecen-1-ol was carried out at ICRISAT, Hyderabad, India, and two experiments conducted in 1977 are described here. Table 3 gives the results of an experiment comparing catches of male *C. partellus* moths in water traps baited with the aldehyde alone, the alcohol alone, the aldehyde plus the alcohol (in the same trap but in different vials) and a virgin female moth, and catches in control, unbaited water traps. The aldehyde and alcohol were tested together in the ratio found in tip extracts, i.e., 7:1, but the aldehyde alone caught significantly more moths than any other treatment ($P < 0.001$). The catches in traps baited with (*Z*)-11-hexadecen-1-ol were not significantly different from those in the control traps, and addition of this compound to the aldehyde caused a signifi-

cant reduction in catches. Very few female moths were caught in any of the traps.

The coefficient of variance in this experiment, using transformed data, was 32%. Further analysis of the data showed that there were significant differences in catches over nights, and these were not due to aging of the attractant sources since fluctuations were also observed in the catches in virgin female and control traps. Peak catches were associated with rain, drizzle, or irrigation of the fields.

A longer experiment, in which the three synthetic pheromone treatments—aldehyde alone, alcohol alone, and aldehyde plus alcohol—were tested at five sites, gave similar results, shown in Figure 1. Traps baited with the aldehyde alone caught more than four times as many moths as those baited with the aldehyde and alcohol in a 7:1 ratio.

These observations and further field experiments will be reported in detail elsewhere.

DISCUSSION

We believe that the GLC, EAG, and chemical data taken in conjunction with the preliminary field results establish that (*Z*)-11-hexadecenal and (*Z*)-11-hexadecen-1-ol constitute the female sex pheromone of *C. partellus*. The major aldehydic component alone, when dispensed from polyethylene vials, is comparable in attractiveness with the female moth and is currently being used in traps to monitor populations of *C. partellus* at ICRISAT.

TABLE 3. CATCHES OF *Chilo partellus* MOTHS IN TRAPS BAITED WITH (*Z*)-11-HEXADECENAL (Z11-16:CHO) AND (*Z*)-11-HEXADECEN-1-OL (Z11-16:OH) COMPARED WITH THOSE IN VIRGIN FEMALE AND UNBAITED TRAPS: 3 TRAPS PER TREATMENT RUN OVER 15 NIGHTS, MARCH-APRIL 1977

Treatment	Total catch		Male moth catch per trap night	
	Males	Females	Mean	Transformed mean[a]
437.5 μg Z11-16:CHO	2194	14	48.8	3.68 a
437.5 μg Z11-16:CHO + 62.5 μg Z11-16:OH	848	9	18.8	2.55 b
62.5 μg Z11-16:OH	74	9	1.6	0.76 c
Virgin female moth	1381	13	30.7	2.64 b
Unbaited trap	102	8	2.3	0.85 c

[a]Means followed by the same letter are not significantly different at the 5% level.

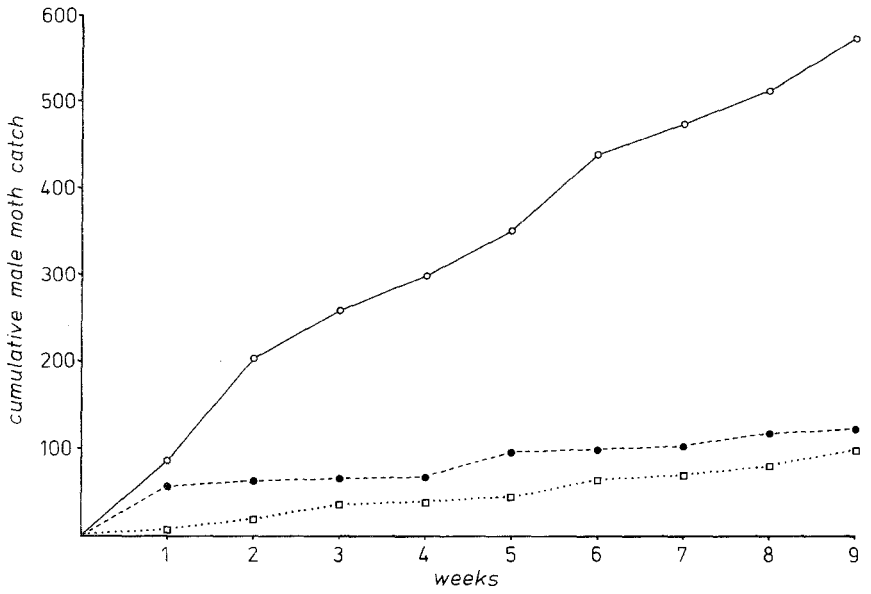

FIG. 1. Cumulative catches of male *Chilo partellus* moths in traps baited with 437.5 μg (*Z*)-11-hexadecenal (○——○), 437.5 μg (*Z*)-11-hexadecenal + 62.5 μg (*Z*)-11-hexadecen-1-ol (●------●), and 62.5 μg (*Z*)-11-hexadecen-1-ol (□·····□) over 9 weeks (January 20 to March 16, 1977); 5 sites; 1 set of 3 treatments per site; vials renewed weekly.

However, the exact function of the alcohol is uncertain. During the initial field trials reported here, addition of (*Z*)-11-hexadecen-1-ol to (*Z*)-11-hexadecenal in the 1:7 ratio found in tip extracts reduced trap catches to the level of the unbaited traps. The two compounds were dispensed separately from adjacent polyethylene vials, and our release rate data for moth pheromones with different functional groups would suggest that the alcohol-to-aldehyde ratio actually emitted by the combined source should also have been approximately 1:7 (Campion et al., 1978). This ratio was chosen for initial trials rather than the lower alcohol-to-aldehyde ratio found in entrained volatiles, because tests with nanogram quantities of synthetic (*Z*)-11-hexadecen-1-ol and (*Z*)-11-hexadecenal have shown that the percentage recovery of the alcohol is much lower than that of the aldehyde in our entrainment system. However, further field trials are in progress to compare the attractiveness of a range of alcohol/aldehyde ratios.

The detection of the alcohol in airborne volatiles from a "calling" female *C. partellus* moth indicates that the alcohol is actually emitted by the moth, and would suggest that it has a definite role in premating behavior, despite the observed effect on trap catches. The situation with *C. partellus* thus seems

to be different from that reported for the tortricid moth, *Choristoneura fumiferana*. In the latter, the main attractant component of the female sex pheromone was identified as (*E*)-11-tetradecenal (Weatherston et al., 1971) and the attractiveness of the synthetic aldehyde to male moths was shown to be reduced by addition of (*E*)-11-tetradecen-1-ol (Sanders et al., 1972). Both the aldehyde and alcohol were found in tip extracts, but only the aldehyde was detected in washings from jars that had held female moths (Weatherston and Maclean, 1974) and in volatiles entrained from female moths and trapped on Porapak Q (Weatherston et al., 1975). Weatherston and Maclean (1974) concluded that the alcohol was merely a biosynthetic precursor to the aldehyde. More recently, reexamination of the pheromone obtained by rinsing out female moth containers showed it to be a mixture of the *E* and *Z* isomers of 11-tetradecenal in a 96:4 ratio, and addition of a small percentage of the *Z* isomer to pure synthetic (*E*)-11-tetradecenal was found to be necessary to optimize attraction (Sanders and Weatherston, 1976). No comment was made on the isomeric composition of the 11-tetradecen-1-ol in tip extracts.

(*Z*)-11-Hexadecenal has also been identified as the major component of the female sex pheromone of *Chilo suppressalis*, where its attractiveness to male moths is synergized by a second component, the homologous aldehyde (*Z*)-13-octadecenal (Nesbitt et al., 1975b; Ohta et al., 1976; Beevor et al., 1977). In both *C. partellus* and *C. suppressalis*, we have found (*Z*)-9-tetradecenyl formate, a compound structurally related to (*Z*)-11-hexadecenal, to be a potent olfactory stimulant for the male moth (Nesbitt et al., 1975b; cf. also Nesbitt et al., 1977), and with *C. suppressalis* this formate has been shown to disrupt communication between male and female moths in the field (Beevor et al., 1977).

(*Z*)-11-Hexadecenal has also been reported as a female sex pheromone component in the noctuid species *Heliothis virescens*, *H. zea* (Roelofs et al., 1974; Tumlinson et al., 1975), and *H. armigera* (Piccardi et al., 1977; Nesbitt et al., unpublished), and in the plutellid *Plutella xylostella* (Tamaki et al., 1977; Chow et al., 1977). (*Z*)-11-Hexadecen-1-ol has been found as a pheromone component in the clover cutworm, *Scotogramma trifolii* (Underhill et al., 1976).

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MORPHOLOGY OF THE PROTHORACIC DEFENSIVE GLAND OF *Schizura concinna* (J.E. SMITH) (LEPIDOPTERA:NOTODONTIDAE) AND THE NATURE OF ITS SECRETION

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Abstract—The larval defensive gland of *Schizura concinna* (J.E. Smith) is situated in the thorax and consists of two sacs joined by an interglandular neck. Its orifice opens into a transverse invagination of the integument at the cervical margin of the prosternite. The major component of the defensive secretion, formic acid, was identified as its *p*-bromophenacyl ester. Ancillary components decyl acetate, dodecyl acetate, and 2-tridecanone from the anterior portion of the gland were identified by GLC and GS-MS.

Key Words—*Schizura concinna*, Lepidoptera, Notodontidae, prothoracic, defensive gland, allomone, defensive secretion, decyl acetate, dodecyl acetate, 2-tridecanone.

INTRODUCTION

Larvae of several species of the family Notodontidae have a defensive gland located ventrally in the prothorax (Pavan and Valcurone-Dazzini, 1976, and references therein). The morphology and histology of the gland in *Schizura concinna* (J.E. Smith), its relation to other organs, and its tracheal supply and musculature was reported by Detwiler (1922). He also determined, by a comparative analysis of the crystals formed when the secretion was frozen, that the defensive secretion contained formic acid. Recently the analyses of other notodontid defensive secretions have indicated the presence of accessory components in addition to formic acid (Eisner et al., 1972).

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In the present study the morphology of the allomone-producing gland in *S. concinna* is reexamined and differences from the early description noted. The presence of formic acid as the main component in the secretion is confirmed, and the identification of the accessory compounds is reported.

METHODS AND MATERIALS

Insects used in this study were either collected as late instar larvae from apple trees on St. Joseph Island, Ontario, and maintained in the laboratory, or reared in the laboratory on artificial diet (Grisdale, 1970). Insects were killed by freezing and decapitated, and the defensive gland was dissected from the thoracic cavity.

For morphological observations whole mounts of dissected glands fixed in ethanol-glacial acetic acid (3:1) were stained with Grenacher's borax carmine (Humason, 1967), dehydrated in ethanol, and cleared in benzene.

For the study of the orifice of the gland, dissected glands were fixed overnight in ice-cold 5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.3) with 2% sucrose. The tissue was then postfixed with 1% osmium tetroxide in 0.05 M cacodylate buffer containing 4% sucrose for 4 hr. The glands were stained en bloc with 2.5% hot aqueous uranyl acetate, dehydrated in an ethanol series, and embedded in Araldite (Locke et al., 1971). Thick sections (1 μm) of Araldite-embedded material were observed in the light microscope after staining with toluidine blue.

For chemical studies dissected replete glands were stored in either methylene chloride or N sodium hydroxide solution and kept at $-4°$C until needed.

Gas-liquid chromatographic (GLC) analyses were carried out on a Perkin-Elmer 990 instrument fitted with flame-ionization detectors. The GLC columns used in this study were 6 ft $\times$ 1/8 in. stainless steel packed with (1) 5% OV-1 on Chromosorb WHP (80/100 mesh); (2) 10% Carbowax 20 M on Chromosorb W (80/100 mesh); (3) 3% OV-25 on Chromosorb WHP (80/100 mesh); and (4) 3% OV-225 on Chromosorb WHP (80/100 mesh) operated at 130, 175, 130, and 190° C, respectively. Helium was used as the carrier gas at a flow rate of 40 ml/min.

Gas chromatography-mass spectrometry (GC-MS) was carried out on a Hitachi RMS 4 mass spectrometer, coupled to a Perkin-Elmer 990 gas chromatograph, operated at 70 eV.

Authentic dodecyl acetate was obtained from Eastman Organic Chemicals, Rochester, New York, and 2-tridecanone from Chemical Samples Corporation, Columbus, Ohio. Decyl acetate was prepared by reacting 1-decanol (Chemical Samples Corp.) with acetic anhydride in dry pyridine.

The *p*-bromophenacyl derivative of the major component of the secretion was prepared as follows. Fourteen replete glands in 1 ml of N sodium hydroxide solution were refluxed over 0.5 hr with 2,4'-dibromoacetophenone (277 mg) in ethanol (3 ml). The reaction mixture was taken to dryness on the

rotavapor, dissolved in ether (10 ml), and the ethereal solution washed with water (3 × 3 ml). After drying the solution over magnesium sulfate, the drying agent was removed and the solution volume adjusted to 15 ml before GLC and GC-MS analyses.

RESULTS

Location and Morphology of the Gland. Defensive glands are present in all instars of *S. concinna*, although in laboratory-reared insects the dorsal, red metathoracic projection, which gives the insect its common name (the red-humped caterpillar), is present only during the last larval (fourth) instar (Figure 1). The gland is situated in the thorax and consists of two sacs, referred to in this report as anterior and posterior glands, joined by an interglandular neck. The gland orifice opens into a transverse invagination of the integument at the cervical margin of the prosternite (Figure 2).

The orifice (Figure 3) is a highly complex structure, having a median projection on the posterior lip and two lateral projections on the anterior. The lateral projections are directed towards the centre where their free ends overlap. There is extensive cuticular development around the orifice, particularly superior to the anterior lip.

The anterior gland is pear-shaped and is about 2.2 mm long. Near the orifice, the gland takes the shape of a cylindrical secretory duct. It increases in diameter posteriorly and is invaginated where it receives the interglandular neck. This entire region of the anterior defensive gland has two lateral folds extending along its length and part of the interglandular neck. Both the anterior gland and the interglandular neck have a distinctly corrugated appearance due to annular cuticular folds in the lining of the lumen.

The posterior gland is approximately 4.3 mm in length. When filled, it extends obliquely to the right posterior dorsal margin of the metathorax. It has an irregular shape when empty. The end proximal to the orifice has a slight invagination where it comes in contact with the anterior gland and usually obscures the interglandular neck. The cuticular lining of the posterior gland is composed of irregularly shaped, truncated papillae having intricately furrowed apices.

Musculature of the gland is restricted to the area surrounding the orifice where bilaterally paired muscles have their insertion. In his description of the musculature and tracheation, Detwiler (1922) described a "small median lump-like structure" near the orifice. Even after repeated examination of several glands, such a lump could not be found.

Chemistry of the Secretion. When the insect is induced to spray, the pungent odor of formic acid is easily discerned. The presence of formic acid in the secretion was unambiguously demonstrated by the formation of its *p*-bromophenacyl ester as described above. On GLC analysis (3% OV-225 at 190°), the insect-derived ester had a retention time of 224 sec. The R_t of authentic *p*-

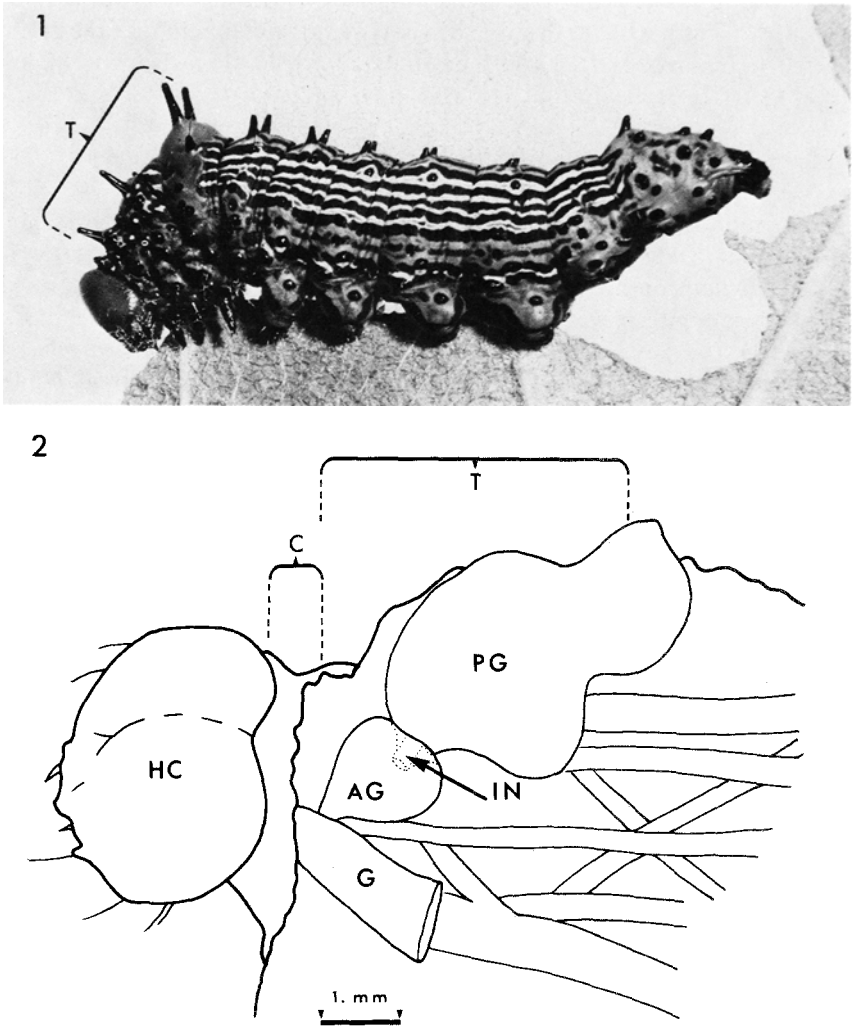


FIG. 1. Larva of the red-humped caterpillar *Schizura concinna* (J.E. Smith).
FIG. 2. Diagrammatic representation of location and size of the prothoracic defensive gland in a dissected insect. Anterior gland, AG; gut, G; head capsule, HC; interglandular neck, IN; cervicum, C; thorax, T; posterior gland, PG.

bromophenacyl formate under the same conditions was 224 sec. The mass spectra of the derivatives were almost identical (Figure 4A and B), both exhibiting as doublets, the molecular ion at m/e 242/244 and diagnostic ions at m/e 183/185 and m/e 155/157 resulting from α cleavage about the keto group.

GLC analysis of the secretion on OV-1 (Figure 5) showed the presence of four compounds: component A with R_t 232 sec, component B, R_t 396 sec, component C, R_t 662 sec, and a fourth component, R_t 464 sec, present only in trace amounts. This last compound was not investigated further. GC-MS of components A and C resulted in spectra (Figures 6 and 7) consistent with acetates, having base peaks at m/e 43 and diagnostic peaks at m/e 61 from protonated acetic acid. Component A gave a $M^+ - 60$ peak at m/e 140, while C exhibited this fragment ion at m/e 186, leading to the conclusion that A is decyl acetate and C is dodecyl acetate. The mass spectrum of component B (Figure 8) had M^+ at m/e 198 and intense fragments at m/e 43 and m/e 58, suggesting that it could be 2-tridecanone.

That the structural assignments are correct is illustrated by the data summarized in Table 1 and a comparison of Figures 6, 7 and 8 with the spectra obtained from authentic decyl acetate, dodecyl acetate, and 2-tridecanone.

GLC analysis of the secretion obtained separately from the anterior and posterior glands showed that lipophilic components came from the anterior gland, although a very small amount of decyl acetate was seen in the trace of the posterior gland secretion. The ratio of the lipophilic compounds in the anterior gland was A:B:C, 92:2:9.

DISCUSSION

Morphology of the Gland. In both *Heterocampa manteo* (Eisner et al., 1972) and *S. concinna*, the prothoracic gland consists of anterior and posterior glands. Those in *Notodonta anceps*, *Cerura vinula* (Hintze, 1969), *Datana ministra*, *Heterocampa guttivitta*, and *Schizura leptinoides* (Percy and MacDonald, unpublished observations) are pouch-shaped glands. With the exception of *D. ministra* (Herrick and Detwiler, 1919), all of the larvae spray formic acid; therefore, the morphology of the gland is not a reflection of the nature of its defensive secretion. This is also apparent from the study of various glands of other insects which secrete formic acid. The formicine ants utilize the poison gland for production of formic acid. The gross structure of this gland differs from the prothoracic defensive gland in that the reservoir is completely surrounded by muscles (Wilson and Regnier, 1971; Hermann and Blum, 1967). The pygidial glands of *Helluomorphoides ferrugineus* and *H. latitarsus* (Eisner et al., 1968), as well as those of other carabid beetles, also produce formic acid. The structure of these glands is typical of pygidial glands, in that the gland cells form a distinct entity separate from the reservoir (for review see Weatherston and Percy, 1978).

When larvae of *S. concinna* are disturbed, they eject the defensive secretion and assume a characteristic posture, throwing the head up. In this manner they are able to eject the spray in any direction, and to a distance of about eight inches (Detwiler, 1922). From our observations we surmise that the many folds, spikes, and projections of the cuticle have an atomizing function,

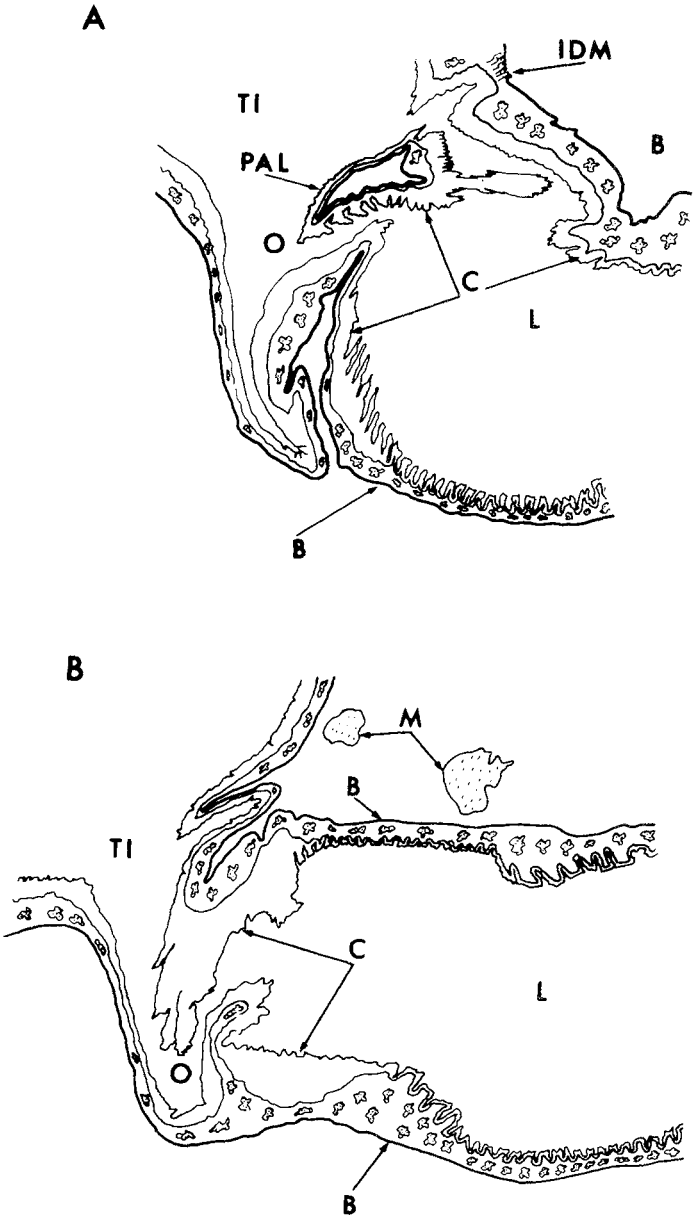
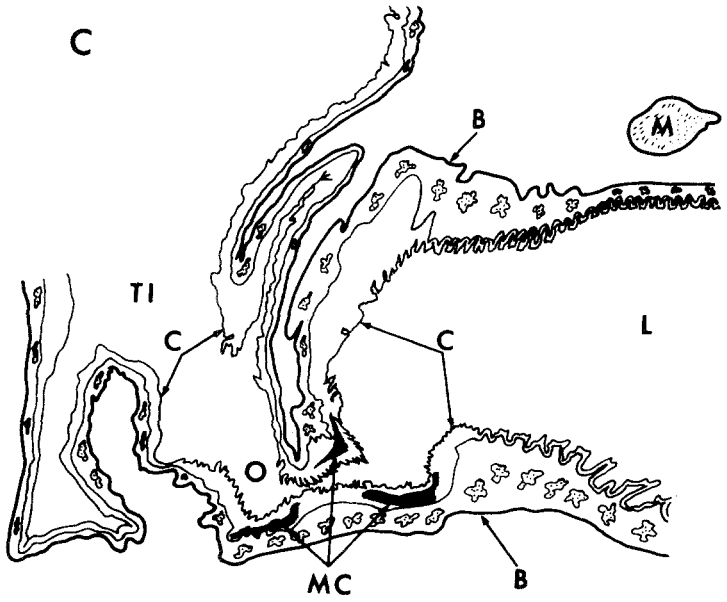


FIG. 3. Camera lucida drawings of sagittal sections of the defensive gland of *S. concinna* through the orifice from median (A, above) to lateral (D, facing page) position. For description see text. Basement membrane, B; cuticle, C; insertion dilator muscle, IDM; gland lumen, L; muscle, M; mesocuticle, MC; orifice, O; projection anterior lip, PAL; transverse invagination, TI.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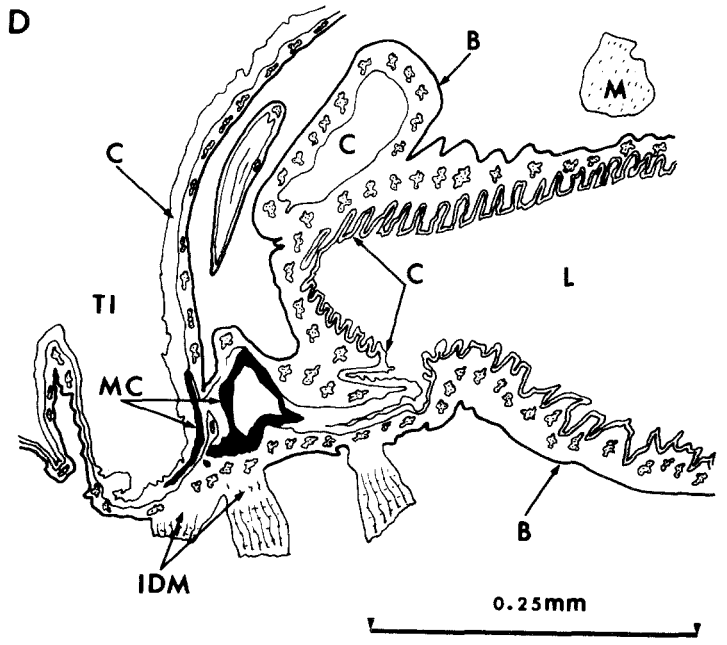
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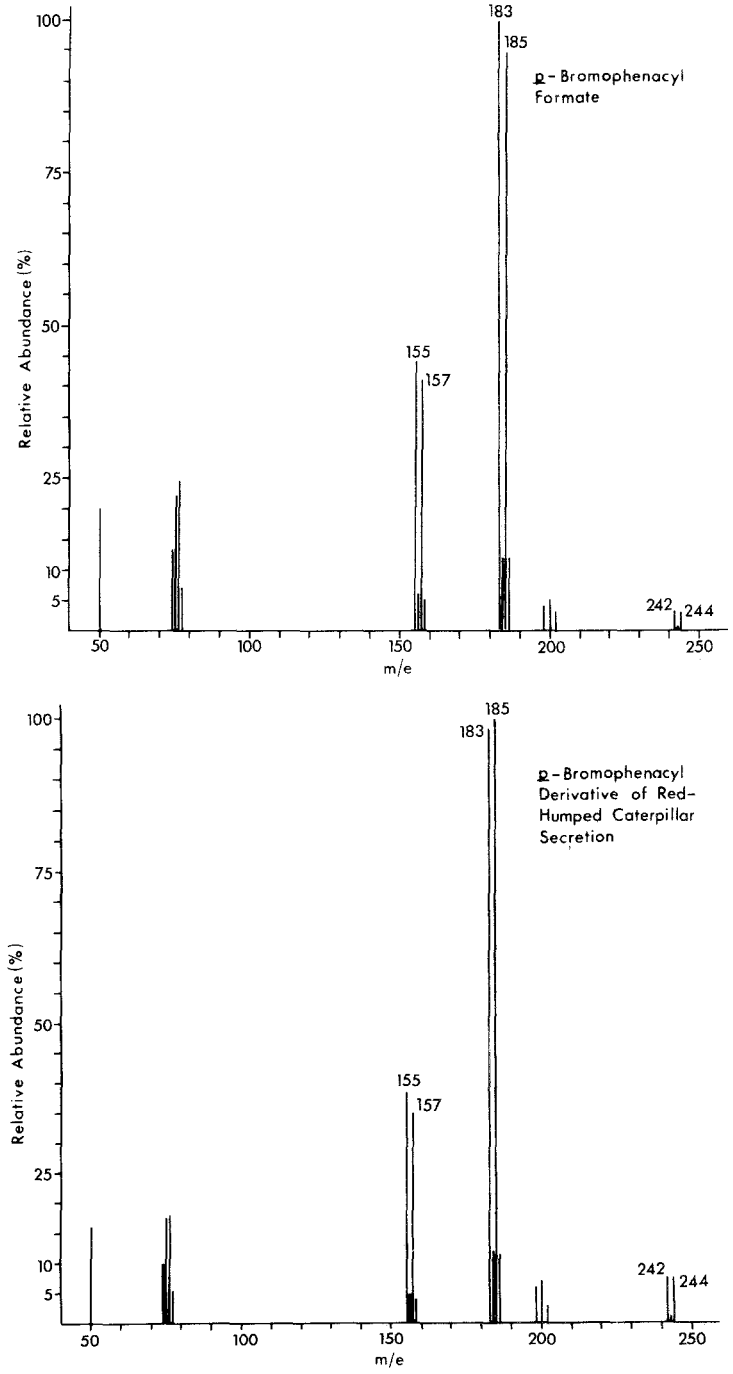

FIG. 4. Mass spectra of *p*-bromophenacyl formate: (A, above) authentic sample; (B, below) insect derived material.

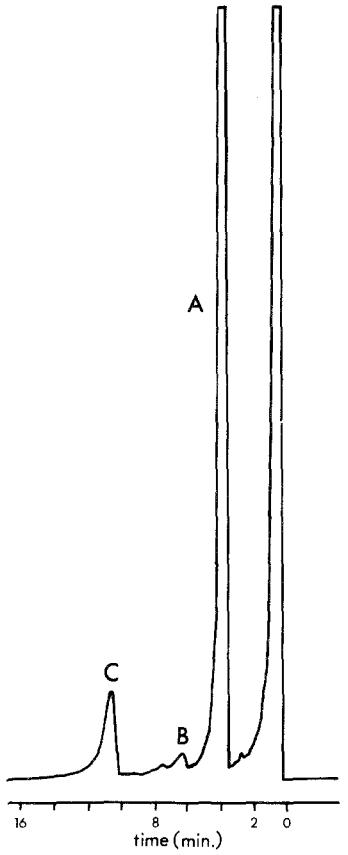

FIG. 5. Gas chromatogram of methylene chloride extract of dissected glands on 5% OV-1 at 130°C.

breaking the ejected fluid into a fine spray. This is undoubtedly aided by the muscles in this region which are positioned dorsolaterally. When the insect is disturbed, they contract and open the orifice. Some of the muscles run posterior to the gland, and these, along with the tracheal air supply, could force the secretion from one gland to the other and through the orifice.

Chemistry and Function of the Secretion. This is the first report of alkanol acetates in the prothoracic defensive secretion of lepidopteran larvae, although 2-tridecanone has been shown to be a minor component in the secretion from *H. manteo* (Eisner et al., 1972). Decyl acetate, dodecyl acetate, and a trace of 2-tridecanone are present in the Dufour's gland secretion of several species of slave-making ants (Regnier and Wilson, 1971). In the ants, however, the acetates are not present in the same gland as the formic acid, the latter being produced by the poison gland. To date the larvae of nine species

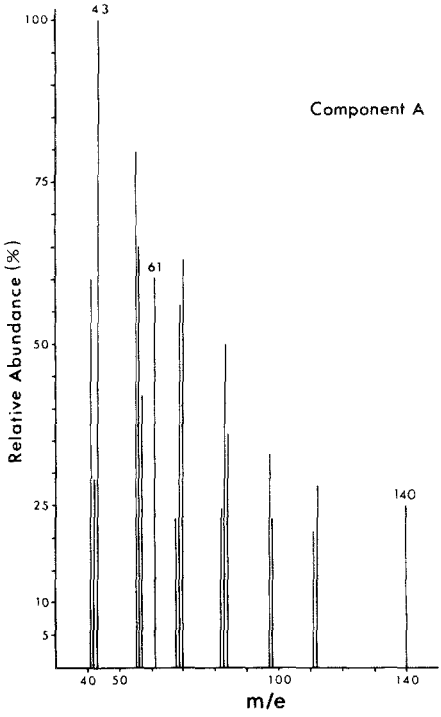

FIG. 6. Mass spectrum of insect component A.

of Notodontidae are known to produce formic acid in prothoracic glands (Pavan and Valcurone-Dazzini, 1976). A reinvestigation of these species might reveal secondary components although Eisner et al., (1961) reported that *S. leptinoides* secreted 20% aqueous formic acid with "no additives." In the case of *H. manteo*, Eisner et al. (1972) have shown that, in addition to formic acid, the defensive secretion of this species contains two minor neutral components accounting for 1.4% of the secretion. These proved to be 2-undecanone and 2-tridecanone present in the ratio 3:1. Entire glands were used in the analysis, and therefore it is not known whether the compounds were located in both the anterior and posterior parts of the gland.

The very small amount of decyl acetate shown to be present in the posterior gland of *S. concinna* is most probably the result of contamination from the anterior gland. The glands were removed from the larvae by the duct leading to the orifice; hence, some of the solution could have run, or been forced into, the posterior gland. Both the anterior and posterior glands were shown to contain formic acid. Formic acid was identified unambiguously as its *p*-bromophenacyl derivative. There are fifteen small carboxylic acids which

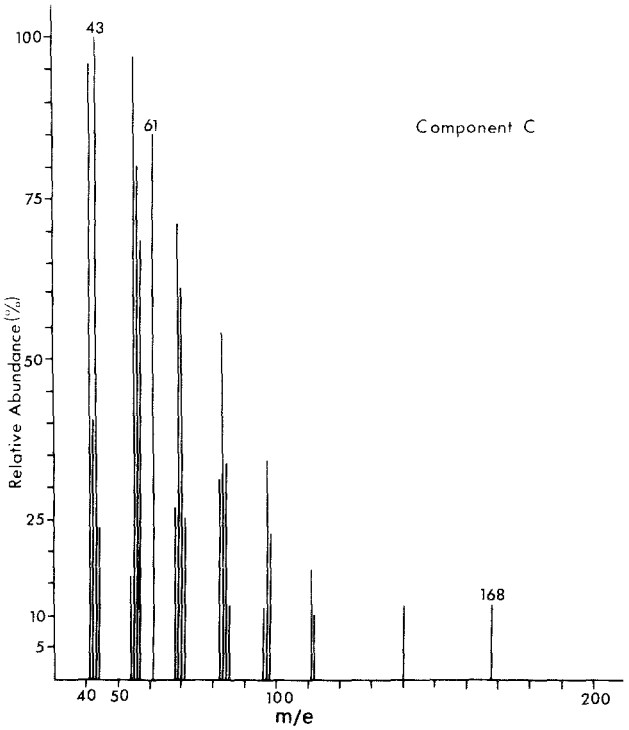

FIG. 7. Mass spectrum of insect component C.

TABLE 1. GLC DATA FOR THE ANTERIOR GLAND SECRETION OF THE RED-HUMPED CATERPILLAR *Schizura concinna*

Compound	Retention time (sec) on		
	3% OV-25	10% Carbowax 20 M	5% OV-1'
Decyl acetate	97	153	232
Dodecyl acetate	242	304	655
2-Tridecanone	164	235	399
Insect component			
A	97	153	232
B	167	236	662
C	248	302	396

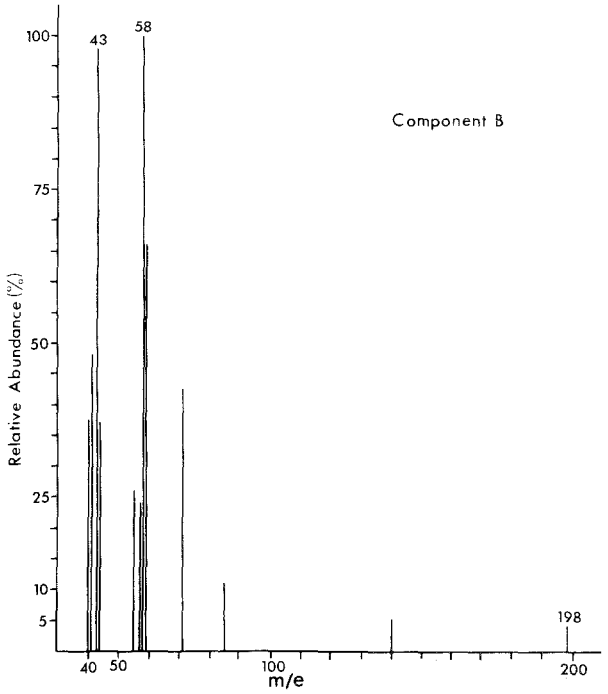

FIG. 8. Mass spectrum of insect component B.

have been found in arthropod defensive secretions. Often their identification has been tenuous, but the use of the *p*-bromophenacyl ester has led to a method for the definitive identification of these small acids (Weatherston et al., 1978).

S. concinna, in common with the other notodontids, can aim its spray accurately and make several ejections. There is general agreement that the function of the formic acid is defensive in nature. The function of the ancillary components in the *S. concinna* secretion is probably similar to that of nonyl acetate in the carabids *H. ferrugineus* and *H. latitarsus* (Eisner et al., 1968) and the acyclic ketones in the notodontid larva *H. manteo* (Eisner et al., 1972). In these instances, it has been shown that the nonyl acetate and ketones act as spreading agents and aid in the penetration of the hydrophilic formic acid through the cuticle. These authors suggest that in notodontid larvae, the ketones also have a defensive function, since by themselves they caused a defense reaction in other insects tested. The ancillary compounds in *S. concinna*, which comprise approximately 3.5% of the total secretion, may also have this alternate function.

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MACROCYCLIC POLYETHERS AS PROBES INTO PHEROMONE RECEPTOR MECHANISMS OF A SCIARID FLY, *Lycoriella mali* FITCH

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Abstract—A series of experiments was undertaken in an effort to understand the possible role of chemical shape and, in particular, the steroid shape in the sex pheromone system of the sciarid fly, *Lycoriella mali* Fitch. A broad spectrum of compounds including *n*-alkanes and macrocyclic polyethers (crown ethers) were tested on sciarid males which were significantly attracted to several test compounds, including *n*-heptadecane (the natural pheromone), cycloheptadecane, and a newly synthesized 17-crown-5 isomer which apparently can adopt the steroid shape. The apparent relationship of shape to activity suggests that the steroid template may indeed be of some consequence in insect olfaction.

Key Words—Diptera, Sciaridae, *Lycoriella mali*, pheromone reception, *n*-alkenes, macrocyclic polyethers, *n*-heptadecane, cycloheptadecane.

INTRODUCTION

Mechanisms by which odors are detected and recognized at the molecular level are not well understood. A number of theories have been proposed which describe olfaction in terms of chemical "receptor sites" (see Roderick, 1966; Moncrieff, 1967; Davies, 1971, for extensive reviews). In this regard Moncrieff (1967, 1970) and Amoore et al. (1952, 1964, 1970) have suggested that odors are related to and can be classified according to molecular shape. The shape of odor molecules which elicit behavioral and/or electrophysiolog-

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ical responses appears to be very important in insect olfaction where the effect of various chemical structures on a given biological response has been most frequently studied because of their versatility and the accessibility of large numbers of insects for replicate analyses (Blum, 1966; Moser, 1968; Silverstein, 1968; Vick, 1969; Vité, 1969, 1974). Many investigators have focused primarily on the activity of various "pheromone-like" chemicals on excised insect antennae by the electroantennagram technique (Priesner, 1969; Schneider, 1977) and by single-cell recording (Kafka, 1974; Schneider, 1977). Recently, numerous studies have shown that insects can discriminate between enantiomeric compounds indicating that the subtle shape differences associated with chirality may be important in insect olfaction (Chapman et al., 1978, Kafka, 1973; Iwaki, 1974; Lensky, 1974; Riley, 1974; Vité et al., 1976). Notwithstanding numerous elegant experiments, the effect of chemical shape(s) on the behavior of whole insects remains to be elucidated.

We report here a series of experiments which were undertaken to elucidate the possible role of chemical shape in the sex pheromone system of the mushroom-infesting sciarid fly, *Lycoriella mali* Fitch.

Heptadecane, the major sex attractant identified in female sciarid flies (Kostelc, 1977), when appropriately folded, outlines the shape of an unsubstituted steroid (II), i.e., adopts the perhydrocyclopentanophenanthrene shape (see Figure 1). Other sex pheromones identified in our laboratory (tetradecenyl acetates, ambrettolides) in the appropriate conformation can assume the steroid shape. Numerous authors (Kerschbaum, 1927; Ruzicka et al., 1930, Luttringhaus, 1937; Prelog et al., 1944, Theimer, 1967) have stressed the importance of the steroid structure in the activity of musks, the largest class of odors known to man. However, to our knowledge, the question of whether or not compounds having the ability to fit a steroid shape are of con-

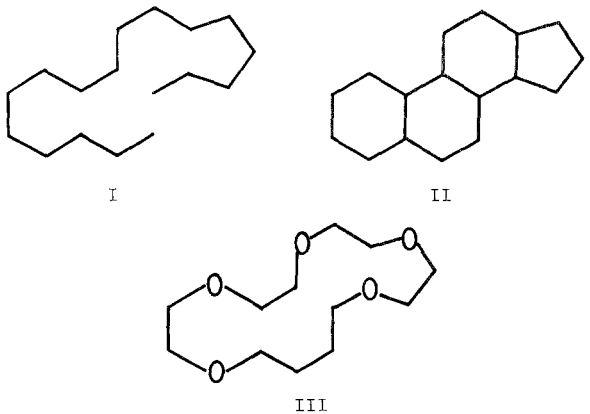

FIG. 1. Possible structural relationships in insect olfaction. Heptadecane (I), a sex attractant of the sciarid fly; steroid skeleton (II); 17-crown-5 (the 2,2,2,2,4 isomer, (III).

sequence in insect olfaction as well as whether a steroid template defines the shape of some chemoreceptor sites has not hitherto been experimentally addressed.

We have tested the steroid hypothesis by examining the response of sciarid males to a broad spectrum of structural classes of organic molecules including the natural pheromone and several macrocyclic polyethers (crown ethers) (Gokel, et al., 1976). The crowns seemed particularly suitable for examination because (1) they are unknown in nature; (2) they are substantially different in polarity and functionality from the natural pheromone; and (3) they have the potential for assuming the steroid shape. The latter was judged in three-dimensional terms by comparing CPK space-filling models of 17-crown-5 (III) and the steroid skeleton.

METHODS AND MATERIALS

Insect Rearing. A culture of sciarid flies, *L. mali*, was initially collected on a mushroom farm near Kennett Square, Pennsylvania, in 1970. A stock culture of sciarids was subsequently maintained from these field-collected flies on a natural medium of mushroom spawn and compost. Adult sciarid males, 1–3 days old, were isolated from stock cultures and kept over water and spawn for 24 hr prior to testing.

Biological Assays. Biological assays were conducted in a Plexiglas® chamber (8.3 cm^3), a modification of an apparatus used by Browne et al. (1969) and described in detail by Girard et al. (1974) and Kostelc (1977). Prior to each test, the bioassay chamber was rinsed with pesticide-grade hexane and spectrograde methanol and dried with Kimwipes®. Ten test males were used in each bioassay; each male was tested only once. Bioassays were conducted at room temperature (24° ± 3°); overhead cool white fluorescent and Gro-lux® lamps provided the lighting. During assays, compressed air (Air Products, Inc., Allentown, Pennsylvania), which passed through a glass tube (300 × 28 mm) filled with Drierite®, flowed through all compartments of the chamber at 200 cc/min. The number of males trapped in each compartment was recorded after a 20-min period. The positions of the samples in the bioassay apparatus as well as the position of the apparatus in the testing area were randomized between each assay.

The percentage of males attracted to the sample compartment for each chemical was subjected to a single-factor analysis of variance and mean separation was executed using Duncan's modified (Bayesian) least-significant-difference test.

Analytical Techniques. Standard test compounds were subjected to gas chromotography (GC) and/or gas chromatography-mass spectrometry (GC-MS) using polar and nonpolar columns in order to determine the purity of each standard. Gas chromatography was carried out on a Finnigan 9500

flame-ionization gas chromatograph, and GC-MS analyses were performed using a Finnigan 3200 gas chromatograph-mass spectrometer with an interactive 6000 data system. Glass U-tube columns (1.8 m × 2 mm ID) were packed with 3% OV-1 on 100/120 mesh Gas Chrom Q (Applied Science Laboratory, Inc., State College, Pennsylvania) or 10% diethyleneglycol succinate on 100/120 mesh Chromsorb WAW (Supelco, Inc., Bellefonte, Pennsylvania). Various temperatures were employed and the flow rate of helium as the carrier gas was 40 cc/min in most cases. For GC-MS, electron impact spectra were recorded from 30-450 atomic mass units (amu) at 69 eV using helium as the carrier gas.

Standard Compounds. Standard test chemicals were obtained from the following sources and the purity of each chemical as determined by GC and/or GC-MS is indicated: *n*-hexadecane (99%), *n*-heptadecane (99%), *n*-octadecane (99%) and palmitic acid (96%) (Aldrich Chemical Co., Inc.); *n*-pentadecane (99%) (Humphrey Chemical Co.); 1-heptadecene (96%) (I.C.N. Pharmaceuticals, Inc.); cycloheptadecanol (97%) (Farchan Division, Story Chemical Co.); 2-methylheptadecane (99%) (Chemical Samples Co.).

Cycloheptadecane (95%) was prepared from cycloheptadecanone by the Huang-Minlon modification of the Wolff-Kishner reduction (Huang-Minlon, 1946). The product had chemical and spectral characteristics similar to those reported by Schröder et al. (1973), for this substance. IR (CCl_4): 2935-2860, 1460 cm^{-1}; MS: *m/e* 210 ($M^+ - 28$); NMR (CCl_4): 8.67τ (s); MP: 64-68°C.

Tetradecyl acetate (99%) was synthesized by the acetylation of tetradecanol.

18-Crown-6 (99%) was prepared as described by Gokel et al. (1974). Monoaza-18-Crown-6 (99%) was prepared as described by Gokel et al. (1977). 17-Crown-5 (2,2,2,2,4) (99%) was prepared from the dianion of tetraethylene glycol (NaH/DMF) and the ditosylate of 1,4-butanediol. IR (neat): 2900, 1450, 1350, 1125 cm^{-1}; NMR ($CDCl_3$, ppm δ): 1.68 (M,4H), 3.67 (m, 21H); Anal. calc'd for $C_{12}H_{24}O_5$: C, 58.06; H, 9.67; Found: C, 58.06, 57.97; H, 9.66, 9.68.

1-Chlorohexadecane (97%) was obtained from the Aldrich Chemical Company and purified by preparative gas-liquid partition chromatography (1/4 in. × 5 ft 10% SE-30 on Chromosorb P, NAW).

RESULTS AND DISCUSSION

An examination of the results of the biological assays (Table 1) indicates that male sciarid flies were statistically attracted above control to several compounds which could possibly fit all or part of the steroid template. The two most active compounds, aside from the natural pheromone (heptadecane),

TABLE 1. SCIARID MALE ATTRACTION IN A LABORATORY BIOASSAY TO VARIOUS CHEMICALS

Compounds	Percent male attraction (%)[a]
1. *n*-Heptadecane[b]	62.5a
2. 17-Crown-5 (2,2,2,2,4)	48.2b
3. Cycloheptadecane	45.0b
4. *n*-Octadecane[c]	42.0b,c
5. Monoaza-18-crown-6	39.1b-d
6. 2-Methylheptadecane[d]	38.7b-d
7. *n*-Hexadecane[d]	38.0b-d
8. 18-Crown-6	30.0c-e
9. Palmitic acid	30.0c-e
10. 1-Heptadecene[d]	26.4d-f
11. 1-Chlorohexadecane	23.6e-g
12. Cycloheptadecanol	17.0e-h
13. *n*-Pentacosane	16.0f-h
14. 1-Tetradecyl acetate	11.0h
15. Control	15.0g,h

[a]Percent male attraction are means of the number of males attracted to the test compounds based on ten males per replication. At least 10 replications were performed with each test compound. Means followed by the same letter are not significantly different at $P < 0.05$.
[b]A major sciarid fly sex attractant.
[c]Octadecane was tested at the 0.1 μg level; all other test compounds were used at the 1.0 μg level.
[d]Pheromones identified in other insect systems.

were 17-crown-5 (III) and cycloheptadecane. The relatively strong attraction of sciarid males to the nonnaturally occurring 17-crown-5, compared to heptadecane, may seem surprising since the two substances are not obviously related chemically (open chain vs. ring; saturated hydrocarbon vs. polyheteroatom system; 53 atoms vs. 41 atoms); it seems likely, however, that both compounds can fit the steroid template. The response of sciarid males to cycloheptadecane was significantly higher than to controls, although statistically lower than to the natural pheromone, heptadecane. The slightly lower response of sciarid males to cycloheptadecane may be due to steric demands which are somewhat different in the cyclic molecule than in its open-chained counterpart.

Obviously, the cyclic hydrocarbons and cyclic polyethers will have volumes and preferred conformations different from the model system, perhydrocyclopentanophenanthrene (bisdesmethylandrostane, II). Nevertheless, Corey-Pauling-Koltun (CPK) models of compounds I, II, and III all have a remarkably similar appearance. According to models, each of these molecules can adopt an approximately flat and oval shape. A molecule of possible interest in this study, androstane, has a distinctly bulkier shape. [Bisdesmethylandrostane (II) is clearly the preferred model compound for this study,

but this molecule is not, to the best of our knowledge, a known compound, and it appears that it will be available to us only by total synthesis.] The possibility that heptadecane, 17-crown-5, and cycloheptadecane, as well as the other test compounds, are assuming an approximately three-dimensional steroid shape and thereby interacting, to varying degrees, with the chemoreceptor sites of sciarid males seems an attractive explanation for the observed biological activity.

In conclusion, we make no claims from this preliminary study about the uniqueness of either this mechanism or model, nor do we suggest that all sex pheromones are likely to fit the steroid nucleus (many do not). Nevertheless, if shape, and in particular discrete components of the steroid nucleus, are important in olfaction, intriguing questions arise concerning the molecular and evolutionary basis for chemoreception.

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THE MAJOR COMPONENT OF THE TRAIL PHEROMONE OF THE LEAF-CUTTING ANT, *Atta sexdens rubropilosa* FOREL

3-Ethyl-2,5-Dimethylpyrazine

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Abstract—The major component of the trail pheromone of the South American leaf-cutting ant, *Atta sexdens rubropilosa* Forel, is 3-ethyl-2,5-dimethylpyrazine (II). Methyl and ethyl phenylacetate and methyl 4-methylpyrrole-2-carboxylate (I), which is the major component of the trail pheromone of *A. texana* (Buckley) and *A. cephalotes* (L.), were also identified and may be minor components. The pheromone is stored in the poison gland. *Atta sexdens sexdens* (L.) also responds strongly to the pyrazine, which in large amounts evokes a weak response from *A. texana*, *A. cephalotes*, and *Acromyrmex octospinosus* (Reich). Foraging workers of *Atta sexdens rubropilosa* did not preferentially pick up baits impregnated with the pyrazine. The pyrazine was puffed into the nests of *A. cephalotes*, and a particular response called "milling" was noted.

Key Words—Trail pheromone, 3-ethyl-2,5-dimethylpyrazine, leaf-cutting ants, *Atta sexdens*, *Atta cephalotes*, foraging, bait pickup.

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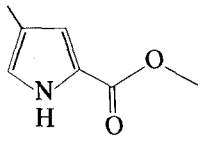
INTRODUCTION

The leaf-cutting ants, typified by the genera *Atta* and *Acromyrmex*, forage along trails defined at least in part by trail pheromones. The trails lead to sources of vegetation that the ants cut and use to cultivate the fungus they eat. In 1964, Blum et al. identified the poison gland as the source of the trail pheromone in some members of the tribe Attini, and later a highly active component, methyl 4-methylpyrrole-2-carboxylate (I) was identified from crushed abdomens of *Atta texana* (Buckley) (Tumlinson et al., 1971) and synthesized (Sonnet, 1972). The same component is also utilized by *A. cephalotes* (L.) (Riley et al., 1974). Robinson et al. (1974) found that *Acromyrmex octospinosus* (Reich)[7] would also follow a trail of I, but *Atta sexdens* (L.) would not. "Lost" workers of *A. sexdens* (ants that had fallen off the nest table and were thus highly motivated to follow a trail) followed trails made from the crushed poison glands of *A. cephalotes* and *A. texana* but not of *Acromyrmex*



I

octospinosus. Given a choice of two such trails, *Atta sexdens*, *A. cephalotes*, and *Acromyrmex octospinosus* generally preferred their own, although *Atta cephalotes* did not discriminate between its trail pheromone and that of *A. sexdens* (Robinson et al., 1974). In the field, ants usually follow trails of their own species, even when those of another species intersect them. For example, workers of *Acromyrmex octospinosus* placed on an *Atta cephalotes* trail invariably left the trail and resumed trail following only after crossing an *Acromyrmex octospinosus* trail (Blum et al., 1964). These results indicate that although similarities exist in the poison sac contents and trails of several species (e.g., the pyrrole), the ants can normally discriminate between trails. To gain a clearer understanding of the similarities and differences between attine trail pheromones, we chose to study the pheromone of *Atta sexdens rubropilosa* Forel.

Atta sexdens is the third most widely distributed attine species (Cherrett and Peregrine, 1976) and is often an agricultural pest. Control is best achieved by a slow-acting pesticide formulated in a bait that foraging ants will pick up and carry into the nest; the ants' activities eventually spread the pesticide throughout the colony.

Cherrett and Lewis (1974) have given a brief history of the development of toxic baits to control leaf-cutting ants. Practical baiting schemes are in op-

[7]Pyrrole I has since been identified in this species (J.H. Cross, R.M. Silverstein, and J.M. Cherrett, unpublished).

eration (Robinson and Aranda, 1975), and Lewis (1973) has shown that blanket spreading of toxic baits is the most effective way to control certain attine species. However, the wisdom of large scale baiting has been questioned (Anon., 1970; Edwards, 1970; Ehrlich and Ehrlich, 1970), since blanket spreading of insecticides can lead to undesirable side effects. Any improvements in the bait that increased bait pickup would make possible the use of smaller quantities of insecticide, thus reducing the amount spread into the environment, the danger to wildlife, and the cost.

Robinson and Cherrett (1973) found that, in the laboratory, citrus pulp baits and sugar impregnated filter paper disks containing the synthetic pyrrole were more readily picked up by *A. sexdens rubropilosa* than controls without the pyrrole, but later field tests were disappointing (unpublished). We reasoned that the authentic *A. sexdens rubropilosa* trail pheromone components might enhance bait pickup by this species.

METHODS AND MATERIALS

The *A. sexdens rubropilosa* ants were obtained from two locations, Rio de Janeiro, Brazil, and Asunción, Paraguay. Although morphological differences were noted between the two populations, Dr. C.F. Gonçalves, the taxonomist who last revised the genus *Acromyrmex*, considered them to be within the same subspecies (Gonçalves, personal communication to S.W.R.). The *A. sexdens sexdens*, *A. cephalotes*, *Acromyrmex octospinosus*, and *A. subterraneus* ants were from colonies maintained in the Bangor laboratory. The *Atta texana* ants were from laboratory colonies of Dr. John C. Moser, USDA-FS, Pineville, Louisiana.

The abdomens were removed from workers of *A. sexdens rubropilosa* and were macerated in methylene chloride (CH_2Cl_2). The supernatant was concentrated and sealed in glass ampoules, and these or the abdomens preserved in CH_2Cl_2 were sent to the Syracuse laboratory. The heads and thoraces of the Brazilian ants were analyzed for the alarm pheromones, which are produced in the mandibular glands (de Oliveira, 1975).

The remaining CH_2Cl_2 was removed in a short-path still and the viscous extract was distilled onto a dry-ice-cooled cold-finger condenser for 3 hr at 70°/0.5 mm Hg. The distillate was washed from the condenser with hexane, and this solution was fractionated on the following GLC columns: column A, 5% SE-30 on Chromosorb G 60/80 mesh, 2.5 m × 4 mm (ID) glass, 50 ml/min He flow rate at 75°C initial temperature for 6 min then programed at 4°/min to 250°C (ethyl phenylacetate retention time = 28 min); column B, 5% Carbowax 20 M on Chromosorb G 60/80 mesh, 5 m × 4 mm (ID) glass, 50 ml/min He flow rate at 170° isothermal (ethyl phenylacetate retention time = 36 min); column C, 5% diethylene glycol succinate (DEGS) on Chromosorb G 60/80 mesh, 5 m × 4 mm (ID) glass, 70 ml/min He flow at 150°C isothermal (methyl phenylacetate retention time = 47 min); column D, 5% Apiezon L on

Chromosorb G 60/80 mesh, 5 m × 4 mm (ID) glass, 50 ml/min He flow at 165°C isothermal (retention time of II = 14.5 min). All glass columns and solid supports were acid washed and treated with dichlorodimethylsilane. Fractions from the Varian model 204B gas chromatograph were collected in glass capillary tubes (30 cm × 2 mm OD) in a thermal gradient collector (Brownlee and Silverstein, 1968).

Nuclear magnetic resonance spectra were obtained ($CDCl_3$, TMS) on a Varian XL 100 (Fourier transform) spectrometer. Infrared spectra were obtained from samples dissolved in spectrograde carbon tetrachloride or carbon disulfide on a Perkin Elmer model 621 double-beam, grating spectrometer equipped with beam condensers and Barnes Engineering 4-μl cavity cells. Mass spectra were obtained on an Hitachi RMU-6 electron impact (70 eV) mass spectrometer, a modification of which allowed introduction of the glass capillaries used with the gas chromatograph.

Commercial methyl and ethyl phenylacetate were washed with $NaHCO_3$ to remove phenylacetic acid and distilled under vacuum. Methyl 4-methylpyrrole-2-carboxylate(I), synthesized by Sonnet (1972), had a melting point of 70–71°C and gave a single peak when chromatographed on column A. A 1:1 mixture of 3-ethyl-2,5-dimethylpyrazine(II) and 2-ethyl-3,5-dimethylpyrazine(III) was a gift of Pyrazine Specialties, Atlanta, Georgia, and was used as received, or the isomers were separated preparatively on column D.

The isolation of the pheromone components was monitored by bioassays based on the ability of the ants to follow a circular trail. Partially purified fractions were diluted with hexane to concentrations of 10, 1, and 0.1 mg whole ant equivalents/μl, and 10, 15, or 20 μl of each concentration was streaked with a microsyringe around a circle of 40–50 cm circumference penciled on white paper. Hexane controls were employed. The paper was then introduced to the ants and their responses were noted (Riley et al., 1974; Moser and Blum, 1963); trail-following behavior was distinct from alarm or food-investigating behavior. Some variations in bioassay practice occurred between laboratories, especially in respect to the origin and numbers of the ants and the manner in which they were introduced to the trail. Strongly active fractions and pyrazine II always elicited a marked effect that was reproducible when tests were repeated by more than one laboratory.

RESULTS

Isolation and Identification of the Pheromone Components

Isolation was started on a crude extract obtained from 4.2 kg (≃280,000) of whole ants from the Brazilian population. Bioassays of this extract and the short-path distillate (one unknown concentration of each) gave results recorded as "positive." A bioassay of one concentration of the distillation resi-

due gave a result recorded as "weakly positive"; considering our later results, we cannot state with certainty whether a nonvolatile component remained in the distillation flask (see Discussion). A single concentration of the distilled CH_2Cl_2 had no activity, and the wash of a liquid-nitrogen-cooled trap from the short-path distillation was inactive in all three standard concentrations (10, 1, and 0.1 mg whole ant equivalents/μl); it seems unlikely that an important component of the pheromone was highly volatile.

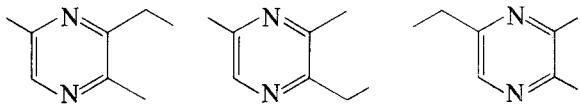
An aliquot of the short-path distillate was collected from column A as a single fraction, which was strongly active in all three standard concentrations. Thus, the pheromone was not thermally labile. The remainder of the short-path distillate was collected in five fractions. The first and fifth fractions were inactive in all three concentrations. The second and fourth displayed weak activity in the two higher concentrations but were inactive in the lowest. The third fraction was "strongly positive" in all three concentrations and was "still weakly positive" after dilution to 0.0001 mg whole ant equivalents/μl. This fraction was then collected in 10 fractions (3-1 through 3-10) from column B. Bioassays (two replicates, but at the same concentration) showed that fractions 3-3 and 3-7 were the most active. Fractions 3-6 and 3-8 also gave good responses; fractions 3-4, 3-5, and 3-9 were weakly active and were not examined further. Fraction 3-3 was fractionated into ten parts (3-3-1 through 3-3-10) on column C. Fraction 3-3-5 was the most active, but an insufficient amount was available for identification. Further fractionation (column C) of fraction 3-6 yielded three compounds, none of which was present in sufficient quantity to identify. Fractions 3-7 and 3-8 were chromatographed on column C; both produced single peaks, which were identified as methyl and ethyl phenylacetate by their NMR and mass spectra and by comparison with authentic samples. The pyrrole I was eluted from column B in fraction 3-10 (retention time = 81 min). The mass spectrum was congruent with that of the synthetic pyrrole. We estimated that 75 μg of methyl phenylacetate (MPA), 150 μg of ethyl phenylacetate (EPA), and 19 μg of pyrrole I were present in the 4.2 kg of whole ants. The synthetic compounds elicited weak to medium trail following from the Brazilian population but only investigatory behavior from the Paraguayan population.

Work was then continued on a second crude extract (4.8 kg of whole ants) of the Brazilian population. Fraction 3 from column A was again considerably more active at all three concentrations than the second and fourth fractions, and it was divided on column B, as reproducibly as possible, into the 10 fractions collected earlier. These fractions (three concentrations) were sent for bioassay against both the Brazilian and Paraguayan populations; both populations responded strongly to a single fraction, 3-2, which had been inactive in the earlier bioassays. The Brazilian population did show a "slight interest" in the fraction that should have contained methyl and ethyl phenylacetate; fractions 3-7 and 3-8 were collected together, because fraction 3-7 (MPA) was

much smaller than fraction 3-8 (EPA) and otherwise much of it would have been lost. Fractions 3-3 and 3-6 were completely inactive.

When refractionated on column D, fraction 3-2 proved to consist mostly of one compound, labeled fraction 3-2-2. The smaller peaks eluting before and after were collected and labeled fractions 3-2-1 and 3-2-3. All three fractions elicited strong trail-following from both the Brazilian and Paraguayan populations, although 3-2-1 and 3-2-3 were tested at much higher concentrations (300 mg/ml vs. 10 mg/ml). Chromatography on a very polar cyanosilicone column, Apolar 10C, failed to split fraction 3-2-2 further. Fractions 3-2-2 from the first and second shipments were identical and were combined to obtain the spectroscopic data.

That fraction 3-2-2 was an isomer of ethyldimethylpyrazine was readily deduced from its NMR and mass spectra. The NMR spectrum (Figure 1, top) showed two methyl groups on an aromatic ring (2.48δ, s, 3H and 2.52δ, s, 3H), an ethyl group on an aromatic ring (2.8δ, q, 2H and 1.27δ, t, 3H), and a single aromatic proton at 8.1δ. The mass spectrum (Figure 1, bottom) agreed with those published for 3-ethyl-2,5-dimethylpyrazine(II) and 2-ethyl-3,5-dimethylpyrazine(III) (Stenhagen et al., 1969; Goldman et al., 1967; and Friedel et al., 1971).



II III IV

The substitution pattern of the aromatic ring was determined from the fingerprint region of the IR spectrum by comparison with authentic samples of II and III. The IR spectrum of authentic II agreed well with that of fraction 3-2-2 (Figure 2), while that of authentic III was noticeably different. The published absorptions of IV are much different (Gelas and Rambaud, 1968). Coinjection of fraction 3-2-2 and authentic II on columns B and D produced on each column a single peak (retention times: column B, 9.7 min and column D, 14.5 min), but coinjection with III gave two peaks on each column (retention times: column B, 9.7 min and 10.3 min; column D, 16.5 min and 17.3 min; II eluted first). Column A (SE-30, 135° isothermal) would not separate isomers II and III, although Friedel et al. (1971) have reported the separation of III and IV on SE-30 and Carbowax 20 M packed columns; IV eluted after III. We concluded that fraction 3-2-2 was II. The quantity of II in the original 4.2-kg sample of ants was about 100 μg.

We collected the compounds in fractions 3-2-1 and 3-2-3 individually from column D. Two were present in quantities estimated to be 10% of pyrazine II, and the other seven were estimated to be between 3 and 7%. Although

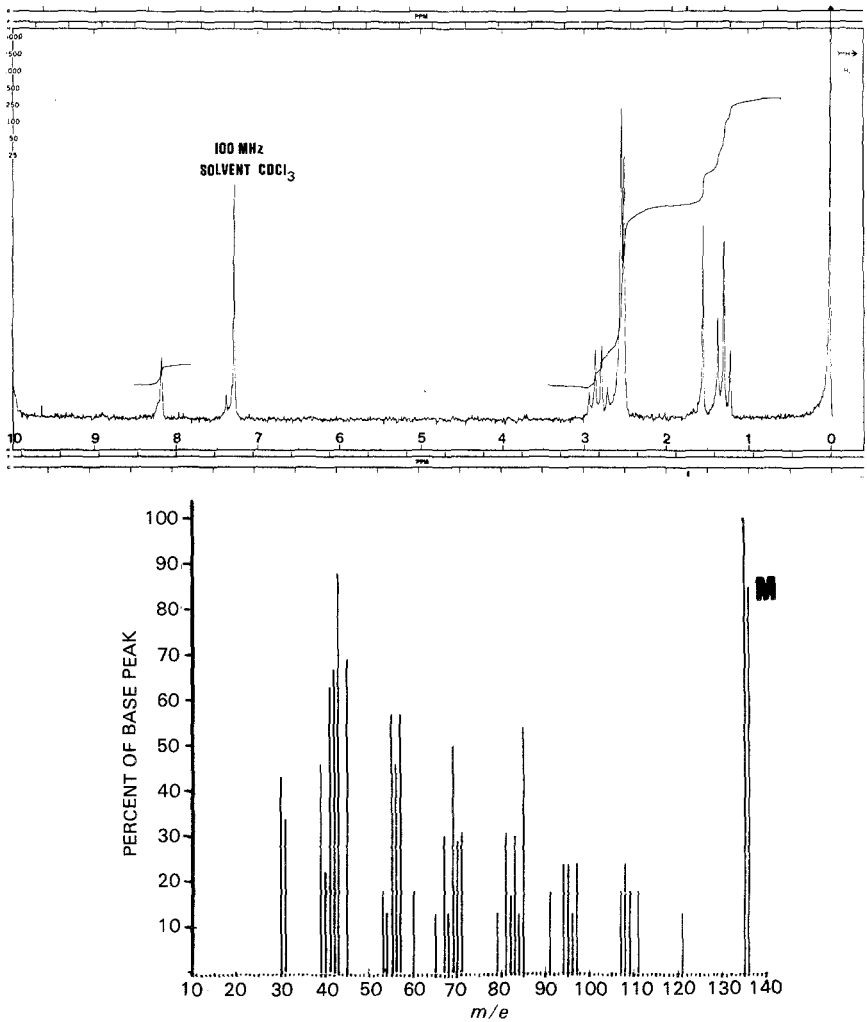


FIG. 1. NMR and mass spectra of fraction 3-2-2 (3-ethyl-2,5-dimethylpyrazine). H_2O peak at 1.56δ.

one appeared to be a methyl ester, none was identified. We were unable to confirm or refute the presence of other pyrazines.

Two samples of the Paraguayan population of *A. sexdens rubropilosa* were worked up as described and examined for the trail pheromone components. Pyrazine II and ethyl phenylacetate were found, but methyl phenylacetate and pyrrole I were not detected.

To test whether or not the main trail pheromone components were now isolated, we prepared from the Paraguayan population a bioassay set consist-

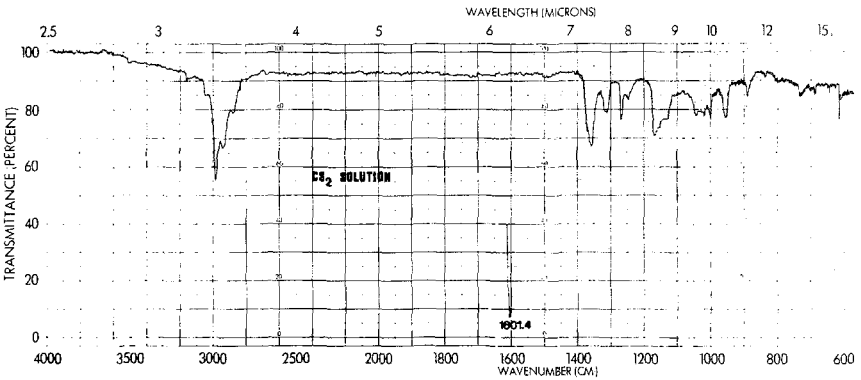


FIG. 2. Infrared spectrum of fraction 3-2-2 (3-ethyl-2,5-dimethylpyrazine).

ing of three samples: the cold-finger condensate, the pyrazine region from column D, and the total material eluted from column D minus the pyrazine region. Surprisingly the cold-finger condensate proved to be inactive. The pyrazine region from column D was moderately active at 10 mg/μl and weakly active at 1 mg/μl in both populations. The Paraguayan population also responded weakly at 0.1 mg/μl. The remainder of the effluent from column D elicited no response from the Paraguayan population and only a weak response at 10 mg/μl from the Brazilian population. These results indicated that no other compound had an activity equal to that of the pyrazine. The question of synergistic relationships was not resolved because of the inactivity of the cold-finger condensate (see Discussion).

Bioassays of the Synthetic Compounds

Synthetic pyrazines II and III were bioassayed separately, and II was obviously the more active (Tables 1 and 2, columns 6 and 7; Table 3, columns 2 and 3). Since pyrazine IV was neither found in the ant nor readily available, it was not tested. The commercially available 1:1 mixture of II and III is as active as II alone within limits of the bioassays; therefore III is not an inhibitor of the natural isomer (Tables 1 and 2, columns 6 and 8). MPA, EPA, and pyrrole I, singly or in combination, elicited weak and often variable responses. One solution (1:1:1), tested in Paraguay and Brazil, gave positive results (Tables 1 and 2, columns 5), but was much less active than the pyrazine. This solution and the pyrazine II did not interact synergistically when tested against the Paraguayan or the Brazilian populations (Tables 1 and 2, columns 5 and 6 or 8 vs. 9). Additive effects would probably not be detected with this bioassay. Other, independently prepared solutions of MPA, EPA, and pyrrole I were completely inactive in numerous bioassays conducted in Paraguay. Solutions prepared in the ratio found in the short-path distillate, I = 1, MPA = 4 and

TABLE 1. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS AGAINST THE BRAZILIAN FIELD POPULATION

Quantity[a,b] (ng)	Compounds tested[c]								
	MPA	EPA	I	MPA + EPA + I (1:1:1)	II	III	II + III (1:1)	MPA + EPA + I + (II + III) (1:1:1:1)	I + MPA + EPA (1:4:7.5)
10^4	0	0	0	0	0	0	0	W	0
10^3	0	0	0	0	0	0	M	M	0
10^2	0	I	0	W	W	0	S	S	0
10^1	I	I	0	M	S	0	M	S	I
10^0	0	I	0	M	S	0	I	M	0
10^{-1}	0	0	0	W	M	0	0	W	0
10^{-2}	0	0	0	0	W	0	0	I	0
10^{-3}	—	—	—	—	0	0	—	0	—
10^{-4}	—	—	—	—	—	—	—	0	—

[a]Total quantity of chemical used to make a 50-cm-circumference circle.
[b]In mixtures, the quantity is that of each compound.
[c]Fifteen ants/test. 0 = no response; I = investigation; W = weak following (a few ants); M = medium following (<50%); S = strong following (>50%).

TABLE 2. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS AGAINST THE PARAGUAYAN FIELD POPULATION

Quantity[a,b] (ng)	Compounds tested[c]							
	I	I + MPA (1:1)	I + MPA + EPA 1:4:7.5	I + MPA + EPA 1:1:1	II	III	II + III (1:1)	I + II + EPA + MPA (1:1:1:1)
0, control	0	0	0	0	0	0	0	0
10^5	0	—	R	—	—	—	—	—
10^4	0	W	R	W	0	0	W	0
10^3	0	W	0	M	M	0	M	M
10^2	0	W	W	M	S	0	S	S
10^1	0	W	0	M	S	0	S	S
10^0	0	W	0	0	S	0	W	S
10^{-1}	—	—	0	0	W	0	0	W
10^{-2}	—	—	—	0	0	0	0	0

[a] Total quantity used to make 50-cm-circumference circle.
[b] The quantity is that of each compound.
[c] Responses scored as in Table 1. R = repellent.

TABLE 3. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS AGAINST LABORATORY COLONIES OF THE PARAGUAYAN FIELD POPULATION

Quantity[a] (ng)	Compounds tested		
	II	III	I + II + MPA + EPA (1:1:1:1)
10^2	9/40[b]	5/60	—
10^0	34/40	10/40	37/60
10^{-2}	29/40	5/60	49/60

[a]Total quantity of each compound used to make a 40-cm-circumference circle.
[b]Number of ants following trail/number of ants tested.

EPA = 7.5, were less active in both populations than the 1:1:1 mixture (Table 1, column 10; Table 2, column 4). They did not elicit trail-following, but the largest quantities tested repelled the Paraguayan population.

Pretreatment of Ants

For the bioassays conducted in Paraguay, ants were normally taken from the field and kept for 2–8 hr with nothing but water before any experiments were run. The ants were then calm and relatively easy to handle during the experiments. If left for a longer period, they became quite clearly agitated and anxious to follow a trail. In these circumstances the ants seemed to be willing to follow a trail of anything that was not actually repellent or to follow trails of the trail pheromone component II at much lower concentrations than they normally would (Table 4).

Source of Pheromone

Poison sacs and Dufours glands from *A. sexdens rubropilosa* were dissected out, separated, placed on the tips of sharpened match sticks and drawn along the 40-cm circumference of a circle (1 gland/circle). Four replicates with 10 ants each were made with Dufour's glands and three replicates were made with poison sacs. No ant followed the Dufour's gland trail for 90°, but 73% of them followed the poison sac trail for at least 90°.

Responses of Other Species in the Laboratory Bioassay

The responses of several attine species and subspecies to the commercial pyrazine mixture (1:1 ratio of II and III) are shown in Table 5. Table 6 shows responses of *A. texana* to the *A. sexdens rubropilosa* compounds. In addition, *Acromyrmex landolti* collected and tested in Paraguay responded strongly to a 1:1:1:1 solution of MPA, EPA, I, and the commercial pyrazine mixture; we did not bioassay I and II separately for this species.

TABLE 4. EFFECT OF LENGTH OF PRETREATMENT ON RESPONSES OF *Atta sexdens rubropilosa*, PARAGUAYAN FIELD POPULATION

Quantity[a] (ng)	Compounds tested[b]					
	II + III (1:1)			I + MPA + EPA (1:1:1)	EPA	I
0, control	0	0	0	0	0	0
10^5	—	—	—	—	R	—
10^4	W	—	—	—	WR	M
10^3	W	—	0	—	S	—
10^2	M	0	0	W	S	S
10^1	M	W	W	M	M	—
10^0	W	S	S	S	M	S
10^{-1}	0	M	S	S	M	—
10^{-2}	0	S	S	M	W	S
10^{-3}	0	S	S	W	—	—
10^{-4}	0	S	M	—	—	—
Length of pretreatment (hr)	2[c]	36[c,d]	36[d]	20	30	30

[a] Total quantity of each component used to make a 50-cm-circumference circle.
[b] Responses scored as in Table 1.
[c] Same solutions.
[d] Same ants.

TABLE 5. EFFECT OF COMMERCIAL PYRAZINE MIXTURE (II AND III) ON TRAIL-FOLLOWING OF SEVERAL ATTINE SPECIES IN THE LABORATORY

Quantity of each compound	% of ants following through 90°[a]				
	Atta sexdens rubropilosa	*Atta sexdens sexdens*	*Atta cephalotes*	*Acromyrmex octospinosus*	*Acromyrmex subterraneus*
50 ng	100.0	95.0	93.3	93.3	100.0
0.5 ng	100.0	95.0	0	0	Not measured
5 pg	33.3	15.0	0	0	Not measured

[a] Six replicates of 10 ants at each point, except for the grass-cutter, *Acromyrmex subterraneus*—1 test of 10 ants.

TABLE 6. LABORATORY BIOASSAYS OF *Atta sexdens rubropilosa* COMPOUNDS ON *Atta texana*[a]

Component	Concentration (ng/μl)[b]		
	0.04	0.4	4.0
Methyl phenylacetate	0	0	0
Ethyl phenylacetate	0	0	0
3-Ethyl-2,5-dimethylpyrazine (II)	++	+	+R
2-Ethyl-3,5-dimethylpyrazine (III)	0	—	0
Methyl 4-methylpyrrole-2-carboxylate (I)[c]	++++	—	—

[a]Bioassays by John Moser, USDA-FS, Pineville, Louisiana. The modified "lost ant" procedure was described by P.E. Sonnet and J.C. Moser, 1973 (*Environ. Entomol.* 2:851-854).
[b]++++ = very strong; ++ = weak but definite; + = weak; 0 = no response; R = repellent; — = not tested.
[c]The major component of the trail pheromone of this species.

Response of Field Nests of Atta cephalotes to Pyrazine Vapor

When the vapor of pyrrole I was blown into nest entrances of *Atta cephalotes* just before they were to begin their daily period of foraging, it triggered a foraging response (Robinson and Cherrett, 1975). This test was repeated using pyrazine vapor. One mg of the commercial pyrazine mixture was placed on a small wad of cotton wool in a glass container, and air was passed over this and into an entrance of a nest of *A. cephalotes* for 15 sec. Behavior was assessed after a further 300 sec. Control experiments were performed by passing air into an entrance for 15 sec. The pyrazines caused the ants to leave the nest entrance holes and to mill around on the nest surface. The response was statistically significant (Table 7).

Bait Pickup

Soybean baits, to which soybean oil with and without the commercial pyrazine mixture had been added, were dyed for identification and the baits

TABLE 7. FIELD RESPONSES OF *Atta cephalotes* TO VAPOR OF II AND III BLOWN INTO THE NEST ENTRANCE[a]

Test	Number of nests "milling"	Number of nests not "milling"
II + III	22	17
Air (control)	4	32

[a]$\chi^2 = 15.02$ with 1 *df*, $P < 0.001$

TABLE 8. PICKUP OF PYRAZINE-IMPREGNATED BAIT BY *Atta sexdens rubropilosa* IN THE FIELD IN PARAGUAY

Concentration of mixture of II + III (ppm)	Number of trials	Total number of bait pieces carried into nest (%)		P^a
		Tests with II + III	Control	
1	11	146 (26)	416 (74)	<0.001
10^{-2}	7	113 (49)	117 (51)	—
10^{-4}	11	175 (50)	174 (50)	—

[a] χ^2 test with 1 *df*.

were scattered together in areas where *Atta sexdens* ants were foraging. Individual bait pieces were counted as they were carried into the nest (Table 8). The pyrazine was repellent at a high concentration and is not shown to enhance pickup at lower concentrations.

DISCUSSION

Pyrazine II consistently elicits trail following activity. The Brazilian and Paraguayan populations of *A. sexdens rubropilosa* and a laboratory colony of *A. sexdens sexdens* are capable of detecting very small quantities of it (Tables 1 through 5). No other component or mixture is as active. This species is also more sensitive to it than are several closely related species (Tables 5 and 6). These results are good evidence that II is a major component of the pheromone.

It should be emphasized that a considerable quantity of II was present in the first shipment of Brazilian ants, although the fraction that contained it was inactive in the bioassay. Six other fractions, including those with MPA and EPA, were active. An explanation that fits these results is that these samples were made with relatively large quantities of material. Tables 1, 2, and 3 show that in large quantities the pyrazine is inactive but the minor components are active. By this reasoning, the bioassay samples made from the second shipment were made with smaller amounts of material and only the pyrazine was active. In retrospect, it is apparent that trail substances should be tested over a wide range of concentrations.

The results in Table 4 show that the ants' responses could be influenced by the time for which they were held before bioassay. When the time was long, the ants responded strongly to compounds that wider experience showed to be less important. This factor, and perhaps other undiscovered ones, caused some variability in our results. For an example, one may compare two bioas-

says of the commercial pyrazine mixture (Table 2, column 8 with Table 4, column 2).

The inactivity of the cold-finger condensate resulted from the preparation of the samples, since it was inactive in two independent bioassays. It may have been due to the concentration phenomenon discussed above, although three concentrations were tested. Nevertheless, the pyrazine-containing fraction isolated from this condensate was active. This fraction and the total-minus-pyrazine fraction were collected from the same chromatographic injection; their relative activities were probably correct.

As is the case with the other attine species examined (Blum et al., 1964), the source of the trail marking pheromone is the poison sac. Blum (1974) has postulated that the trail pheromones of myrmicine ants were originally trace constituents of the venom that were exploited as the function of the gland changed. The poison sac contents are rich in amino acids, which probably can react enzymatically to form pyrazines (cf. Maga and Sizer, 1973). Pyrazines have been identified previously in ants. They function as alarm pheromones in certain species of ponerine ants (Wheeler and Blum, 1973) and have been identified in the heads of the Argentine ant, *Iridomyrmex humilis* Mayr (Dolichodinae) (Cavill and Houghton, 1974).

The role of MPA, EPA, pyrrole I, and the compounds in the other less active fractions is not clear. They are active only in relatively large quantities and usually elicit weak or medium responses. Until the identification of the pyrazine, which consistently elicits strong responses, we thought we might be dealing with two subspecies of *A. sexdens*. The compounds may be minor constituents of the trail pheromone. These eusocial ants rely heavily on pheromones to regulate their colonies and may be able to take advantage of the increased information available from multicomponent pheromones (Silverstein and Young, 1976). The observation (Blum et al., 1964) that *A. cephalotes* and *Acromyrmex octospinosus* avoided each other's trails suggests that these species use multicomponent pheromones, because for both species pyrrole I is a major component (Riley et al., 1974; J.H. Cross, R.M. Silverstein, and J.M. Cherrett, unpublished). The work of Robinson et al. (1974), described in the introduction, supports this hypothesis also.

On the other hand, these compounds may simply be recognized by the ants as being from their species; the parent acid of MPA and EPA, phenylacetic acid, has been identified in *Atta sexdens* (Schildknecht and Koob, 1971) and is apparently associated with anabiosis.

We conducted two behavioral studies with the pyrazine. *A. cephalotes* uses pyrrole I as the major component of its trail pheromone (Riley et al., 1974). Robinson and Cherrett (1975) showed that I also releases foraging activity in a nest whose workers are preparing to forage. Since *A. cephalotes* can detect the pyrazine (Table 5), we repeated the experiment with the commercial pyrazine mixture. The ants responded to it by leaving the nest entrance

holes and actively milling around on the nest surface. Foraging behavior, however, was not observed. We cannot reach a firm conclusion about this response, because the pyrazine has not been sought in this species.

In the second study, bait pickup with and without the pyrazine was measured. Robinson and Cherrett (1973) had found that *A. sexdens* in the laboratory would more readily pick up baits that contained pyrrole I. In the field, however, Robinson (unpublished) observed no difference. Since discrepancies between laboratory and field results are often due to the use of imperfect pheromone compositions, we repeated the bait pickup experiment after confirming the activities of pyrazine II and the commercially available mixture of II and III, which was used for the experiment. The results in Table 8 demonstrate that the pyrazines did not enhance bait pickup, and in fact, larger quantities caused decreased pickup. This may be related to the fact that the ants do not follow trails made with large quantities of II. It seems unlikely that the results would differ if the experiment were repeated with pure II.

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SEX PHEROMONE CROSS-ATTRACTION AMONG FOUR SPECIES OF PINE TIP MOTHS, *Rhyacionia* SPECIES[1,2]

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Abstract—Field tests utilizing crude pheromone extracts from *Rhyacionia frustrana*, *R. rigidana*, *R. subtropica*, and *R. buoliana* showed that *R. subtropica* and *R. buoliana* were mutually attractive. *R. frustrana* was attracted in low numbers to both *R. subtropica* and *R. buoliana*, but *R. subtropica* and *R. buoliana* did not significantly respond to *R. frustrana*. *R. rigidana* did not respond to pheromone extracts from any other *Rhyacionia* spp., nor was it attractive to the others. Cross-attraction occurred among those species which were largely allopatric and shared few tree hosts.

Key Words—*Rhyacionia* spp., tip moths, sex pheromones, cross attraction, pheromone specificity, Lepidoptera, Tortricidae.

INTRODUCTION

Rhyacionia spp. moths are pests of various *Pinus* spp. and some spp. may cause serious damage.

The Nantucket pine tip moth, *R. frustrana* (Comstock) is the most common species in the eastern U.S. and infests a variety of pines (Yates, 1960). In different parts of its range, *R. frustrana* is sympatric with and may share hosts with several other *Rhyacionia* spp. (Figure 1). *R. frustrana* overlaps with the pitch pine tip moth, *R. rigidana* (Fernald) over much of its range (Miller and Neiswander, 1959; Berisford, 1974a; Baer and Berisford, 1975; Powell and

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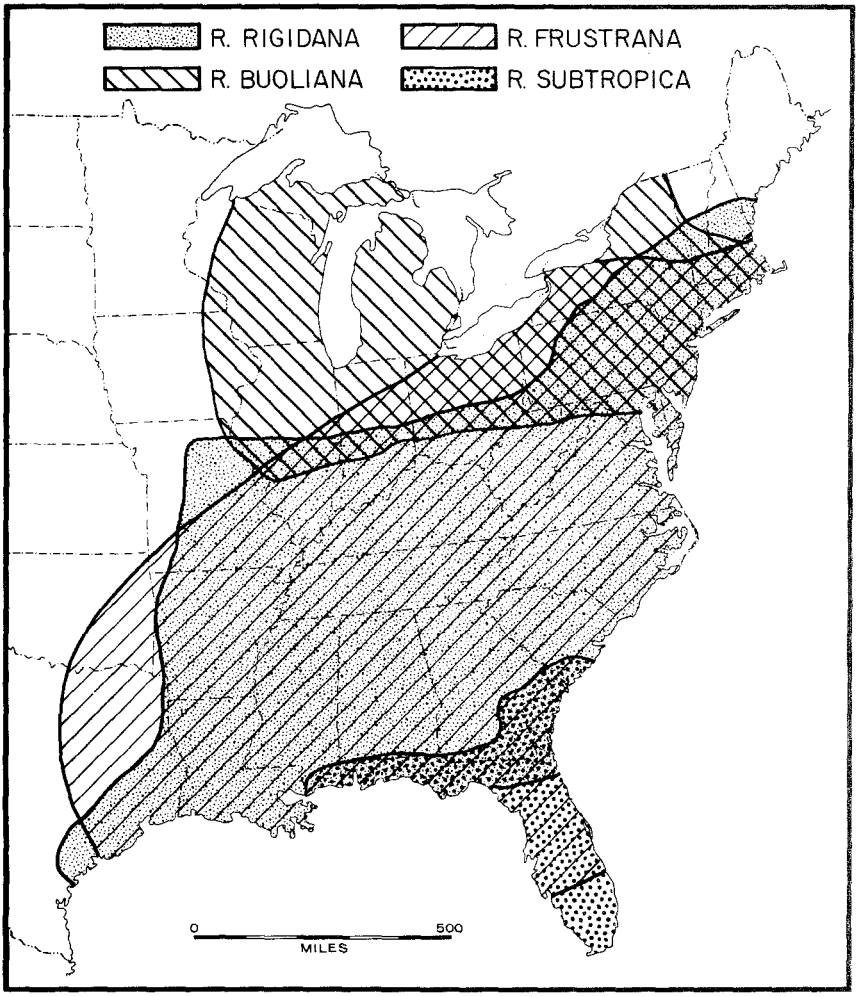

FIG. 1. Ranges of *Rhyacionia frustrana*, *R. rigidana*, *R. subtropica*, and *R. buoliana* (compiled from various sources).

Miller, 1978); the European pine shoot moth, *R. buoliana* Schiffermuller (Powell and Miller, 1978); and the subtropical pine tip moth, *R. subtropica* Miller, (Miller and Wilson, 1964; McGraw, 1975).

The life cycles of some of these *Rhyacionia* spp. may be synchronous at times (Berisford, 1974), particularly during emergence from the overwintering stages. However, much of the time the life cycles are asynchronous.

The pheromone for *R. buoliana* has been identified as *trans*-9-dodecenyl acetate (Smith et al., 1974), and the pheromone of *R. rigidana* is *trans*-8, *trans*-10-dodecadienyl acetate (Hill et al., 1976).

R. frustrana and *R. rigidana* are not cross-attractive, but are, in fact, mutually inhibitory (Berisford and Brady, 1973; Berisford et al., 1974; Berisford, 1974b, 1977). However, no data have been available on relationships among the other species. In 1973, a study was initiated to determine if cross-attraction occurs among *R. frustrana*, *R. rigidana*, *R. subtropica*, and *R. buoliana*.

Rhyacionia spp. pheromones may be useful for biological studies, population monitoring, aids for timing insecticide applications, and perhaps as communication disruption agents. Data on possible cross-attraction may expedite identification of the pheromones of other *Rhyacionia* spp. and allow researchers to anticipate any cross-attraction which may occur if emergence and female calling periods of 2 or more of these species overlap.

METHODS AND MATERIALS

Infested shoots of loblolly pine, *P. taeda* L., containing pupae of *R. frustrana* and *R. rigidana*, were collected in Oconee County, Georgia. Shoots of red pine, *P. resinosa* Ait., infested with *R. buoliana*, were collected in Allegany County, Maryland. Typical slash pine, *P. elliotii* var. *elliotii* Engelmann, shoots infested with *R. subtropica* were collected in Glades County, Florida. The infested shoots were placed in lighted rearing cages and adults were collected as they emerged (Berisford and Brady, 1972) except for *R. subtropica*. *R. subtropica* pupae were removed from the shoots and placed in rearing cages because the pupae had difficulty forcing through the hardened resin of the shoots after they were removed from the trees.

Crude pheromone extracts were prepared in hexane and ether (50/50) from the abdominal tips of females (Berisford and Brady, 1972).

Tests for cross attraction were conducted during field emergence of adult moths in the areas where pupae were collected. Extracts of all 4 *Rhyacionia* spp. were tested simultaneously against adult populations present. Since *R. frustrana* and *R. rigidana* emerge simultaneously in the spring (Berisford, 1974a), tests were run against both at the same time (March 1974). Tests were run against *R. buoliana* in June 1974 and 1976 and on *R. subtropica* in July 1974 and 1976.

Pheromone extracts were placed on filter paper (5 female equivalents (FE) per trap) and deployed in pherocon 1-C® traps (Hill et al., 1976). At least 25 trap nights on which some moths were trapped were used to determine response for each *Rhyacionia* spp. Five to 10 traps per night were baited with each pheromone and hung in alternate trees in a single row. At least 5 unbaited traps served as checks each night.

Data were analyzed by a chi-square test of independence using a 4×2 contingency table.

RESULTS AND DISCUSSION

Results of trapping are given in Table 1. *R. frustrana* males responded significantly ($P > 0.05$) to the pheromones of both *R. buoliana* and *R. subtropica* pheromone but at a substantially lower level than to their own attractant.

R. rigidana males responded only to their own pheromone and none of the other *Rhyacionia* spp. tested were attracted to *R. rigidana* pheromones.

R. buoliana and *R. subtropica* exhibited strong reciprocal attraction, and neither was significantly more attracted to its own pheromone than to the other species. Neither *R. buoliana* nor *R. subtropica* was attracted to *R. frustrana* or *R. rigidana.*

The sex pheromone of *R. rigidana* (*E,E*8,10-12:Ac) is apparently different from all of the other moths tested. *R. rigidana* belongs to the same species group as *R. subtropica* (Powell and Miller, 1978), but they apparently do not share tree hosts nor pheromone components. They are allopatric in distribution except for small parts of Florida, Georgia, Alabama, and South Carolina (Figure 1).

The pheromone of *R. buoliana* (*E*9-12:Ac) is apparently very similar or identical to the attractant of *R. subtropica*, although the species are apparently not closely related. *R. buoliana* is an imported species and even in the U.S. its range and host trees do not overlap with *R. subtropica.*

Although pheromones can serve as efficient species-isolating mechanisms for *Rhyacionia* (Berisford, 1974b), *R. buoliana* and *R. subtropica* have spatial and temporal differences which obviate the need for different sex pheromones (Figure 1). The range and host plants of *R. frustrana* overlap with both *R. buoliana* and *R. subtropica*, and *R. frustrana* males are attracted to the pheromones of both of these species. Simultaneous emergence of *R. subtropica* and *R. frustrana* from slash pine was noted as they emerged from overwintering pupae, and a few males of both species were trapped by their respective pheromones. It appears that their females may call at different times,

TABLE 1. NUMBERS OF *Rhyacionia* SPP. CAUGHT PER TRAP NIGHT BY CRUDE PHEROMONE EXTRACTS OF *R. frustrana*, *R. rigidana*, *R. subtropica*, AND *R. buoliana*

Responding species	Source of crude extracts				
	R. frustrana	*R. rigidana*	*R. subtropica*	*R. buoliana*	Check
R. frustrana	4.40	0	1.03	0.57	0.06
R. rigidana	0	1.38	0	0	0
R. subtropica	0	0	2.40	1.92	0.03
R. buoliana	0.20	0.04	1.84	1.23	0.03

thereby avoiding confusion of *R. frustrana* males since they are attracted to *R. subtropica* pheromone. However, populations of either *R. frustrana* or *R. subtropica* are frequently low in areas where both species occur, perhaps due in part to interruption of communication by pheromones from calling females. Pheromone specificity is generally highest among those species which have the greatest overlap in tree hosts and ranges, as in *R. frustrana* and *R. rigidana.* Those species which do not share tree hosts or have range overlap (*R. buoliana* and *R. subtropica*) are mutually attractive. The range and tree hosts of *R. frustrana* overlap slightly with *R. subtropica* and *R. buoliana.* There is a corresponding slight attraction of *R. frustrana* to the pheromones of both of these species. Also, *R. frustrana* may be the only member of the 4 spp. studied here which has a multicomponent sex pheromone.

The sex pheromones of these four *Rhyacionia* species have little relation to their taxonomic species groups, but may be relative to temporal and spatial overlaps as well as tree host preferences. However, any apparent relationships involving *R. buoliana* may be coincidental since it is not a native North American species.

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SEX PHEROMONE OF THE CRANBERRY GIRDLER, *Chrysoteuchia topiaria* (ZELLER) (LEPIDOPTERA: PYRALIDAE)

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Abstract—*Z*-11-Hexadecen-1-al (Z11-16:A1), free of the *E* isomer, was identified in extracts of female *Chrysoteuchia topiaria* (Zeller) abdominal tips. In commercial grass seed fields, traps baited with synthetic Z11-16:A1 were almost as attractive as female-baited traps. An alcohol, thought to be *Z*-11-hexadecen-1-ol on the basis of gas chromatographic retention times, was also isolated from the extract.

Key Words—*Chrysoteuchia topiaria*, cranberry girdler, sex attractant, sex pheromone, sod webworm, *Z*-11-hexadecen-1-al, *Z*-11-hexadecen-1-ol.

INTRODUCTION

The cranberry girdler, *Chrysoteuchia topiaria* (Zeller) (also referred to as a sod webworm in grasses), is a sporadic but destructive pest in commercial production of grass seed and cranberries in the Pacific Northwest. In the Willamette Valley of Oregon, adult flight occurs during late June and early July when most grasses are fully headed. During the flight period, females lay eggs that hatch in 12 days and the subsequent larvae burrow into the crown of grasses (Kamm, 1973a). Insecticides are not effective on the larvae because of the protection afforded by the foliage, and infestation becomes apparent only after damage to the fields. Identification of the sex pheromone would make possible the use of traps to detect and assess the adult population and thus provide a tool for a pest management program using appropriate control measures.

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METHODS AND MATERIALS

Collection of Insects and Bioassay. Experimental insects were collected as diapausing prepupae from a commercial field of bluegrass near Albany, Oregon, during the autumns of 1974 and 1976. The prepupae were placed in polyethylene freezer containers (the bottoms of which were lined with moist peat moss) and stored in a cold frame outdoors. Adults were obtained from these prepupae throughout winter and spring by procedures previously reported (Kamm, 1973b).

The adults emerged and were conditioned in controlled environment chambers at 20°C and a cycling regimen of 16 hr light, 8 hr dark. Males were segregated from females within 30 min of emergence and placed in 0.25-liter Mason jars. Three males were placed in each jar to minimize the effect of an active moth on the others (1 jar of moths was considered a replicate). The Mason jars served as a bioassay chamber when the lid was replaced with 16-mesh screen with a hole in the center just large enough to receive and hold a glass rod 3 mm in diameter. To conduct a bioassay, males were removed from the chamber to the laboratory 30 min after the beginning of the light period. After another 15–30 min in the laboratory, males were exposed to extracts of abdominal tips of females or synthetic preparations by dipping a glass rod into the preparation, evaporating the solvent, and inserting the rod into the jar through the screen. The number of males responding (4 replicates) and the relative intensity of response were used as criteria for assessing a test preparation. The type of behavioral activity was observed and scored for 15 min. Initially, the following categories of response were recorded for test materials: 0 = no response; + = limited antennae waving and walking, and 1–5 males responding, ++ = moderate antennae waving, walking, and wing fanning, and 6–9 males responding; +++ = intense antennae waving, walking, and wing fanning, and 10–12 males responding. During the exploratory phase of the project we found that consistent results could be obtained only by interpreting the +++ category as positive and the other categories as negative, although the other categories provided useful information for subsequent tests. Several jars of control insects were exposed to the laboratory environment before and during a bioassay; normal activity of these males scored no higher than + and was usually 0.

Collection of Pheromone. Pheromone was extracted from abdominal tips of females by a procedure similar to that of Sower et al. (1973), except that the abdominal tips were allowed to steep for 0.5–1.5 hr in methylene chloride that had been distilled through a 50-cm Vigreux column. Pheromone was also obtained by collecting the effluvia of calling females by trapping on Porapak-Q® by a procedure similar to that of Cross et al. (1976), except that a cylindrical copper screen was inserted in place of paper in the holding chamber for the moths to perch on. The cylindrical copper screen was covered on

the ends with more screen so it could be removed from the chamber without escape of insects. The insects in the screen cylinder were placed in the collection apparatus from 0900 to 1200 hr during which time about 50% could be observed to be calling. About 50 females were used per run. The holding chamber was 30 cm long by 6.5 cm in diameter; the collection tube was 1.8 cm in diameter and was packed with Porapak-Q to a length of 16 cm. The flow rate of compressed air (purified by passage through molecular sieves and Porapak-Q) was 1 liter/min. A control experiment with 1 μg of tetradecyl acetate on a watch glass showed that 60% of the evaporated acetate could be obtained by washing the holding chamber and 40% could be obtained by washing the Porapak-Q column. Consequently, for the pheromone collection both the holding chamber and Porapak-Q were washed with dichloromethane.

Purification of Pheromone. Purification of pheromone from extracts of tips or effluvia collected from calling females was effected by collection from a Hewlett-Packard[1] (model 5711) gas chromatograph equipped with a flame ionization detector and a column effluent splitter (one part of effluent to detector and 10 parts to collection trap). The column was 2.3 mm $\times$ 2 m stainless steel packed with 3% polydimethyl siloxane (SE-30) on 80/100 mesh Gas Chrom Q® and was operated at 170°C. Pheromone was collected in stainless-steel capillary tubing cooled in dry ice–acetone. Collection efficiency was 40–70%.

Gas Chromatography. The following analytical columns were used: (A) 2.3 mm $\times$ 2 m stainless-steel column containing 3% SE-30 on 80/100 mesh Gas Chrom Q; (B) 2.3 mm $\times$ 2 m stainless steel column containing 5% polyethylene oxide (Carbowax® 20M) on 80/100 mesh Gas Chrom Q; (C) 2.3 mm $\times$ 6.1 m stainless-steel column containing 15% polycyanopropyl phenyl siloxane (OV-275) on 100/120 mesh Chromsorb® P, AW-DMCS (Supelco, Inc., Bellafonte, Pennsylvania); (D) 2.3 mm $\times$ 7.3 m column with a Hi-Pack® Carbowax 20M liquid phase (Hewlett-Packard Co., Avondale, Pennsylvania).

Liquid Chromatography. Liquid chromatography of extracts was conducted with a 1.25 cm $\times$ 1 m column of 40 μm silica gel. Fractions were eluted with hexane–ether (99:1) to separate hydrocarbons from moderately polar compounds (esters, aldehydes, ketones, and epoxides are included in this group). Then 100% ether was used to elute more polar compounds (alcohols are in this group). Elution volumes of the three classes were determined with model compounds prior to the use of female extract.

Synthetic Chemicals. Candidate attractants used in this study were either purchased from Farchan Division of Story Chemical Co., Willoughby, Ohio, and used as received or synthesized by standard procedures (Schwartz and Waters, 1972). Synthesized candidates were free of alcohol or alkyne precurs-

[1]Mention of the proprietary products does not constitute an endorsement by the USDA.

ors by gas chromatographic analysis. A sample of Z11-16:Al was kindly furnished by Dr. J.H. Tumlinson, USDA-SEA, Gainesville, Florida.

Other Procedures. Ozonolysis was conducted in predistilled methylene chloride at −70°C, and the ozonide was reduced with triply recrystallized triphenyl phosphine. Mass spectra were determined on a Du Pont gas chromatograph-mass spectrometer at the Oregon Graduate Center (Beaverton, Oregon) by Drs. R.G. Smith and G.D. Daves.

RESULTS AND DISCUSSION

Exploratory Studies. The first experiments were conducted to characterize liquid chromatographic mobilities of pheromone in extracts of female abdominal tips (10-20 tips/test). The hydrocarbon fraction and the alcohol fraction gave borderline (++) and negative responses, respectively. The moderately polar fraction (aldehydes, ketones, esters, or epoxides) gave an intense positive response, which was as strong as the response to the original extract.

Isothermal gas chromatography of extracts was conducted at various temperatures (150-230°C) with collection of fractions of eluent so as to cover the volatility range from zero retention time to that of a 22-carbon ester. Initially, 10-min fractions were collected to obtain an approximation of the volatility range of active fractions. Finally, 1 min fractions were collected to more precisely determine the retention times. Fractions, with the approximate retention time of tetradecyl acetate (14:Ac) on either SE-30 (column A) or Carbowax 20M (column B), consistently gave intense responses. In addition, a later-eluting fraction sometimes gave an intense response, but was not consistent and could not be characterized.

To further characterize the pheromone, extracts of female abdominal tips were subjected to chemical tests. Potassium hydroxide in methanol (test for esters) and hydrogen bromide in acetic acid (test for epoxides) did not decrease response in the laboratory bioassay. Ozonolysis (test for unsaturation) or phenylhydrazine plus acetic acid (test for aldehyde or ketones) caused a loss of response in the bioassay.

In addition to these tests some monounsaturated acetates and aldehydes were bioassayed. In general, these compounds gave positive results at some concentration. Compounds which required a concentration of 10^{-4} g/ml or higher to produce a positive bioassay were *Z*-7-, *E*-7-, *Z*-8-, *Z*-9-dodecenyl acetates and *E*-7-, *Z*-9-, *Z*-11-tetradecenyl acetates. In contrast, monounsaturated 16-carbon aldehydes gave responses at the indicated minimum levels in g/ml: *Z*-7-, 10^{-8}; *Z*-9-, 10^{-7}; *Z*-11-, 10^{-6}; and *Z*-13-, 10^{-11}.

Collectively, these exploratory data indicated that one component of the sex pheromone was probably an unsaturated hexadecenal or hexadecenone and that possibly a second less volatile component was present. Because of the strong response of the insect to Z13-16:Al we initially thought this compound might be a pheromone component.

Isolation and Structure Determination. Figure 1 shows a gas chromatographic trace of an extract of female abdominal tips on SE-30. Component A, retention time 11.8 min, elicited a positive response in the bioassay and had a retention time equal to 0.97 of 14:Ac. Thus it appeared to be the pheromone component which was characterized in the exploratory studies. Component B, retention time 17.7 min, elicited no response, whereas component C, retention time 21.8 min, sporadically elicited a positive response when collected during various experiments.

In the laboratory, females could be observed calling between 1000 and 1200 hr and have been observed to mate between 0800 and 1200 hr in commercial grass seed fields. When female abdominal tips were extracted between 1000 and 1200 hr on the day of emergence, 4.3 ng of component A/female was obtained. Between 1300 and 1500 hr of the first day, 1.2 ng/female was obtained, and for two experiments between 1000 and 1200 of the second and third days of emergence, 1.9 and 1.4 ng/female were obtained. Consequently, in subsequent experiments, the extractions were made between 1000 and 1200 hr on the first day of emergence, and we typically obtained 3.8–4.6 ng of component A/female. Component B occurred in about the same ratio to

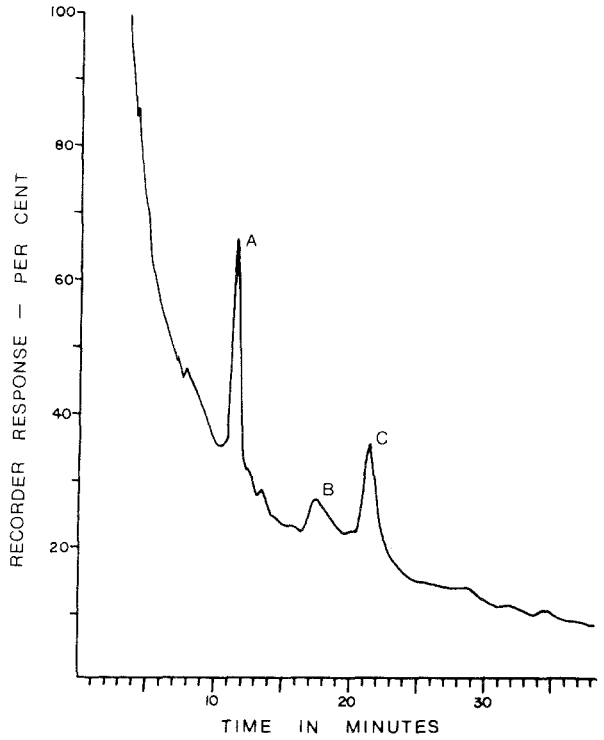

FIG. 1. Gas chromatogram (SE-30) at 150°C of a dichloromethane extract of abdominal tips of female *C. topiaria.*

component A, as shown in Figure 1 from the various extracted samples, whereas the amount of component C varied erratically.

Each of the components, A, B, and C, was purified by collection from the gas chromatograph (SE-30 column). Then each component was injected on the Carbowax 20M column (column B). Relative to 16:A1 the retention times of component A were 0.93 on SE-30 and 1.11 on Carbowax 20M, which are identical to the values of Z11-16:A1. Component B had the same retention time as Z11-16:OH on both SE-30 and Carbowax 20M. On SE-30, component C had a retention index (I_x) (Kovats, 1965) of 1925, and on Carbowax 20M, I_x was 2660.

Hill et al. (1975) and Cross et al. (1976) found that pheromone components that were not recoverable from extracts could be successfully isolated by collecting the effluvia of calling females. Because of this consideration we collected the effluvia of calling female *C. topiara*. The gas chromatographic analysis (Figure 2) showed that components A and C were detected, but not component B. However, if component B were present in the proportion in which it occurred in the tip extract, it would not have been detected. About 0.5

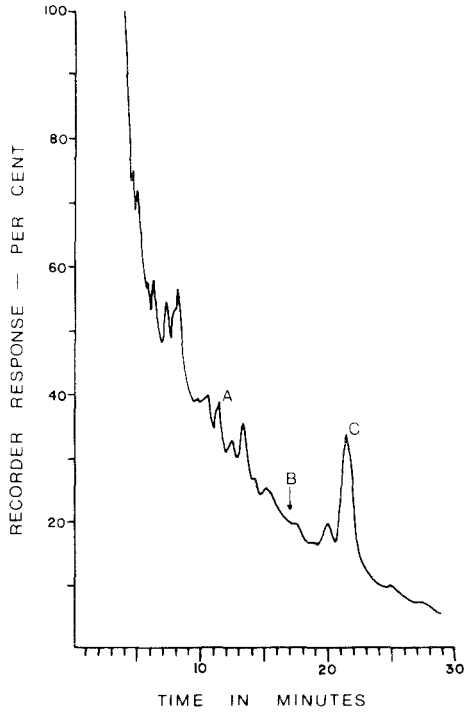

FIG. 2. Gas chromatogram (SE-30) at 150°C of a dichloromethane extract of the effluvia of calling female *C. topiaria*, which had been collected on Porapak-Q.

ng of component A/female was obtained from the collected effluvia. Bioassay of gas chromatographic fractions from the collected effluvia did not indicate the presence of a pheromone component not found in the extracts of female abdominal tips.

The mass spectrum of 500 ng of component A showed the following diagnostic peaks: 238 (M^+), 220 (M^+-18), and 194 (M^+-44). Therefore, component A is a monounsaturated 16-carbon aldehyde. Component C was identified as di-*n*-butyl phthalate (DNBP) from its mass spectrum. DNBP is a ubiquitous plasticizer. We do not know the source of this contamination since our extracts did not come into contact with plastics and our solvents were distilled and showed no DNBP upon concentration. The occurrence of DNBP in extracts from insects has been noted previously (Jones et al., 1973). We did not obtain enough of component B for mass spectral identification.

Two separate ozonolysis experiments were conducted, one with 50 ng and the other with 200 ng of component A, and both indicated Δ^{11} unsaturation by gas chromatographic analysis on SE-30 (column A) and Carbowax 20M (column B). A peak occurred near pentadecane on SE-30 and between eicosane and docosane on Carbowax 20M as expected for the dialdehyde and had the same retention times as the peaks from synthetic *Z*11-16:Al. Temperature programing was used for the SE-30 experiment, and either or both a mono- or dialdehyde product could have been detected for any position of the double bond. On Carbowax 20M, analyses were conducted at three different temperatures and unsaturation could have been detected for any positions except Δ^{12} or higher.

Although the ozonolysis experiments established a Δ^{11} double bond, they did not necessarily rule out the possibility of a small percentage of a positional isomer. Further information on this point was obtained by analysis of the pheromone on the high-efficiency Carbowax 20M column (column D). On this column at 210°C, both synthetic and natural *Z*11-16:Al had a retention time of 35.9 min (peak width, 2.3 min); *Z*7-16:Al had a retention time of 34.6; *Z*9-16:Al, 35.3; and *Z*13-16:Al, 38.1. There was no other peak in the trace of the pheromone sample nor any asymmetry or broadening of the natural *Z*11-16:Al peak.

Z-*E* isomer analysis of the pheromone on OV-275 (column C) indicated the pheromone was pure *Z* isomer (<0.5% *E*). On this column at 200°C, synthetic *Z*11-16:Al had a retention time of 20.2 min (peak width, 1.8 min) and synthetic *E*11-16:Al had a retention time of 18.3 min.

We interpret the weak laboratory bioassay response of *Z*11-16:Al relative to *Z*13-16:Al to be a reflection of the fact that the laboratory bioassay is is not a measure of attractancy per se. It does not seem reasonable that males can detect *Z*13-16:Al at a lower concentration than *Z*11-16:Al. Probably they are equally or more sensitive to *Z*11-16:Al but do not have available to them an appropriate response to low concentrations of *Z*11-16:Al while in

the bioassay jar. The strong response to Z13-16:Al, however, does indicate that this compound has some significance to male *C. topiaria.*

Field Tests. Preliminary field tests were successful when Z11-16:Al impregnated on rubber septa (1 mg/septum) (Maitlen et al., 1976) was placed in Pherocon® 1C traps and exposed to field populations just above the grass canopy. In one series of tests, captures of males averaged 13.5 males/trap-day in the traps baited with synthetic Z11-16:Al (containing 2.3% E11-16:Al) and 15.3 males/trap-day in traps baited with one live female (controls, 0.3 males/trap-day). Other field tests including tests of Z11-16:OH and Z13-16:Al will be reported separately.

Z11-16:Al is a pheromone component of several moth species. In field tests *C. topiaria* and *Heliothis armigera* (Piccardi et al., 1977) are captured in traps baited with Z11-16:Al alone, whereas the presence of a second pheromone component is required in order to trap the other species. Both Z11-16:Al and Z9-14:Al are necessary for trap capture for *Heliothis virescens* (F.) (Roelofs et al., 1974; Tumlinson et al., 1975), and both Z11-16:Al and Z13-18:Al are necessary components for *Chilo suppressalis* (Walker) (Nesbitt et al., 1975; Ohta et al., 1976). Tamaki et al. (1977) found Z11-16:Al plus Z11-16:Ac to be pheromone components of *Plutella xylostella* (L.). In addition, Z11-16:Al, although unattractive to *Heliothis zea* (Boddie), appears to be a component of this insect's sex pheromone (Roelofs et al., 1974; Sekul et al., 1975). Underhill et al. (1977), Steck et al. (1977), and Ando et al. (1977), on the basis of field screening experiments, have implicated Z11-16:Al as a pheromone component in combination with other compounds of several moth species.

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SEX PHEROMONE OF THE AMERICAN COCKROACH, *Periplaneta americana*

Isolation and Structure Elucidation of Periplanone-B

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Abstract—The sex pheromone of the American cockroach, *Periplaneta americana*, consists of two compounds, which we have named periplanone-A and periplanone-B. The two compounds can be isolated from fecal material in a ratio of 1:10, and periplanone-B can also be isolated from intestinal tracts. A total amount of 200 μg of periplanone-B has been isolated from both fecal material and alimentary tracts. By means of gas chromatography–mass spectrometry, hydrogenation, infrared analysis, and NMR analysis, we were able to identify periplanone-B as (1*Z*,5 *E*)-1,10(14)-diepoxy-4(15),5-germacradien-9-one. The overall structure was confirmed by comparison with a synthetic, biologically active epimer of periplanone-B.

Key Words—Sex pheromones, cockroach, *Periplaneta americana*, periplanone-B, sesquiterpenoid, germacrane derivative.

INTRODUCTION

The structure elucidation of the sex pheromone of the American cockroach, *Periplaneta americana*, is a problem of long standing. In 1963 Jacobson and coworkers presented evidence to the effect that the pheromone is 2,2-dimethyl-3-isopropylidenecyclopropyl propionate (Jacobson et al., 1963).

At about the same time, Wharton and coworkers (Wharton et al., 1962, 1963) published data (mainly gas-chromatographic retention times) which cast some doubt on Jacobson's structure, although this could not be disproved. The enormous difference in gas-chromatographic retention times between Jacobson's and Wharton's materials (6 and 145 min, respectively, under comparable conditions) suggests that the two groups were working on entirely different substances. At the time, however, this was not taken as an indication that the pheromone might consist of several different substances. When

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Jacobson's compound was later synthesized and found to be biologically inactive (Day and Whiting, 1964), Jacobson withdrew the proposed structure (Jacobson and Beroza, 1965). Jacobson's compound was found to be entirely different from the natural product (Day and Whiting, 1966; Wakabayashi, 1967). Apparently, Jacobson's material consisted mainly of inactive compounds contaminated with minute amounts of active material.

No further structures have been proposed for the pheromone, although several attempts have been made to isolate and identify it (Chen, 1974; Chow et al., 1976; Takahashi and Kitamura, 1976b; Kitamura and Takahashi, 1976). All these attempts have so far been abortive. The presence of two biologically active compounds (called periplanone-A and periplanone-B) in extracts of feces obtained from virgin females, and their isolation, purification, and molecular formulas have been described before (Persoons et al., 1974; Ritter and Persoons, 1975) and a tentative structure of periplanone-B has been proposed (Persoons et al., 1976, Persoons, 1977).

In this article the structure elucidation of periplanone-B is described in detail. A tentative structure for periplanone-A and its possible relationship with periplanone-B has been published elsewhere (Talman et al., 1977).

METHODS AND MATERIALS

Mass Rearing of Periplaneta americana. The insects were reared at 30°C (±1°C) and a relative humidity of 50–60% under a day/night regime of 17 hr light and 7 hr darkness. Water and food (Bonzo Puppy) were supplied in abundance. Once a month the egg capsules were collected from the stock cultures. Nymphs hatching from egg capsules within a week were collected in plastic containers measuring 70 × 30 × 40 cm and provided with an abundance of shelters. Ten to fifteen containers housing 2000–2500 insects each were used simultaneously.

At the end of the larval stage, the male and female nymphs were separated, because copulation decreases pheromone production considerably (Roth and Willis, 1952; Wharton and Wharton, 1957). Male nymphs were reared to adulthood for the required bioassays. After reaching adulthood, the females were used for the production of the starting materials. From 15,000 to 40,000 virgin females were reared at the same time.

Behavioral Test. For monitoring the successive purification steps of the pheromone, we used tests developed by Wharton and coworkers (Wharton et al., 1954a,b) and Takahashi and Kitamura (1972) and based on the sexual excitation of the males.

A pasteur pipette was rinsed with a solution of the pheromone. The solvent was evaporated and air was blown for 1–2 sec through the pipette into glass jars containing 15–25 male cockroaches. A sample was considered to be active when at least 50% of the males showed signs of sexual excitement (wing

raising, copulatory attempts). The tests were performed irrespective of the time of day (Lipton and Sutherland, 1970), although other investigators prefer to carry them out in the dark, when the males are more excitable (Buts and Aranoff, 1970; Takahashi and Kitamura, 1972; Hawkins and Rust, 1976; Chow et al., 1976).

Electroantennographic Test. The electroantennographic (EAG) tests were used as a supplement to the behavioral tests. The technique was as described by Roelofs and Comeau (1971). The signals were displayed on an oscilloscope (Philips, P.M. 3200), and a UV recorder (Honeywell, 906 T visicorder) was used to record the signals.

Extraction of Excrement-Soiled Filter Paper. The technique was a modification of that used by Roth and Willis (1952) and by Wharton et al. (1962). Young virgin females were collected in plastic boxes measuring 70 × 30 × 40 cm immediately after the last moult (600–650 insects per box). About 20 rolls of Whatman No. 3 chromatographic paper (45 × 3 cm) were placed in each container. Every 3–4 weeks the excrement-soaked rolls were collected and replaced with new rolls until the insects died. Up to ten containers were used at the same time. The collected material was stored at −15°C before being processed. The paper containing the excrement of one batch of 600 females was soaked in 10 liters of water to which lead nitrate was added. The paper was ground and the resulting slurry centrifuged. This procedure was repeated three times.[1] Fifty percent of the aqueous extract was distilled, and the distillate extracted four times with hexane. The extracts were combined, dried for 24 hr over anhydrous magnesium sulfate, concentrated to a volume of 20–30 ml, and stored at 2–3°C.

Extraction of Feces. Initially the excrement was scraped off the filter paper rolls, after which the filter paper and excrement were processed separately. This method of collecting excrement was later modified in such a way that the feces of thousands of insects could be collected easily. To this end 15,000–20,000 virgin females were confined in a large cage (1 × 1 × 1 m), in which 15 pieces of nylon cloth (1 m^2 each) were stretched vertically in such a way that they could readily be removed. The bottom of the container consisted of a gauze screen, below which a drawer was placed to collect most of the excrement. The nylon sheets were replaced with new ones every 2–3 weeks. The soiled sheets were rinsed with water, to which was added the excrement that had collected in the drawer. The resulting aqueous suspension was processed for extraction of the pheromone.

Extraction of Alimentary Tracts. In a pilot experiment the alimentary tracts of 20 virgin females (2–3 weeks old) were collected. To avoid contamination of the other parts of the gut with active material via the feces, the insects were starved for ten days (Cornwell, 1968), during which they were kept

[1]The large-scale processing of the starting material was carried out at the Pilot Plant of the Department of Chemical Technology of the Technical University, Delft.

individually in jars without access to water or food. The dissected tracts were each divided into seven parts, as shown in Figure 1. The corresponding parts were pooled, homogenized in acetone, centrifuged, and concentrated to 0.5-ml aliquots. Each fraction was bioassayed by the behavioral test.

During this investigation a total of 32,000 complete alimentary tracts were dissected and processed in batches of 2000 each. The tracts were collected without solvent, freeze-dried at −180°C, ground, and some water was added. The pheromone was codistilled with water. During the distillation water was added dropwise. The distillate was extracted four times with hexane. The extracts were combined and dried over anhydrous magnesium sulfate.

Precipitation of Crude Extracts in Acetone at −20° C. The various hexane concentrates from the filter paper, feces, and alimentary tracts were concentrated, and the residue was dissolved in as little acetone as possible and transferred to a centrifuge tube. This solution was stored overnight at −20°C, and the precipitate which had separated was removed by centrifugation (5–10 min, 3000 rpm). The supernatant was collected, the residue redissolved in acetone and again stored overnight at −20°C. This procedure was repeated four times. The supernatants were combined, concentrated, and subjected to gel-permeation chromatography.

Gel Permeation on Sephadex LH 20. Samples of 0.5 ml were applied on a glass column (100 × 1.4 cm ID) loaded with Sephadex LH 20 by means of a three-way valve. Freshly distilled acetone was used as the eluent; 5-ml fractions were collected and assayed as described above. Dilution series were made for location of maximum activity. Active fractions from all runs were combined, concentrated, and stored at 2–3°C.

High-Pressure Liquid Chromatography (HPLC) on Silica Gel. The combined active fractions collected from the Sephadex column were taken up in hexane–2% ethyl acetate and prepurified on a preparative silica gel column: column stainless steel, 50 × 1 cm ID, loaded with silica gel (Merck HR 60), particle size 5–20 μm, working pressure 90–100 atm, flow rate 20 ml/min, eluent 2% ethyl acetate in hexane (both freshly distilled), sample size 1 ml in-

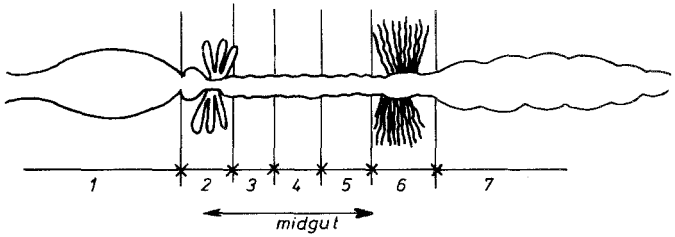


FIG. 1. Alimentary tract of *Periplaneta americana* (schematic). The numbered sections were investigated separately: 1, crop + esophagus; 2, ceca + proventriculus; 3, 4, 5, ventriculus; 6, ileum + malpighian tubules; 7, colon + rectum.

jected by means of a high-pressure injection valve. Fractions of 7.5 ml were collected and bioassayed. Fractions containing periplanone-B were combined, concentrated, and rechromatographed with an HPLC system incorporating analytical columns, under the following conditions: Three columns (25 × 0.5 cm ID) were loaded with Lichrosorb Si 60 (particle size 5-8 μm) and connected by capillary tubing; pressure 230 atm, flow rate 2.3 ml/min, eluent 1% ethyl acetate in hexane (freshly distilled), sample size 300 μl, detection with a DuPont UV spectrophotometer with flow cell, set at 254 nm; 2-min fractions were collected (= 4.5 ml).

Gas Chromatography of Purified Extracts. Corresponding active fractions from various HPLC runs were combined, and further analyzed by gas chromatography. All gas-chromatographic analyses were carried out with a Pye Unicam gas chromatograph, model 104.

To locate the activity of the compounds on various columns, 1-min fractions were collected from the gas chromatograph in chilled capillaries (Burson and Kenner, 1969) and these capillaries assayed by the behavioral test or the EAG method, or both. After location of the activity, 10 to 50-μl aliquots of the samples were injected on a polar column (5% DEGS, diethyleneglycol succinate on Chromosorb W (AW), 80-100 mesh, 160°C, 200 × 0.4 cm ID, N_2 = 45 ml/min, or 10% Carbowax 20 M on Chromosorb GAW-DMCS, 180°C, 250 × 0.4 cm ID, N_2 = 45 ml/min). The previously determined area of activity was collected and reinjected on a nonpolar column (5% OV 101 on Chromosorb G (AW-DMCS), 80-100 mesh, 200 × 0.4 cm ID, 180°C, N_2 = 45 ml/min). Those parts containing the activity were again collected for further analysis.

Mass Spectrometry. The mass spectrum of periplanone-B was run on a combined gas chromatograph-mass spectrometer (LKB 9000), fitted with a 2-m, 5% OV 101 column and operated at 180°C. Mass spectra were taken at different spots in the gas chromatographic peak as a check of the purity of the compound. A compound was considered to be pure when its mass spectra taken from different spots were identical.

Mass Spectrometry of Hydrogenated Periplanone-B. The active compound was hydrogenated and subjected to mass spectrometry in the GC-MS combination (LKB 9000) fitted with an OV 225 column. The top of the column (7 cm) was loaded with palladium chloride as the hydrogenation catalyst. Hydrogen was used as the carrier gas and mass spectra were taken at different spots of the chromatographic peak.

Infrared Analysis. A sample containing 7 μg of periplanone-B was subjected to infrared analysis in KBr on a double-beam grating spectrometer (Perkin-Elmer, model 421).

Ultraviolet Analysis. A sample of 1.5-2.0 μg of the substance was dissolved in 150 μl of hexane, and the spectrum recorded with a double-beam grating UV spectrophotometer (Cary, model 4).

NMR Analysis. A first NMR spectrum was run with a sample containing about 100 μg of periplanone-B in 10 μl of $CDCl_3$ on a Varian 220 MHz spectrometer. As the substance appeared to decompose rather rapidly in $CDCl_3$, a second sample of about 70 μg was collected, and its spectrum taken in 10 μl of CS_2, $CS_2 + C_6D_6$ (10:1), and in C_6D_6. These spectra were run on a Varian HR 300 and a Varian SC 300 spectrometer, the latter with a digital resolution better than 0.2 Hz over a spectral width of 3000 Hz (Fourier transformation of a 32 K data table).

RESULTS

Collection and Purification of Crude Extracts. From the work by Bodenstein (1970), Takahashi and Kitamura (1976a), and this work, it is obvious that the pheromone is produced in the midgut and excreted with the feces. The pheromone could only be recovered from that section of the midgut which comprises the proventriculus and the ceca (section 2, Figure 1).

Separation on a Sephadex column usually afforded 3–4 active fractions. The most active fractions could, as a rule, be diluted by a factor of 10,000 without loss of activity.

Prepurification on a preparative HPLC column of the combined active fractions from the Sephadex column resulted in isolation of two active compounds (referred to as periplanone-A and periplanone-B, respectively) within 20 min. Periplanone-A eluted after 3–5 min and periplanone-B after 13–18 min. Upon rechromatography over analytical columns, periplanone-B eluted after 64–68 min.

From the gas chromatographic results it appeared that periplanone-B was the most abundant compound. Extracts of feces were found to contain about 10 times less of periplanone-A than periplanone-B, whereas the alimentary tracts contained hardly any periplanone-A at all. For this reason we concentrated our efforts primarily on the structure elucidation of periplanone-B.

Mass Spectrum. Peak matching of the parent peak in the mass spectrum of periplanone-B shows that it corresponds to $C_{15}H_{20}O_3$ (calculated 248.1412; found 248.1386 and 248.1343). The spectrum agrees very well with that of a compound isolated by Chen (1974; Nakanishi, personal communication), and with that published by Takahashi and Kitamura (1976b) and Kitamura and Takahashi (1976). The molecular formula of periplanone-B suggests that the compound is a sesquiterpenoid.

Mass Spectrum of Hydrogenated Periplanone-B. The highest m/e value found for hydrogenated periplanone-B was 250 (originally 248), indicating that periplanone-B contains at least one double bond. The peak at m/e 234 (= M − 18) suggests a molecular weight of 252, though no parent ion was

found at this value. The fact that some groups of peaks in the mass spectrum of hydrogenated periplanone-B are moved 2–4 mass units up the m/e scale with respect to the spectrum of periplanone-B, constitutes strong evidence that the molecule of periplanone-B contains two double bonds.

Infrared Spectrum.[2] An absorption band at 1705 cm^{-1}, which can be attributed to a nonconjugated keto group, accounts for one of the three oxygen atoms in the molecule of periplanone-B indicated by the mass spectrum. The position of this band suggests that this keto group occurs either in an open chain or in a ring of at least six carbon atoms.

Comparison of the IR spectrum of periplanone-B with those of germacrone, germacrone diepoxide, and curdione[3] leads to the conclusion that the presence of another keto group in periplanone-B can be ruled out. The broad band around 3400 cm^{-1} has about the same intensity as that in the IR spectra of curdione, germacrone, and germacrone-diepoxide due to water present in the KBr pellet. Since, in addition, the IR spectrum shows no bands assignable to ether, ester of carboxyl groups, the two remaining oxygen atoms probably belong to epoxide groups. It is likely that one of these is an *exo*-epoxide responsible for the absorption at 3030 cm^{-1}, and for the —C—O— stretch absorption near 1250 cm^{-1} (Borrow and Searles, 1953; Bellamy, 1958; Jones and Sandorfy, 1956). Since the IR spectrum of periplanone-B does not show the C—H stretch absorptions at 3000 cm^{-1} of the trisubstituted epoxide groups of germacrone diepoxide, the remaining oxygen atom probably forms part of a (*Z*) or an (*E*)-epoxide group (bands at 890 cm^{-1} and 830 cm^{-1}) (Shreve et al., 1951). The band at 1605 cm^{-1} indicates that the two double bonds, derived from the mass spectrum of hydrogenated periplanone-B, constitute a conjugated system.

A strong band at 970 cm^{-1} shows that one of these double bonds has the *E* configuration. Another strong band at 905 cm^{-1} accounts for a vinylidene group, confirmed by a weak band at 3075 cm^{-1}. Other absorptions in this region (2925 cm^{-1} and, very weak, 2850 cm^{-1}) indicate the presence of aliphatic or alicyclic methylene groups. Two strong bands at 2870 cm^{-1} and 2950 cm^{-1} show the presence of two methyl groups which as indicated by a doublet of medium intensity at 1360 cm^{-1} and 1380 cm^{-1}, occur in an isopropyl configuration.

Ultraviolet Spectrum. The UV spectrum of periplanone-B shows a maximum absorption at 226 nm, confirming the presence of a conjugated diene system and agrees with that calculated (229 nm) for a conjugated diene system

[2]For comparison a number of infrared spectra of various germacrane derivatives were kindly supplied by Dr. Morikawa, The Institute of Food Chemistry, Osaka, Japan; Dr. Takeda, Shionogi Research Laboratory, Osaka, Japan, and Dr. Yamamura, Meijo University, Nagoya, Japan.
[3]Germacrone was provided by Dr. Sutherland, University of Manchester, England; germacrone diepoxide was provided by Dr. Herout, Prague, Czechoslovakia; and curdione was provided by Dr. Hikino, Sendai, Japan.

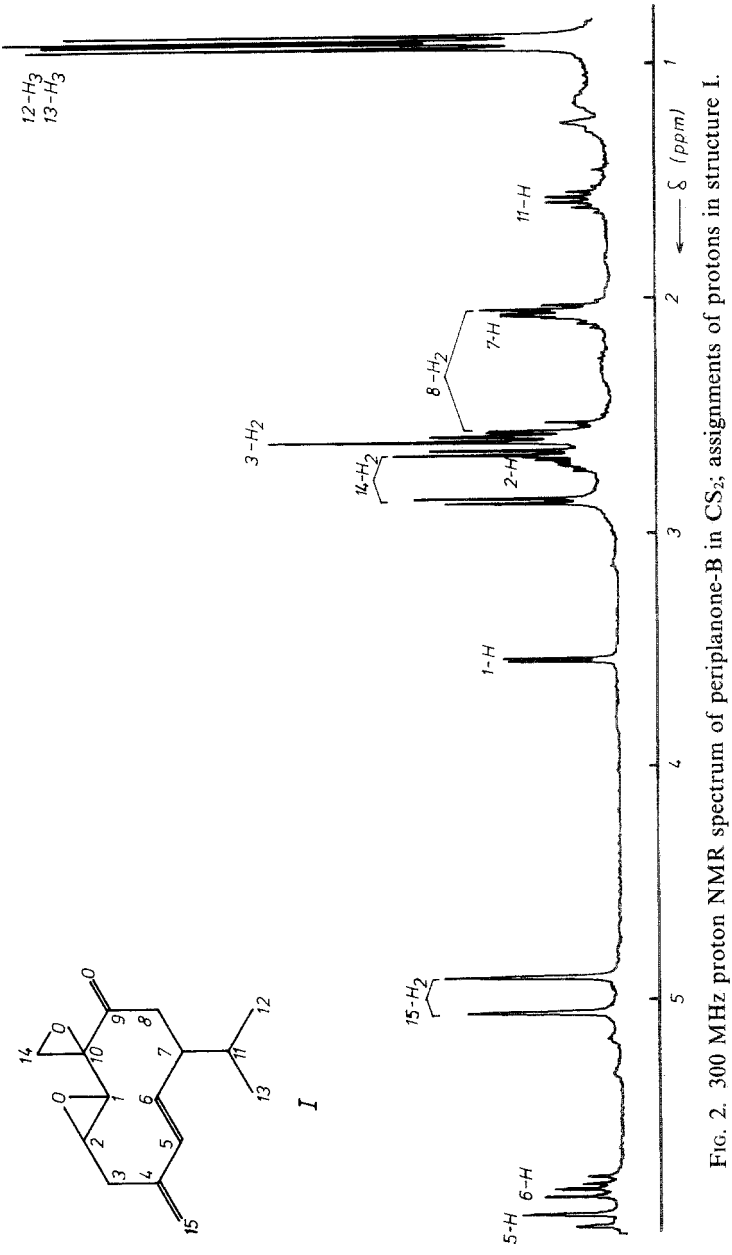


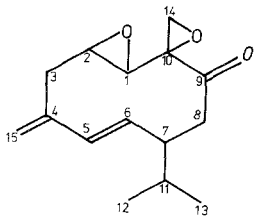
FIG. 2. 300 MHz proton NMR spectrum of periplanone-B in CS_2; assignments of protons in structure I.

with two alkyl substituents or two ring residues (Woodward, 1942; Scott, 1964).

NMR Spectrum of Periplanone-B. The spectrum in (CS_2) is shown in Figure 2, and the chemical shifts of the various protons in different solvents are listed in Table 1. On the basis of mass spectrometry and peak matching, the molecular formula was concluded to be $C_{15}H_{20}O_3$. In satisfactory agreement with this, the signals in the NMR spectrum integrate to a maximum of 21 hydrogen atoms. The mass spectrum of hydrogenated periplanone-B, as well as its infrared spectrum (band at 1600 cm^{-1}) and UV spectrum (absorption at λ^{max} = 226 nm) indicate the presence of a system of conjugated double bonds. That this is a conjugated diene $H_2C{=}C(H){-}C(H){=}C(H){-}C(H){-}H$ (E) is borne out by

TABLE 1. PROTON CHEMICAL SHIFTS OF PERIPLANONE-B IN DIFFERENT SOLVENTS (TOP), AND NUMBERING OF C ATOMS IN STRUCTURE I (BELOW)

	Solvent	
Protons	$CDCl_3$ (Varian HR 220, 20°C)	CS_2 (Varian HR 300, 30°C)
1-H	3.81	3.52
2-H	2.94	2.68
3-H_2	2.69	2.58
	2.76	2.58
15-H_2	4.98	4.87
	5.11	5.02
5-H	6.05	5.91
6-H	5.90	5.78
7-H	2.20	2.06
11-H	1.63	1.56
12-H_3	0.88[5]	0.87
13-H_3	0.91[5]	0.89
8-H_2	2.68	2.55
	2.20	2.04
14-H_2	2.83	2.63
	3.04	2.84



the NMR signals of four olefinic hydrogen atoms (Figure 2) at $\delta = 4.87$ (s, broadened), $\delta = 5.02$ (s, broadened), $\delta = 5.91$ (d, 16 Hz, broadened) and $\delta = 5.78$ (dd, 16 and 10 Hz). Saturation of the proton at $\delta = 5.78$ showed that it is coupled to a multiplet at $\delta = 2.06$, which is part of an AB-type system ($\delta = 2.04$ dd, 5.5 Hz and (−) 11.5 Hz; $\delta = 2.06$ ddt, 5.5 Hz, 7.5 Hz and 10 Hz).

Saturation of this two-proton AB multiplet in turn causes the triplet-like signal at $\delta = 2.55$ (with $J = 10$ Hz and (−) 11.5 Hz) to collapse into a singlet. Therefore, a CH_2 ($\delta = 2.04$ and $\delta = 2.55$) must be attached to the allylic CH ($\delta = 2.06$). Saturation of a one-proton multiplet at $\delta = 1.56$ (octuplet ~ 6.5 Hz) showed that it is coupled to two methyl groups ($\delta = 0.87$ d, 6.5 Hz; $\delta = 0.89$ d, 6.5 Hz) as well as to the allylic CH ($\delta = 2.06$ Hz). These data are in accordance with the partial structure shown in Figure 3.

Computer simulations of the NMR pattern of this system are in good agreement with the observed spectrum. The AB pattern at $\delta = 2.63$ and $\delta = 2.84$ (J = 6 Hz) is attributed to an *exo*-epoxide group, $>C\overset{O}{<\!\!-\!\!>}CH_2$, because the other plausible explanation, viz., a CH—CH group without further vicinal couplings is impossible in view of the other structural elements present (see below).

The strong IR absorption at 1705 cm^{-1} constitutes evidence for a nonconjugated ketone. The one-proton doublet at $\delta = 3.52$ (d, 4 Hz) accounts for the third oxygen atom, which forms part of the epoxide group in $-CH_2-CH\overset{O}{<\!\!-\!\!>}CH-$.

Evidence for the proton sequence in this partial structure was obtained by saturation of the one-proton doublet at $\delta = 3.52$, which resulted in simplification of the one-proton multiplet at $\delta = 2.68$ (ddd, 8 Hz, 6 Hz, and 4 Hz). The signal at $\delta = 2.68$ is strongly coupled to a CH_2 signal at $\delta = 2.58$ (J_{gem} = (−) 12 Hz).

Spectrum simulation of this ABCX system (for the CS_2 as well as for the $CDCl_3$ solution of the compound, the spectra of which have quite different patterns) confirmed the CH—CH_2 couplings. The vicinal coupling constant of 4 Hz indicates that the epoxide has the *Z* configuration. Rerunning of the spectrum on an SC 300 spectrometer revealed several small couplings that had not been observed before. These provided important additional information on the structure. One result of saturation of the protons around $\delta = 2.5$–2.6

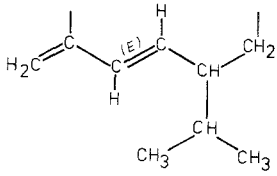


FIG. 3. Partial structure of periplanone-B.

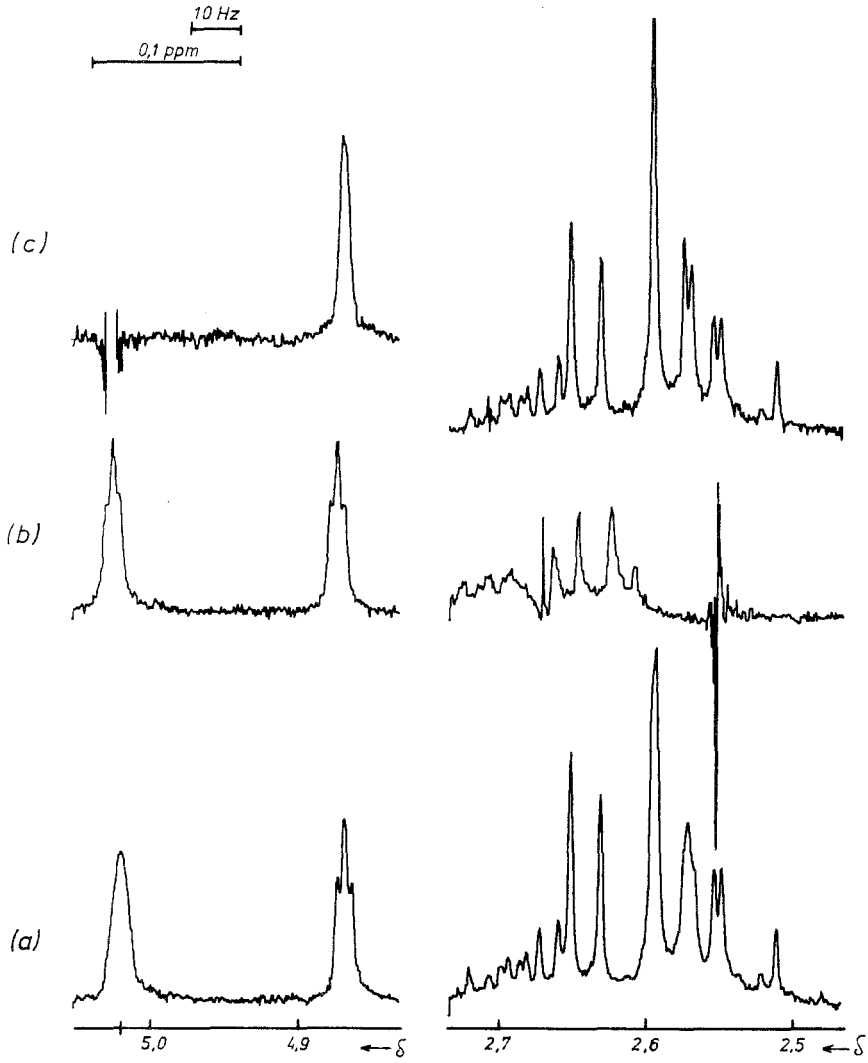


FIG. 4. Partial proton NMR spectra of periplanone-B in CS_2: (a) without saturation; (b) saturation around $\delta = 2.5$–2.6; (c) saturation of $\delta = 5.02$.

was that the broadened singlet at $\delta = 5.02$ was changed into a narrow triplet (Figure 4, trace b). Saturation of the olefinic proton at $\delta = 5.02$ in turn sharpened the signal at $\delta = 2.58$ (Figure 4, trace c). The demonstrated coupling constitutes evidence that the methylene group in $—CH_2—CH\overset{O}{\diagup\!\!\diagdown}CH—$ must be allylic to the vinylidene double bond, and the partial structure shown in Figure 5 emerges.

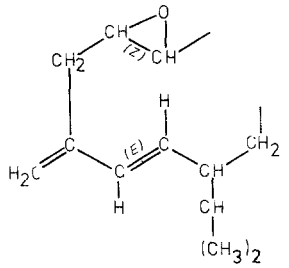


FIG. 5. Partial structure of periplanone-B.

Combination of this partial structure with the remaining ketone and epoxide groups leads to two possible structures, I and II (apart from stereochemical differences), both possessing a ten-membered alicyclic ring (Figure 6). Only structure I has the germacrane-type skeleton, which obeys the head-to-tail isoprene rule. Structure II is also composed of three isoprene units, but in addition to a head-to-tail coupling, it also contains a tail-to-tail arrangement. The chemical shifts expected for $-C\underline{H}_2-CH-CH(CH_3)_2$, in structure II are about 0.5 ppm lower than those found for periplanone-B, whereas the expected values in structure I are in good agreement with those found (Figure 7).

DISCUSSION

The compound described in this paper has no spectroscopic resemblance to the compound proposed by Jacobson et al. (1963) for the American cockroach sex pheromone. The fact that the pheromone of *P. americana* contains more than one component is now firmly established (Chen, 1974; Persoons et al., 1974; Chow et al., 1976, Takahashi and Kitamura, 1976b; Kitamura and Takahashi, 1976; Persoons, 1977). Although it is difficult to compare the experimental data, it seems certain that periplanone-B has been isolated by Chen (1974; Nakanishi, personal communication) and also by Takahashi and Kitamura (1976b) and Kitamura and Takahashi (1976). The mass spectra of

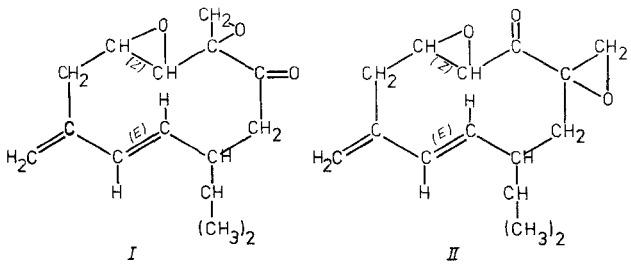


FIG. 6. Two possible structures of periplanone-B derived from the spectral data.

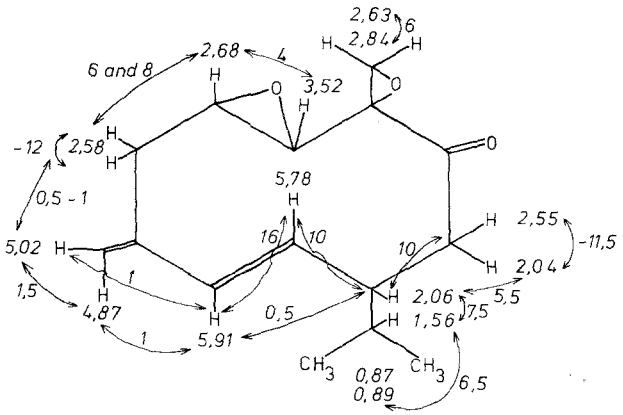


FIG. 7. Proton chemical shifts and coupling constants of periplanone-B in CS_2, as assigned to structure I. (1*Z*, 5*E*)-1,10(14)-diepoxy-4(15),5-germacradiene-9-one).

the compounds they isolated are in good agreement with the mass spectrum of periplanone-B. The amounts they were able to isolate so far have been insufficient for a complete structure elucidation. The molecular formulas of periplanone-A and periplanone-B ($C_{15}H_{20}O_2$ and $C_{15}H_{20}O_3$, respectively) (Persoons et al., 1974) might suggest that the latter is an oxidation product of the former. However, attempts to oxidize periplanone-A to periplanone-B were unsuccessful. If periplanone-B is a simple oxidation product of periplanone-A, the latter would probably be present in the insect's gut in much larger amounts. However, the fecal extracts contain periplanone-A and periplanone-B in a ratio of about 1:10, and extracts of alimentary tracts mainly contain periplanone-B with very little periplanone-A. It seems more likely now that periplanone-A is a derivative from periplanone-B (Talman et al., 1977).

The finding that the pheromone is produced in the midgut conflicts with a statement by Stürckow and Bodenstein (1966) that the pheromone is synthesized in the head, although they were unable to find a pheromone gland there. Their experiments are not convincing, because biologically active material can be obtained from all parts of the insect by extraction with an organic solvent. This finding may be explained by diffusion of the pheromone into the wax layer of the insect's cuticle as the pheromone is excreted. Our finding that the pheromone of *P. americana* is produced in the midgut rather than in the head confirms the work of Bodenstein (1970) and of Takahashi and Kitamura (1976a). Our own attempts to find a gland or a glandlike organ in the region where the pheromone is produced were unsuccessful. Takahashi and Kitamura (1976a) found marked differences between the midgut epithelium of males and that of pheromone-producing females. For the cockroach *Byrsotria fumigata*, Moore and Barth (1976) proposed the genital atrium as the main point of pheromone release and, possibly, production.

Interspecific responses exist between various *Periplaneta* sp., (Frazier, 1970, Schafer, 1977, Kawasaki et al., 1977). Moreover *P. americana* and *P. japonica* also respond manifestly to germacrene-D (Tahara et al., 1975; Takahashi and Kitamura, 1976b). Since it has been found that the major component of the sex pheromone of *P. americana* is a germacrane derivative, these interspecific responses might suggest that the sex pheromones of *Periplaneta* cockroaches are all germacrane derivatives.

Structure I of periplanone-B was confirmed by comparison of its spectral data with those of a synthetic, biologically active epimer of periplanone-B recently synthesized by Still (Still, personal communication).[4] Its spectral data were almost identical with those of the natural pheromone. The differences observed may be attributed to stereochemical differences between the synthetic and the natural compound.

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[4]Note added in proof: Meanwhile periplanone-B itself (*dl* form) has been synthesized. Its spectral properties and biological activity are completely identical with those of periplanone-B (Still, 1978).

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CHIRAL SPECIFICITY OF THE SEX PHEROMONE OF THE RED-HEADED PINE SAWFLY, *Neodiprion lecontei*

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Abstract—The stereospecificity of the sawfly pheromone 3,7-dimethyl-2-pentadecanol acetate against *Neodiprion lecontei* was studied. Two *erythro* and a 1:1 mixture of *threo* isomers (C-2 and C-3) were synthesized for this purpose. It was found that only one isomer with (−)-*erythro* configuration (2*S*, 3*S*) had biological activity. The potency of this synthetic pheromone was roughly identical to the one shown by the naturally occurring pheromone in this species.

Key Words—Sawflies, pheromone, stereospecificity, (−)-*erythro* configuration, enantiomers, chirality, optical isomers, *Neodiprion lecontei.*

INTRODUCTION

Earlier we have reported (Jewett et al., 1976) the identification of the chemical structure of the sex attractant of diprionid sawflies. In brief, females of many species of sawflies use either the acetic or propionic ester of 3,7-dimethyl-2-pentadecanol as the pheromone to attract the males. The pheromone is said (Casida et al., 1963) to be one of the most potent chemical attractants, being able to attract males from a few hundred meters away. During the process of identification, a racemic mixture of the above alcohol was synthesized by our group (Jewett et al., 1976). Soon three other groups (Kocienski and Ansell, 1977; Magnusson, 1977; Place et al., 1978) also independently synthesized it.

We have since tested the synthetic pheromone in the field against four different species and found (Jewett et al., 1978) that it is not as active as the naturally occurring pheromone in every case, although it did attract males. For instance, in a test against *Neodiprion sertifer*, traps baited by the racemic synthetic pheromone sporadically caught one or two males, while those baited by

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the natural pheromone constantly collected large numbers of males. Furthermore, when the synthetic pheromone was added adjacent to the natural pheromone in a trap, the total catch was drastically reduced.

We have interpreted the above information to mean that the naturally occurring pheromone has a rigid optical requirement and that some of the optical isomers in the synthetic racemic mixture were actually inhibitory to the attractiveness of the pheromone (Jewett et al., 1976). There is some supporting evidence. First, the natural pheromone of *N. lecontei* that we isolated showed a proton magnetic resonance pattern which is compatible with an *erythro* arrangement of two hydrogens on carbon 2 and carbon 3. Second, naturally occurring pheromones from different species, despite being identical in the overall chemical structure, and even after they were highly purified, elicited varying degrees of responses from males of other species.

To prove the above hypothesis, we have synthesized three stereochemically pure pheromones with respect to carbon 2 and 3 positions. These two positions were chosen because of the known *erythro* configuration in *N. lecontei* and the prediction that the chirality at the sites close to the functional group (in this case the ester moiety) is likely more important than that far removed from it.

METHODS AND MATERIALS

Bioassay. To test the efficacy of the synthetic pheromones against *Neodiprion lecontei* males, a field experiment was conducted in Sault Ste. Marie, Ontario, in a jack pine stand during the summer months of 1977. The trap used was a 3M Co. trap, 20 × 20 cm, with sticky inner surfaces. A known quantity of synthetic pheromone in 2 ml of ether was added to a 5-cm piece of cotton dental wick. After evaporation of the solvent, the wick was placed at the lower center of one side of the sticky surface. The traps were hung from appropriate branches at the approximate height of 1.5 m above the ground and 4 to 5 m apart from each other. The catches were recorded at one week intervals until the adult populations in the field disappeared (after 6 weeks). Two lots separated by a road but adjacent to each other, were chosen. Each lot was used for one series of each dilution for those three compounds.

Synthesis of Optical Isomers of Pheromone. The overall scheme of synthesis is shown in Figure 1.

Preparation of C_{12} Unit. For 2-bromodecane (I): to 127 g of 2-decanol, obtained by the reaction of nonanal and CH_3MgI, 240 g PBr_3 was added with stirring so that the temperature was kept under 5° C. This required about 2 hr. After the solution was allowed to stand at room temperature overnight, it was heated at 90° C for 1.5 hr, cooled to room temperature, and poured into 300 g crushed ice. The oily layer was separated, and the aqueous layer was extracted with two 100-ml portions of ether. The extract combined with oil was washed

successively with 30 ml conc. H_2SO_4 and 100 ml of water, then dried over $CaCl_2$. After the ether was removed, the residue was distilled to yield 154 g of (I), bp 110–115°C/20 mm Hg. Found: C, 54.03; H, 9.169%. Calc: C, 54.30; H, 9.57%.

For 3-methylundecanoic acid (II): to a solution of sodium methoxide in methanol, which was prepared from 15.2 g Na and 500 ml methanol, was added 88 g dimethyl malonate in one portion. To the mixture, 133 g of (I) was added at such a rate as to keep the temperature at 60–65°C. After the addition was completed, the reaction mixture was refluxed for 24 hr. Upon removal of methanol under reduced pressure, 150 ml water was added and the mixture was extracted with 200 ml ether. After the extract was dried over $CaCl_2$, the ether was removed by distillation, leaving 98 g crude dimethyl 1-methylnonylmalonate (bp 115–120°C/0.5 mm Hg). This was added to a mixture of 70 g KOH, 30 ml water, and 300 ml methanol. The reaction mixture was refluxed overnight and then concentrated to dryness under reduced pressure. The residue was acidified with 200 ml of 12 N HCl and refluxed overnight in an oil bath. The product was extracted with three 100-ml portions of ether. The combined extract was dried over anhydrous $MgSO_4$ and then concentrated under reduced pressure to give an oil which was distilled to yield 59 g (II), bp 131°C/1.2 mm Hg. Found: C, 71.60; H, 12.22. Calc: C, 71.95; H, 12.08%.

For 3-methyl-1-undecanol (III): to the solution of 22 g $LiAlH_4$ in 500 ml ether, 58 g (II) was added dropwise. After the addition was completed, the solution was refluxed for 1 hr, cooled to room temperature, hydrolyzed with 50 ml water, and acidified with 150 ml 6 N HCl. The alcohol in the aqueous layer was extracted with 100 ml ether.

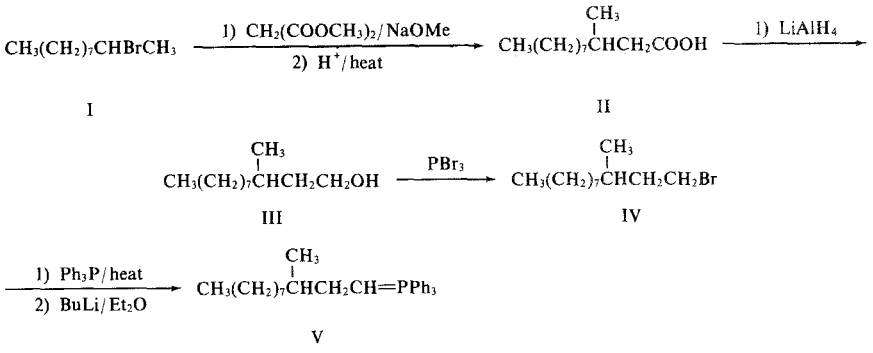
The extract was concentrated to 150 ml and dried over $MgSO_4$. After the ether was removed, the residue was distilled to yield 53 g (III), bp 97–98°C/0.8 mm Hg. Found: C, 77.01; H, 14.19%. Calc: C, 77.35; H, 14.07%.

For 1-bromo-3-methylundecane (IV): bromination of (III) with PBr_3 was carried out under the same procedure as that applied for the preparation of (I). From 52.5 g (III), 55.4 g (IV) was obtained; bp 95–96/1 mm Hg. Found: C, 57.62; H, 10.46. Calc: C, 57.90; H, 10.00%.

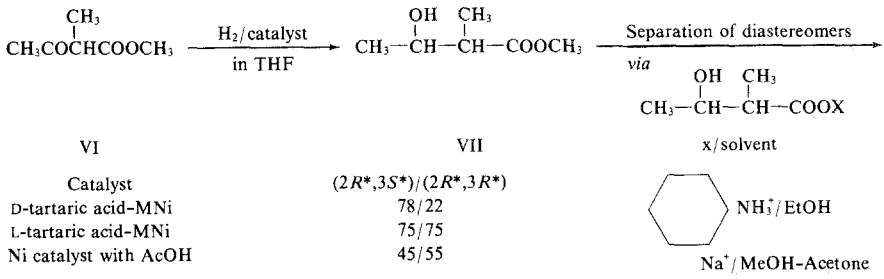
NMR δ = 0.90 and 0.91 (6H, overlapped distorted d and t, $-\underset{\underline{C}H_3}{\underset{|}{C}}H-$ and $C\underline{H}_3-CH_2-$) 1.25 (14H, m, $-(C\underline{H}_2)_n-$), 1.75 (4H, overlapped m, $-C\underline{H}_2-CH_2-Br$ and $-\underset{CH_3}{\underset{|}{C}}\underline{H}-$) 3.75 (2H, t, $-CH_2-C\underline{H}_2$ Br). IR, 2950, 2900, 2810, 1460 cm^{-1}.

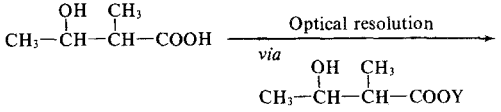
For 3-methylundecanyltriphenylphosphonium ion (V): the mixture of 40 g triphenylphosphine and 38 g (IV) was placed in a flask filled with dry nitro-

C_{12} unit



C_5 unit





VIII

Major enantiomer/
Optical purity
(+)-(2*S*,3*R*)/56%
(−)-(2*R*,3*S*)/60%
(2*R**,3*R**)

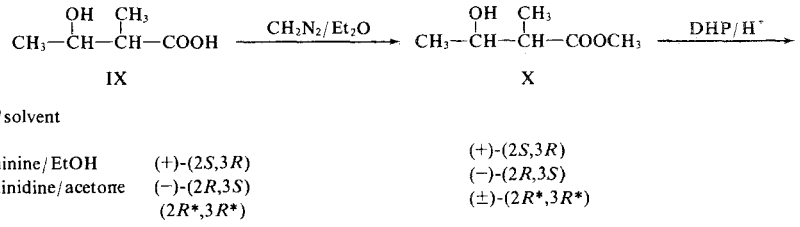


FIG. 1. Synthetic route of 3,7-dimethyl-2-pentadecanol (XV) and its acetic ester (XVI). *Mixture (1:1) of 2*R*,3*S* and 2*S*,3*R*.

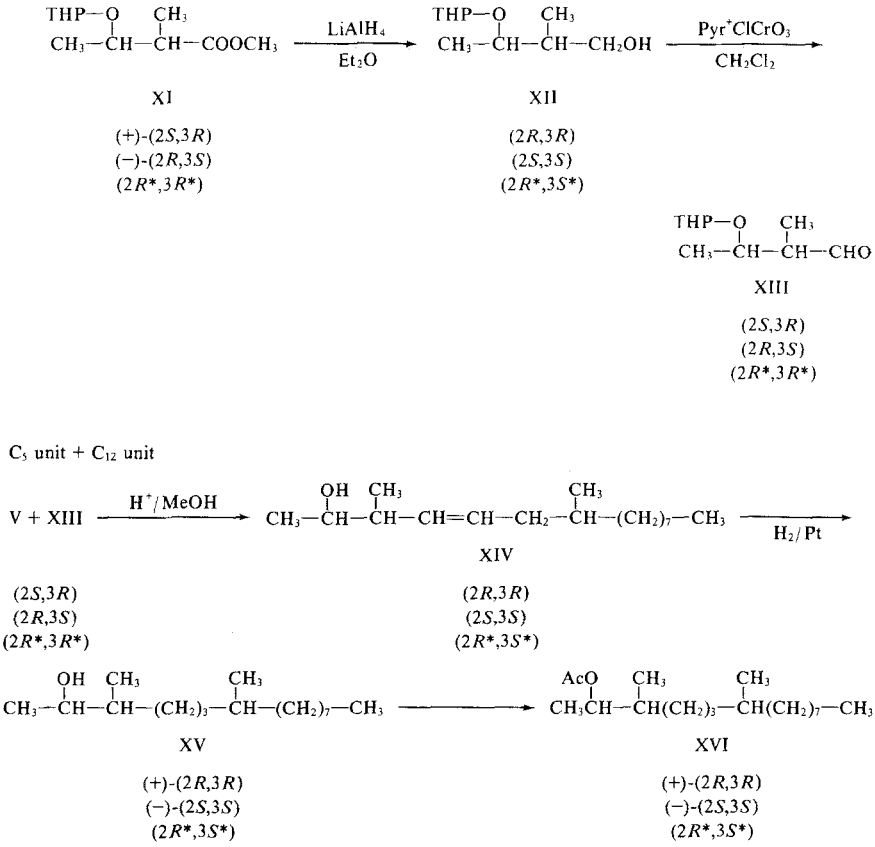


FIG. 1. (*continued*)

gen and heated at 180°C for 30 hr. The viscous mass was cooled to 50°C and mixed with 300 ml ether to form white crystals. The crystals were ground in the ether to make a slurry. The slurry was placed in a centrifuge tube and the crystals were precipitated by centrifugation. After the ether was removed by decantation, *n*-hexane was added to make a slurry again and centrifuged. This procedure was repeated until almost all the PPh_3 was removed (5 times). The crystals in the centrifuge tube were placed in a desiccator with P_2O_5 under 0.2 mm Hg for 2 days. Then the crystals were ground in a dry box, dried with P_2O_5, and again placed in the desiccator under the reduced pressure for a week to yield 73 g 3-methylundecanyltriphenylphosphonium bromide; mp 60–61°C. Addition of BuLi into a suspension of the phosphonium salt in ether gave a solution of (V).

Preparation of C_5 Unit. For the modified nickel catalyst: well pulverized $NiCO_3$ (10 g) was reduced for 1 hr at 350°C under an 8-liter/hr hydrogen

stream. The resulting reduced nickel (6 g) was soaked for 1 hr at 85°C in 60 ml 1% tartaric acid solution adjusted to pH 4.1 with N sodium hydroxide.

After removal of the solution, the modified nickel catalyst was washed successively with a 60-ml portion of water, two 300-ml portions of methanol, and a 300-ml portion of THF.

For (+)-(2*S*,3*R*)-methyl-3-hydroxy-2-methylbutyrate [(2*S*,3*R*)-VII]: methyl-2-methyl-3-oxobutyrate (100 g) in 200 ml THF and 0.6 ml acetic acid was hydrogenated with 6 g (2*R*,3*R*)-tartaric acid-modified nickel under 110 kg/cm^2 of initial hydrogen pressure at 100°C for 30 hr.

After removal of the catalyst, fractional distillation gave 90 g of diastereomeric VII. GLC (90°C) showed 78% *erythro* isomer, retention time 14.1 min, and 22% of *threo* isomer, retention time 12.8 min. The isolation of (2*S*,3*R*)-VIII was carried out by the method reported before (Tai et al., 1978; Tai and Imada, 1978). The ester was saponified to 3-hydroxy-2-methylbutyric acid (VIII) which was then converted into the cyclohexylammonium salt. Three successive recrystallizations of the salt from ethanol gave the *erythro* isomer. Removal of base with ion exchange resin gave 41 g of *erythro*-VII whose optical purity was 56% [(2*S*,3*R*) enantiomer in excess].

Optical resolution of (+)-(2*S*,3*R*)-VIII was carried out via its quinine salt. Two recrystallizations from ethanol gave optically pure products. At this stage of recrystallization, the optical rotation of the sodium salt of (+)-VIII, derived from the quinine salt, reached a steady value and remained unchanged by three further recrystallizations. The mixed melting point determination of the quinine salts at each crystallization showed that the sample of this stage was homogeneous. After removing the quinine, the liberated acid (IX) was treated with ethereal diazomethane to give 23 g (+)-(2*S*,3*R*)-X, bp 75°C/15 mm Hg, $[\alpha]_D^{20} = 11.35$ (neat). GLC (90°) showed a single peak at 14.1 min. IR and NMR spectra were identical with those of the one prepared before. NMR taken in the presence of Eu(tfmc)$_3$ indicated that the sample was optically pure.

For (−)-(2*R*,3*S*)-methyl-3-hydroxy-2-methylbutyrate [(2*R*,3*S*)-X]: hydrogenation of methyl-2-methyl-3-oxobutyrate (100 g) with (2*S*,3*S*)-tartaric acid-modified nickel by the same procedure as before gave 93 g diastereomeric (VII) from which 42 g *erythro*-VII [optical purity 60%, (2*R*,3*S*) enantiomer in excess] was obtained.

Optical resolution with quinidine (two recrystallizations of the quinidine salt from methanol-acetone), followed by esterification with diazomethane, gave 27 g (−)-(2*S*,3*R*)-X, $[\alpha]_D^{20} = -11.36$ (neat). GLC (90°C) indicated 100% purity. IR and NMR were identical with those of (+)-(2*S*,3*R*)-X.

For (±)-(2*R**,3*R**)-methyl-3-hydroxy-2-methylbutyrate [(2*R**,3*R**)-X]: methyl-2-methyl-3-oxobutyrate (100 g) in 200 ml of THF and 0.8 ml of acetic acid was hydrogenated with unmodified reduced nickel catalyst to give 92 g of diastereomeric (VII) (*threo*/*erythro* = 55:45).

The isolation of *threo* isomer by the published method (Tai and Imada, 1978) gave 29 g of (2*R**,3*R**)-X: bp 75°C/15 mm Hg. GLC (90°C) showed a single peak at 12.8 min. IR and NMR spectra were identical with those of the one prepared before (Tai and Imada, 1978).

For methyl-2-methyl-3-tetrahydropyranoxybutyrate XI: to 15 g of (2*S*,3*R*)-X was added 16.2 g of dihydropyran. On adding a small amount of *p*-toluene-sulfonic acid, the reaction occurred exothermically. After the mixture was allowed to stand for 3 hr at room temperature, 100 ml of ether was added. The solution was washed with 50 ml of 2% aqueous solution of $NaHCO_3$ and dried over $CaCl_2$. After the ether was removed, the residue was distilled to yield 22.5 g of (2*S*,3*R*)-XI, bp 92°C/2 mm Hg.

Both (2*R*,3*S*)-X (15 g) and (2*R**,3*R**)-X (15 g) were converted into (2*R*,3*S*)-XI (22.0 g, bp 83°C/1.5 mm Hg) and (2*R**,3*R**)-XI (19.5 g, bp 95°C/3 mm Hg) by the method described above.

For 2-methyl-3-tetrahydropyranoxy-1-butanol (XII): to the cooled solution of 8 g $LiAlH_4$ in 500 ml ether, 22 g (2*S*,3*R*)-XI was added dropwise. After the addition was completed, the mixture was refluxed for 1 hr, cooled, and hydrolyzed with 5 ml water. The ether layer was concentrated to 50 ml and dried over K_2CO_3. After the ether was removed, the residue was distilled to yield 15.6 g (2*R*,3*R*)-XII, bp 105°C/3.5 mm Hg. Found: C, 63.48; H, 10.92. Calc: C, 63.79; H, 10.71%.

By the same method as above, (2*R*,3*S*)-XI (21 g) and (2*R**,3*R**)-XI (19.0 g) were converted to (2*S*,3*S*)-XII (15.1 g, bp, 93°C/2 mm Hg; found: C, 63.60; H, 10.82%) and (2*R**,3*S**)-XII (13.8 g, bp, 92°C/1.8 mm Hg; found: C, 64.01; H, 11.00).

For 2-methyl-3-tetrahydropyranoxybutanal (XIV): to the mixture of 21.5 g (100 mmol) pyridinium chlorochromate and 9.1 g anhydrous sodium acetate in 150 ml dry CH_2Cl_2, which was cooled at 2°C, was added 10.8 g (2*R*,3*R*)-XII in one portion. After the reaction mixture was stirred at 5°C for 4 hr, 200 ml ether was added. The mixture was passed through a column packed with 200 ml Florisil, and the eluate was concentrated under reduced pressure to give crude (2*S*,3*R*)-XIII.

This was used in the next reaction without further purification. By the same method mentioned above (2*R*,3*S*)-XIII and (2*R**,3*R**)-XIII were prepared from (2*S*,3*S*)-XII and (2*R**,3*S**)-XII, respectively.

Coupling of C_5 and C_{12} Units. For 3,7-dimethyl-2-hydroxy-4-pentadecene (XIV): an *n*-hexane solution of BuLi was dropped into the mixture of 20.5 g (40 mmol) V and 300 ml dry ether in an atmosphere of N_2 on ice bath. After stirring at room temperature for 4 hr, the reaction mixture was red and the white salt had disappeared. To this solution (2*S*,3*R*)-XIII was added dropwise until the red color of the mixture almost disappeared (7.4 g XIII was required). The mixture was stirred at room temperature overnight. The mixture was passed through a short Florisil column, and the eluate was concen-

trated under reduced pressure. The residue was chromatographed on a column (3 × 40 cm) packed with Florisil. The eluate with hexane-ether (95:5) yielded 6.6 g oil upon evaporation of solvent. The oil was suspended in 30 ml methanol containing a trace amount of *p*-toluene-sulfonic acid, and the mixture was stirred for 4 hr at room temperature to give a homogeneous solution. After removal of solvent under reduced pressure, the residue was dissolved in 30 ml ether and washed with 10 ml aqueous $NaHCO_3$ solution. After removal of ether, the residue was vacuum distilled to give 4.6 g (2*R*,3*R*)-XII, bp 115°C/0.1 mm Hg. Found: C, 79.80; H, 13.50%. Calc: C, 80.04; H, 13.47%.

Both (2*S*,3*S*)-XIV and (2*R**,3*S**)-XIV were prepared by the same procedure mentioned above, except for the use of (2*R*,3*S*)-XIII and (2*R*,3*R*)-XIII.

From 25 g (V) and 8.6 g (2*R*,3*S*)-XIII, 7.5 g (2*S*,3*S*)-XIV was obtained, bp 113°C/0.1 mm Hg. Found: C, 79.85; H, 13.71%.

From 9.5 g (V) and 3.2 g (2*R**,3*R**)-XIII, 2.4 g (2*R**,3*S**)-XIV was obtained, bp 100°C/0.05 mm Hg. Found: C, 79.88; H, 13.70%.

For 3,7-dimethyl-2-pentadecanol (XV): in an atmospheric hydrogenation flask filled with hydrogen, 100 mg Pt_2O and 20 ml methanol were shaken for 10 min. Into the mixture, 4.5 g (2*R*,3*R*)-XIV was added, and the mixture was shaken under hydrogen until no more hydrogen was absorbed. After removal of catalyst and solvent, the product was vacuum distilled to give 4.3 g (+)-(2*R*,3*R*)-XV, bp 108°C/0.05 mm Hg. Found: C, 79.11; H, 14.47. Calc: C, 79.61; H, 14.15% $[\alpha]_D^{20} = +10.97$ (neat). NMR (CCl_4, TMS) $\delta = 0.89$ (9H, envelope which can be resolved into two d and one t by the addition of Eu $(dpm)_3$, 3 $C\underline{H}_3$), 1.08 (3H, d, J = 7 Hz, $C\underline{H}_3$—CHOH—) 1.22 [22H, envelope, 10(CH_2) and 2(CH)], 3.55 [1H, two q $\underline{H}$—C(OH)CH_3]. NMR spectra taken in the presence of Eu$(dpm)_3$ and Eu $(hfmc)_3$ showed that the *erythro* isomer (major compound) was optically pure. IR (neat) 3370, 1465, 1380, 1100 cm^{-1}. GLC (180°C) indicated 98% purity, retention time 18.3 min, and 1% unidentified impurities, retention time at 7.9 and 9.1 min.

Both (−)-(2*S*,3*S*)-XV and (±)-(2*R**,3*S**)-XV were obtained by the same method as mentioned above, except for the use of (2*S*,3*S*)-XIV and (2*R**,3*S**)-XIV.

From 7.5 g (−)-(2*S*,3*S*)-XIV, 7.3 g (−)-(2*S*,3*S*)-XV was obtained. Found: C, 79.22; H, 14.41%. Calc: C, 79.61; H, 14.15%, $[\alpha]_D = -11.10$ (neat). GLC (180°C) indicated 98% purity. NMR and IR data were identical with those of (2*R*,3*R*)-XV. From 3.2 g (2*R**,3*S**)-XIV, 3.1 g (2*R**,3*S**)-XV was obtained. Found: C, 79.15; H, 14.45%. Calc: C, 79.61; H, 14.15%, NMR $\delta =$ 0.89 [9H, envelope which can be resolved into two d and one t by the addition of Eu$(dpm)_3$] 1.06 (3H, d, J = 6 Hz), 1.22 (22H, envelope), 3.56 (1H, two q); IR identical with (−)-(2*S*,3*S*)-XV. GLC (180°C) indicated 98% purity with the same impurities as (2*S*,3*S*)-XV.

Preparation of Pheromone. 2-Acetoxy-3,7-dimethylpentadecane (XVI) was prepared by treatment XV with acetic anhydryde and pyridine.

From 0.5 g (−)-(2*S*,3*S*)-XV, 0.45 g (2*S*,3*S*)-XVI was obtained, bp 108°C/0.05 mm Hg. Found: C, 75.71; H, 12.93%. Calc: C, 76.45; H, 12.83%, $[\alpha]_D^{20} = +5.4$ (*n*-hexane c = 17).

NMR (CCl_4TMS) $\delta = 0.87$ (9H, envelope, 3 × C$\underline{H}_3$—), $\delta = 1.10$ [3H, d, J = 6.1 Hz, C$\underline{H}_3$—CH(OH)—], 1.23 [22H, envelope, —$(CH_2)_n$— and —CH—], 1.91 (3H, S, C$\underline{H}_3$C—) 4.63 (m, —C$\underline{H}$OH—), IR (neat), 2950, 2940, 2850, 1735, 1465, 1372, 1240, 1015 cm^{-1}. GLC (170°C) indicated 98% purity, retention time 6.8 min.

From 0.5 g (+)-(2*R*,3*R*)-XV, 0.46 g (2*R*,3*R*)-XVI was obtained, bp 109°C/0.05 mm Hg. $[\alpha]_D^{20} = -5.89$ (hexane c = 9.10). Found: C, 75.64; H, 12.90%.

NMR and IR data were same as that of (2*S*,3*S*)-XIV. GLC (170°C) indicated 98% purity.

From 0.5 g (2*R**,3*S**)-XV, 0.45 g (2*R**,3*S**)-XVI was obtained, bp 108°C/0.05 mm Hg. Found: C, 75.98; H, 13.44%.

NMR (CCl_4 TMS) $\delta = 0.87$ (9H, envelope 3 × C$\underline{H}_3$—) $\delta = 1.08$ (3H, d, J = 6.0 Hz, C$\underline{H}_3$—CHOH—), 1.23 [22H, envelope —$(CH_2)_n$— and —CH—], 1.91 (3H, s, C$\underline{H}_3$CO—), 4.63 (*m*—C$\underline{H}$OH—); IR (neat) same as that of (2*S*,3*S*) isomer. GLC (170°C) indicated 98% purity, retention time 6.8 min.

RESULTS AND DISCUSSION

The results of NMR analyses on 3,7-dimethylpentadecan-2-ol (Figure 2) show that each *erythro* isomer (2*R*,3*R* and 2*S*,3*S*) is free from contamination by the other *erythro* isomer. Thus they can be considered pure as far as the accuracy of the MNR assay method. On the other hand, both isomers contain approximately 5% each of a *threo* isomer. The reason for the introduction of *threo* isomers can be traced to the Wittig reaction (V and XIII; see Figure 1). In this reaction, a small amount of the enol form of the aldehyde is expected to form, and the resulting epimerization would yield a small amount of a *threo* isomer for each *erythro* isomer. Thus, the (+)-*erythro* pheromone (2*R*,3*R*) contains a trace of 2*R*,3*S* isomer, and the (−)-*erythro* pheromone (2*S*,3*S*) is similarly contaminated by 2*S*,3*R* isomer.

Bioassay samples were coded as follows: acetate of (2*R*,3*R*) as A-I; (2*S*,3*S*) as A-II; that of the *threo* mixture (2*R*,3*S* and 2*S*,3*R*) as A-III and corresponding propionate esters as P-I, P-II, and P-III, respectively.

The results of bioassays were summarized in Table 1 and include data only from A-II. Only the traps baited with A-II were active against *N. lecontei* males. The others (A-1, A-III, and controls) caught no males. The results

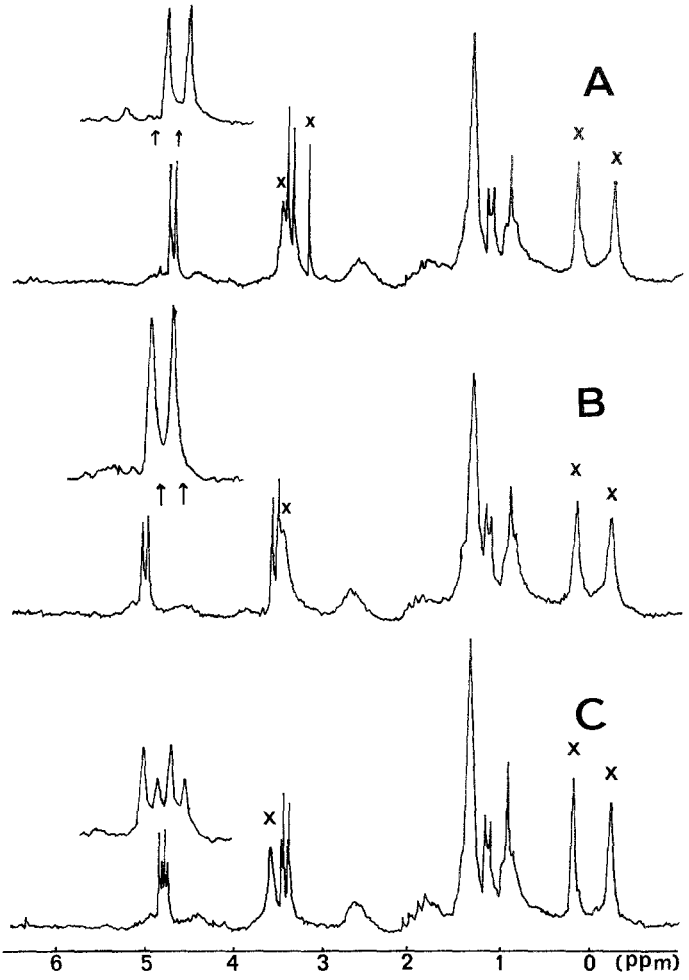


FIG. 2. NMR spectra of (A) 2*R*,3*R*; (B) 2*S*,3*S*, and (C) mixture of A and B of 3,7-dimethyl-2-pentadecanol. The doublets due to $C\underline{H}_3$—CH(OH)— clearly distinguishes 2*R*,3*R* from 2*S*,3*S*. In the case of (C), more 2*S*,3*S* than 2*R*,3*R* was added to distinguish these two isomers. NMR conditions are: sample 15 mg each and solvent $CDCl_3$ 500 μl with Eu(hfmc)$_3$ 50 mg. The measurement was made at 50°C by using JEOL Fx-100 with spectrum width 1000 Hz. The chemical shift values depend upon the concentration of shift agents. The cross-marks above some peaks indicate that they are due to the shift agent (in the case of A, there is one more contaminant peak due to acetone).

clearly indicate that the natural pheromone must have the configuration of (2*S*,3*S*)-*erythro* and that the chirality was the cause for the earlier problem of the inferior performance of the racemic synthetic pheromone. The fact that traps with other optical isomers did not catch any males must indicate the remarkable specificity of the antennae receptors for the pheromone. Also, since the threshold dose of A-II (i.e., 1 μg trap) was identical to the level obtained from the natural pheromone trap (Jewett et al., 1978), it is likely that the chirality at carbon 7 position does not play a significant role in this species.

In the case of gypsy moth pheromone (Cardé et al., 1977), the synthetic racemic mixture did not give a clear-cut dose-effect relationship, whereas optically pure preparations did. With *N. lecontei*, the racemic mixture and the optically pure preparation gave a dose-effect relationship (tested from 0.1 to 1000 μg/trap) (Jewett et al., 1978). The difference is that in the former the threshold concentration was on the order of 100 μg/trap in contrast to the latter case which was about 1 μg/trap. Since approximately 1/8 of the racemic mixture consists of the isomer of the right chirality, the above difference cannot be explained by the amount of the active ingredient alone. Rather, it must mean that other isomers do interact with the antennal receptors to reduce the field effectiveness of the pheromone by at least one order of magnitude.

The strictness of the requirement for pheromone chirality must be species specific. The degree and the nature of interference by optical isomers other

TABLE 1. NUMBER OF *N. Lecontei* MALES CAUGHT IN TRAPS BAITED WITH (−)-(2*S*,3*S*) ISOMER (A-II) OF 2-ACETOXY-3,7-DIMETHYLPENTADECANE[a]

Pheromone (μg)	Trap site	Examination date[b] 6/7	6/14	6/21	7/4	7/11	7/18	Cumulative total
300	A[c]	8	10	2	1	2	0	58
	B	12	12	7	1	1	2	
100	A	3	12	2	2	1	0	36
	B	2	8	4	2	0	0	
30	A	2	0	1	1	0	0	12
	B	3	3	2	0	0	0	
10	A	2	0	1	0	0	0	6
	B	1	1	1	0	0	0	
3	A	3	1	2	0	0	0	11
	B	4	1	0	0	0	0	
1	A	0	0	0	0	0	0	1
	B	1	0	0	0	0	0	

[a] Traps baited with other isomers caught no males.
[b] Only fresh catches have been recorded for each examination date.
[c] Two sites in a jack pine stand, A and B, are separated from each other by a road.

than the true pheromone are expected to vary from one species to another in analogy to the cases with geometric isomers: e.g., in some species the opposite isomer (i.e., *E* isomer to the insect utilizing the *Z* isomer) is inhibitory (Roelofs and Comeau, 1971), while in others it can be innocuous to stimulatory (Klun and Robinson, 1974). Many lepidopterous insects require precise ratios of mixtures of *E* and *Z* isomers for maximum activities (Roelofs and Cardé, 1974).

Research on effects of chiral isomers on antennal receptors lags far behind that of geometrical isomers, largely because of the difficulties in synthetic approaches. Our work here, along with that of others (e.g., Cardé et al., 1977; Chapman et al., 1978; Silverstein, 1978; Anderson et al., 1978; Mori, 1978), indicates that chirality of pheromone isomers plays a very important role. Thus it is likely that phenomenon of intricate isomer interaction should begin to unfold when scientists start using optically pure isomers for pheromone studies.

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SECONDARY PHEROMONE COMPONENTS AND SYNERGISM IN STORED-PRODUCTS PHYCITINAE

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Abstract—Attraction of male *Ephestia cautella* (Walker), in a warehouse population, to traps baited with the synthetic sex pheromone (*Z,E*)-9,12-tetradecadienyl acetate was greatly enhanced in the presence of the synthetic secondary pheromone (*Z*)-9-tetradecenyl acetate. Lower levels of enhancement were observed with five compounds related to the secondary pheromone, and the synergistic effect followed a simple chemical pattern. These results are discussed in relation to the possible role of secondary pheromones in species-recognition by males and in interspecific competition.

Key Words—Secondary pheromone components, synergism, competition, Lepidoptera Pyralidae, Phycitinae, *Ephestia cautella*, (*Z,E*)-9,12-tetradecadienyl acetate, (*Z*)-9-tetradecenyl acetate.

INTRODUCTION

Several closely related Lepidoptera in the subfamily Phycitinae (family Pyralidae) are important pests of stored products. These species, *Ephestia* spp. and *Plodia interpunctella* (Hübner), infest a wide range of commodities, and most of them have a widespread distribution. In spite of the resulting overlap of geographical ranges and preferred foods, it is uncommon to find two or more of these species successfully attacking a particular batch of a commodity simultaneously. (*Z,E*)-9,12-tetradecadienyl acetate (ZETA) has been identified as a female sex pheromone component in five of these species: in *Plodia interpunctella* (Hübner) and *Ephestia cautella* (Walker) (Brady et al., 1971; Kuwahara et al., 1971b), in *E. elutella* (Hübner) (Brady and Nordlund, 1971), in *E. figulilella* Gregson (Brady and Daley, 1972), and in *E. kühniella* Zeller (Kuwahara et al., 1971a). It has been shown to elicit attraction of males (and other male behavioral responses) in these species. The majority of lepi-

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dopteran sex pheromones are complexes of more than one component, and this is the case in some of the stored-products Phycitinae. The secondary components in these complexes may function as species-specific selection mechanisms in premating behavior (Brady and Daley, 1972; Ganyard and Brady, 1971; Grant et al., 1975), although other isolation mechanisms have also been proposed (Grant et al., 1975).

Secondary pheromone components have been reported in those species of Phycitinae which have received the most attention from researchers. The females of *P. interpunctella* release (*Z,E*)-9,12-tetradecadienol (ZETOH) in greater amounts than the attractant ZETA (Sower et al., 1974b). The abdominal glands of *E. cautella* females also contain ZETOH (Kuwahara and Casida, 1973; Read and Beevor, 1976) in small amounts, but it is not yet known whether this compound is released by the females; ZETOH was also found in the female abdominal glands of *E. elutella* and *E. kühniella* by Kuwahara and Casida (1973). The females of *E. cautella* also produce and release another component, (*Z*)-9-tetradecenyl acetate (ZTA), at approximately half the rate of that of the attractant ZETA (Brady, 1973; Read and Beevor, 1976).

The ZETOH released by *P. interpunctella* and found in some *Ephestia* species is a potent inhibitor of attraction behavior of *E. cautella* males (Sower et al., 1974b; Read and Haines, 1976), and *E. cautella* males have separate receptor mechanisms for ZETA and ZETOH (Sower et al., 1974a). The other compound (ZTA) released by *E. cautella* females only caused male stimulatory response at rather high concentrations in bioassay tests (Brady, 1973); it is not attractive to males, but our previously published warehouse trials indicated that it may enhance the attractiveness of the attractant ZETA (Read and Haines, 1976; Haines, 1976). The experiment described below was undertaken in order to provide more satisfactory evidence of the synergistic role of the secondary component (ZTA) and to investigate the structural specificity for synergism by a secondary pheromone component.

METHODS AND MATERIALS

The experiment was conducted in Nairobi, Kenya, on stacks of bagged wheat in the large Government Wheat Store where a light infestation of *Ephestia cautella* was present. This store, the design and use of the cylindrical traps employed, the specification of the ZETA, and the method of release of the pheromones from polythene vials were the same as those used in earlier experiments and reported previously (Read and Haines, 1976). (*Z*)- and (*E*)-9-tetradecen-1-ol were prepared by routine acetylenic routes, and the corresponding acetates and aldehydes were prepared by treatment with acetic anhydride in pyridine, and with Collins' chromium trioxide dipyridine complex, respectively (see Nesbitt et al., 1975, 1977). Gas chromatographic analysis

TABLE 1. CATCHES OF *E. cautella* MALES IN TRAPS BAITED WITH SYNTHETIC PHEROMONES AND RELATED COMPOUNDS

Treatment[a]	Mean no. of moths per trap in 3 days	Mean of transformed counts[b]
ZETA + ZTA	15.67	8.27
ZETA + ETA	8.00	4.87
ZETA + ZTAL	6.00	3.91
ZETA + ETAL	5.00	3.36
ZETA + ZTOH	3.33	2.46
ZETA + ETOH	2.33	1.88
ZETA alone	2.33	1.87
Control	0.33	0.33

[a]The compounds tested were: ZETA: (Z,E)-9,12-tetradecadienyl acetate; ZTA: (Z)-9-tetradecenyl acetate; ETA: (E)-9-tetradecenyl acetate; ZTAL: (Z)-9-tetradecenal; ETAL: (E)-9-tetradecenal; ZTOH: (Z)-9-tetradecen-1-ol; ETOH: (E)-9-tetradecen-1-ol.

[b]Counts were transformed by raising to the power of 0.768 to give acceptable homogeneity of variance. Least significant differences between means of transformed counts are: 2.04 at 5% level, 2.83 at 1% level.

showed that all the compounds were more than 96% pure: the *Z* compounds contained 2.5% of the *E* isomers, and the *E* compounds contained 1% of the *Z* isomers.

The eight treatments used in this experiment are listed in Table 1. Each vial (one vial per trap) in the six "combination" treatments contained 1 mg of the attractant ZETA and 0.5 mg of the secondary compound, and each vial in the "attractant only" treatment contained 1 mg of ZETA; these vials contained an equal weight of 2,6-di-*tert*-butyl-*p*-cresol (BHT) as an antioxidant. The control vials were treated only with the hexane solvent used in preparing all the vials.

Three stacks of wheat were selected as blocks in a randomized block design (one replicate of each of the eight treatments per block). The traps were left on top of the stacks for three days, and the randomized arrangement of traps within each block was changed daily.

RESULTS

The mean numbers of *E. cautella* males caught per trap during the three-day period are shown for each treatment in Table 1, together with the results of the transformation indicated by Taylor's power law. A two-way analysis of variance of the transformed data showed that treatment variance was highly significant ($F = 13.03$ at 7 and 14 *df*, $P = 0.004\%$) and that block variance was not significant ($F = 1.18$ at 2 and 14 *df*, $P = 33.5\%$).

The least significant differences between transformed values for the various treatments are indicated in Table 1; the attractiveness of ZETA is signifi-

cantly enhanced by the presence of (*Z*)-9 acetate, (*E*)-9 acetate, or (*Z*)-9 aldehyde ($P = 0.001\%$, 0.69%, 4.98%, respectively). Furthermore, the addition of the (*Z*)-9 acetate (the compound released by *E. cautella* females) produces a significantly more attractive combination than that with the (*E*)-9 acetate ($P = 0.30\%$). Neither the (*E*)-9 aldehyde nor the two stereoisomers of 9-tetradecen-1-ol produced a combination significantly more attractive than the attractant on its own.

DISCUSSION

It is clear from the above results that (*Z*)-9-tetradecenyl acetate (ZTA), the natural secondary component of the *E. cautella* pheromone complex, is a potent synergist to the major attractant (ZETA) and that ZTA has a significantly greater synergistic effect than five related compounds (including its geometric isomer, ETA). These data confirm our earlier suggestion as to the function of ZTA (Read and Haines, 1976; Haines, 1976). Brady (1973) suggested that, since a combination of ZTA and ZETA was not as effective as crude pheromone gland extracts in laboratory attractancy tests, an additional synergist must be present in the complex but may be nonstimulatory on its own. This conclusion was based on a comparison of 2 and 10 female equivalents of crude extract with a combination of 10 ng ZETA plus 5 ng ZTA, representing 5 female equivalents according to his extraction data. However, his extraction yields are low when compared with the estimates of Read and Beevor (1976), and according to the latter his synthetic test material probably represented only 1 female equivalent. Thus, on the available evidence, there seems to be no reason to postulate a further natural synergist additional to (*Z*)-9-tetradecenyl acetate in *E. cautella*.

Several authors (Ganyard and Brady, 1971; Sower et al., 1974b) have shown that the pheromone produced by *P. interpunctella* females inhibits the sexual responses of *E. cautella* males to females of both species; this inhibition is due to the release by *P. interpunctella* females of relatively large amounts (Sower et al., 1974b) of ZETOH, which is a strong inhibitor of the attraction behavior of *E. cautella* males (Read and Haines, 1976).[2] This mechanism not only prevents males of *E. cautella* from attempted courtship with female *P. interpunctella* but, in a situation where sufficiently large numbers of *P. interpunctella* are present, it will inhibit *E. cautella* males from responding to *E. cautella* females. By contrast, males of *P. interpunctella* respond equally to the pheromones released by females of *E. cautella* and *P.*

[2]Although it has not yet been shown that ZETOH is released from the glands of *E. cautella* females, the slight increase in ZETOH content of the glands after mating (Kuwahara and Casida, 1973) and the presence of a separate receptor mechanism for ZETOH in the males (Sower et al., 1974a) indicate that this component may have a conspecific function, possibly as an inhibitor released by mated females.

interpunctella (Ganyard and Brady, 1971; Grant et al., 1975), thus indicating that the ZTA released by *E. cautella* females does not have an inhibitory effect on *P. interpunctella* males, which are therefore capable of normal response to conspecific females in the presence of *E. cautella* females. However, males of *P. interpunctella* will be at a disadvantage in the presence of *E. cautella* because of the time wasted in fruitless attraction to *E. cautella* females; the effect of this disadvantage will be directly related to the proportion of *E. cautella* in a mixed population.

The above suggestions on "pheromone competition" between *E. cautella* and *P. interpunctella* give rise to some predictions about the outcome of such "competition" in situations favorable to both species. First, the male responses to the pheromone complexes will tend to reinforce any large imbalance of population density between the two species. Second, the presence of *E. cautella* females will interfere with the probability of *P. interpunctella* males finding conspecific females, especially when the proportion of *E. cautella* in the population is high. Third, *E. cautella* males (by their discriminatory response) can locate conspecific females in the presence of *P. interpunctella* females, unless the density of the latter is high enough to inhibit the response of the *E. cautella* males. Finally, if the density of *P. interpunctella* is high, mating of *E. cautella* will be reduced because of the inhibition of males by the *P. interpunctella* secondary pheromone. The existence of such "pheromone competition" in situations suitable for both species would thus lead to one of two alternative states of dynamic equilibrium, depending on relative populations during the early stages of population growth: either (1) *E. cautella* would dominate *P. interpunctella* but not to the complete exclusion of the latter (even when *E. cautella* is present in large numbers), or (2) *P. interpunctella* would dominate, often to the complete exclusion of *E. cautella*, when present in large numbers.

These predictions agree well with general observations of warehouse infestations: viz., in upland Kenya (where both species occur and where conditions are suitable for the successful development of both species) the most common infestations of stored grain are caused by *E. cautella*, often with a small proportion of *P. interpunctella* also present, but occasionally a bulk of grain is found to have a serious infestation of *P. interpunctella* whereas *E. cautella* is absent. Competition is, of course, influenced by many other physical and biotic factors, and mating isolation is also effected by other mechanisms (Grant et al., 1975; Grant and Brady, 1975), but the influence of secondary pheromone components in competition between *E. cautella* and *P. interpunctella* may nevertheless be important in certain situations.

The experimental data presented here show that the order of effect of the six compounds tested (which all have the same "ω distance" between the double bond and the terminal methyl group) follows a simple chemical pattern: the synergism is always greater in each pair of stereoisomers when the double

bond forms the *Z* configuration, and the carbonyl (aldehyde) group has an effect which is intermediate between that of the acetoxyl (acetate) group and the hydroxyl (alcohol) group.[3] This pattern can be compared with the structure-response relationships found by Priesner et al. (1975) in their study of electroantennographic responses of noctuid species. Recent unpublished data (Priesner, personal communication) on electroantennographic responses of *E. cautella* males to highly purified (at least 99% pure) (*Z*)-9-tetradecenyl acetate (ZTA) and (*E*)-9-tetradecenyl acetate (ETA) show that ZTA is about ten times more effective than ETA in eliciting a physiological response in the males.

From the viewpoint of pest management, the data presented here support our previous suggestion (Read and Haines, 1976) that survey traps for *E. cautella* baited with a combination of ZETA and ZTA would be more effective than traps baited with ZETA alone in terms of efficiency and possibly also specificity.

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BUILDING PHEROMONES OF *Vespa orientalis* AND *Polistes foederatus*

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Abstract—Among Vespinae both the queen and the workers build cells of a typical architectural design which are suitable for rearing a brood. In the case of *Vespa orientalis*, but not in the species *V. crabro*, *Paravespula vulgaris*, *P. germanica*, *Dolichovespula saxonica*, or *D. media*, groups of workers of various ages which are kept in artificial breeding boxes in the absence of a queen continue to build new cells and even entire combs. Workers which are deprived of a queen build worker cells but not queen cells. The construction commences from a central point where the workers had congregated for a while. The site of construction can be preselected by intentionally directing the workers to rest in a particular spot. Once the building has been initiated, the workers will persist building in the same spot over and over again, even after any imposed limitation has been removed. In this last respect, *Polistes foederatus* emulates *V. orientalis*. From the above results, it would seem that hornets resting in a particular spot for a length of time deposit there a substance—probably volatile—which induces assembly of hornets in that spot. Furthermore, the hornets deposit there the same or another substance which stimulates the initiation of building. It is proposed to name this substance a building initiating pheromone. A distinction should be made between the building pheromone released by the workers and usually initiating the building and similar pheromones released by the queen which are capable of either initiating or restricting the building.

Key Words—Hymenoptera, Vespidae, *Vespa orientalis*, *Polistes foederatus*, building initiation pheromones.

INTRODUCTION

Building activity of *V. orientalis* in artificial breeding boxes (ABB) that enable observation and experimentation has been extensively described (Ishay, 1964, 1965, 1973, 1975a,b,c, 1976a,b; Ishay et al., 1967; Ishay and Sadeh, 1975, 1977; Kisliuk and Ishay, 1977; Motro, 1977; Kugler et al., 1979; Motro et al.,

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1979). From the listed studies it is possible to summarize the onset of building by the Oriental hornet as follows: groups of workers introduced into the ABB on the first day after eclosion concentrate under the roof of the ABB and remain there for many hours. If the groups consist of 5–10 workers only, these aggregate in a single spot. However, if more numerous, then the workers usually split up into several subgroups which arrange concentrically around one or two workers that are situated centrally and remain resting almost motionless in this position for a day or two. Individual workers from these subgroups occasionally descend from their perch to feed themselves as well as to bring food and drink to their stationary mates. At the site where the workers congregate they also commence building on the 2nd–3rd day of life. The building proceeds as follows: one (or more) of the workers in the subgroups imbibes considerably (for 3–5 min) from the sugar solution offered it *ad libitum*, then approaches the clump of clay soil provided for building purposes, scrapes off with its mandibles a number of morsels which it moistens with its saliva and kneads into a soft, muddy pulp. The masticated building material is next deposited in the resting place of the group, usually the center of the roof. This is followed by additional mud clumps, provided by one or more workers, which together are molded into the pedicle and the cells of the comb. Within the latter the workers deposit unfertilized eggs which ultimately hatch (male hornets).

In *V. orientalis* and other Vespinae described (Montagner, 1964; Wilson, 1971; Spradbery, 1973), the building is clearly geotactic, commencing usually at the vertex of the nest and proceeding downwards in the direction of the earth's center of gravitation. Even if for any reason the building does not start from the top but rather from a pedicle fastened to the side of the nest, the cells making up the subsequent comb are directed towards the gravitational force.

In the ABB the workers of *V. orientalis* build on a fixed substrate and so also do various wasp species in nature. There are, however, some exceptions. For instance, *D. media* in Eurasia and species of *Dolichovespula* and *Paravespula* in the U.S.A. fasten the nest to the branches of trees as well as to the stems of perennial plants. Wasps of genus *Polistes* ordinarily construct the comb underneath the drain pipes of houses (i.e., fastened to a fixed substrate) in which case the cells open downwards, towards the center of gravity as in Vespinae, but they may also attach the combs to the stems of annual plants or to shrubs as *Verbascum sinuatum* and *Inula viscosa*, all of which sway in the wind. Such combs as are attached to plants are constructed at right angles to the stem of the plant so that the cell apertures are usually in a line parallel to the surface of the earth. Thus in genus *Polistes* we may encounter a gamut of comb construction wherein at the one extreme the cells face down and at the other they face out tangentially to the earth's surface. For *P. foederatus*, comb building under laboratory conditions has been described by Perna (1976) and Perna et al. (1979): under conditions in nature there is only one female and she is the one who initiates every new cell, but for wasps kept in captivity, the

hierarchy is not always clear. However, if there is a clear one, the α female starts the building and dominates the other females. In fact, dominance and hierarchy in the *Polistes* species is reflected in two phenomena: (1) the α female initiates the cells, and (2) she dominates the other females by means of "antennal clashing."

Upon removal of a nest built under laboratory conditions either by workers of *V. orientalis* or by α females of *P. foederatus*, the wasps build anew in the same spot as before. We presume that at the resting place of the wasps there is deposition upon and within the contact substrate of some hornet worker or wasp female secretion. We further assume that this secretory material is responsible for the initiation of building at this site. The present paper describes observations and experiments which corroborate these assumptions.

METHODS AND MATERIALS

With *V. orientalis*, the observations and experiments were carried out in Israel in a vesparium of such dimensions as to allow clear viewing of the breeding boxes under optimal temperature and humidity as detailed previously (Ishay et al., 1967; Ishay and Ruttner, 1971; Ishay, 1973). Work with the other species of Vespinae, namely *V. crabro*, *P. vulgaris*, *D. saxonica*, and *D. media* was carried out in Germany in the Institut für Bienenkunde, Oberursel/Taunus of the Zoological Institute, Frankfurt University. As for the work on *P. foederatus*, this was done in the Institute of Zoology, Florence University, Italy.

The experiments were run in the following manner: in the case of Vespinae (1) entire colonies, including a fertile queen, combs, brood and adult workers, were ether-anesthetized in situ in the field and then transferred to artificial breeding boxes (ABB). The transfer and the subsequent feeding of the wasps were as described by Ishay (1964); (2) within the ABB, groups of 10–300 workers of uniform age were maintained together from the moment of eclosion (from the native translocated comb); in the case of Polistinae, females of *P. foederatus* were transferred from the field, as described before, together with the embryonic combs which they had started to build at the beginning of the season. It should be mentioned that such combs contained brood at various stages of development but no adult workers. The transfer to the laboratory and the subsequent feeding were done as described by Perna (1976) and Perna et al. (1979).

The experiments and observations on Vespinae were run on at least 7 colonies of wasps or hornets and 10 groups of workers per each species. With *P. foederatus*, the observations were made on 32 females which had each constructed an embryonic nest prior to their translocation to the laboratory wherein they were usually kept in groups of 4 per ABB.

The experiments intended to restrain *V. orientalis* workers within a fixed spot of the ABB (in order to start them building there) were carried out as follows: (1) Restraint upon a glass plane: a glass sheet was introduced into the ABB and glued onto any one of the walls except the floor, or alternatively 1–3 test tubes were introduced (with or without sugar solution which is the usual food staple of wasps or hornets in the ABB). (2) Creation of an optimal temperature within a focal point of the ABB: this was accomplished by directing a light beam from a red lamp into the ABB, thereby creating a thermal gradient with a high of 31–32° C vs. the ambient temperature of 28° C. (3) Creation of a stronger magnetic field than that ordinarily extant in the ABB: for this purpose the ABBs were placed on a solenoid container producing a magnetic field of 1.34 oersted (Ö) vs. the normal 0.32 Ö, i.e., producing an additional static magnetic field in the horizontal direction (Kisliuk and Ishay, 1977). (4) Creation of a gravitational resultant higher than that of the earth's, i.e., hypergravity: this was accomplished by placing the wasp-populated ABB on the arms of a specially constructed centrifuge with the resultant at the arm tip during spinning ranging between 1.5 and 2 *g* (Ishay and Sadeh, 1975; Ishay and Sadeh, 1977). Young hornets have a tendency to concentrate in one corner of the ABB, while spinning sometimes even on the floor. (5) Anesthesia: the wasps were anesthetized with ether and left somnolent on the floor of the ABB for several hours. (6) From sites of previous natural comb construction or from sites within the ABB where combs had been constructed, particles of soil or of other building material adhering to the substrate were scraped off and transferred to other locations within a new hornet-inhabited ABB. (7) Crowding: in ABB normally housing 10–20 hornets, 300 workers of uniform age were introduced. Presence of so many individuals within a single ABB limits freedom of movement and consequently the hornets assemble in groups of 10–15 individuals at various sites of the ABB, be it the vertical walls or underneath the roof. After several hours these initially temporary assembly points become permanent, with members of each group spending most of their time at the affixed site. To prevent the hornets from fighting over the food or building material, one places around the permanent assembly joints numerous test tubes with sugar solution, a large number of meat morsels, and an abundance of soil clumps—the essentials for normal nest activity. This enables the hornets to stay in one place most of the time, with only occasional excursions of single hornets to the food or building supplies.

RESULTS

In all instances where colonies of Vespinae were maintained complete and intact, the adult wasps—queens as well as workers—invariably remained "attached" to the combs even when these were not fastened in the correct geotactic orientation. The workers initiated new cells at the edges of the comb, but

only in the vicinity of cells already occupied by brood. Each new cell was built from the start by one or more workers to about 1/3-1/2 its final height, and only after the queen deposited an egg in it did the workers continue to lengthen the cell in accordance with the needs of the developing brood. There was absolutely no building of new cells except in the vicinity of intact, brood-filled cells. Neither was there any building of new cells so long as the comb contained intact cells vacant of brood, as, for instance, cells that had eclosed workers or ones from which the larvae for some reason (disease or stunting) had been ejected. Whenever a cell was vacated, it was oviposited into by the queen within a few minutes—right after the workers cleaned it out. In all species but *V. orientalis*, death of the queen resulted in immediate cessation of new cell building, although there was still construction of envelopes around the combs as well as completion and mending of damaged cells.

Groups of Workers. In all species but *V. orientalis*, when the workers are kept in groups (without combs), they tend to aggregate about a central point which they warm by brooding (Ishay, 1973) and also brood over any pupae of their own or other species that had been removed from their cocoon and offered them (Ishay and Ruttner, 1971). The assembled wasps create at their resting point a uniform temperature of about 30°C, but they do not build combs with cells under these conditions. If such worker groups are offered a brood comb, they show a clear tendency to depart the assembly point, "climb" the comb and attend the contained brood. They continue to nurse the brood until its maturation and eclosion; they also oviposit unfertilized eggs in the vacant cells, build envelopes around the comb, mend existing cells as the need arises, but do not build new cells.

In the case of *V. orientalis* colonies, death or absence of the queen creates an immediate commotion in the nest (Ishay, 1964). Apart from manifestations of aggressive behavior, the workers in the orphan nest display within 1-2 days a sudden "awakening" of building activity; they energetically commence building new cells both around existing combs as well as in the surrounding area and immediately upon completion of the cell foundations, workers can be seen to take possession of various "segments" of the comb, ovipositing therein unfertilized eggs. It is clearly evident that all this building activity is uncontrolled, because it persists despite the fact that the existing combs may contain a plethora of vacant cells.

Groups of *V. orientalis* workers maintained in ABB at a temperature of 28-30°C, tend to aggregate usually right under the roof of the ABB, and after 2-3 days commence building a comb whose pedicle is affixed to the roof and which has a distinct geotactic orientation (Ishay, 1975a; Ishay and Sadeh, 1975, 1977).

All the procedures mentioned in Methods and Materials can induce such hornets to congregate in a particular spot and commence building there. These include the introduction of glass tubes (Figure 1), optimal temperature,

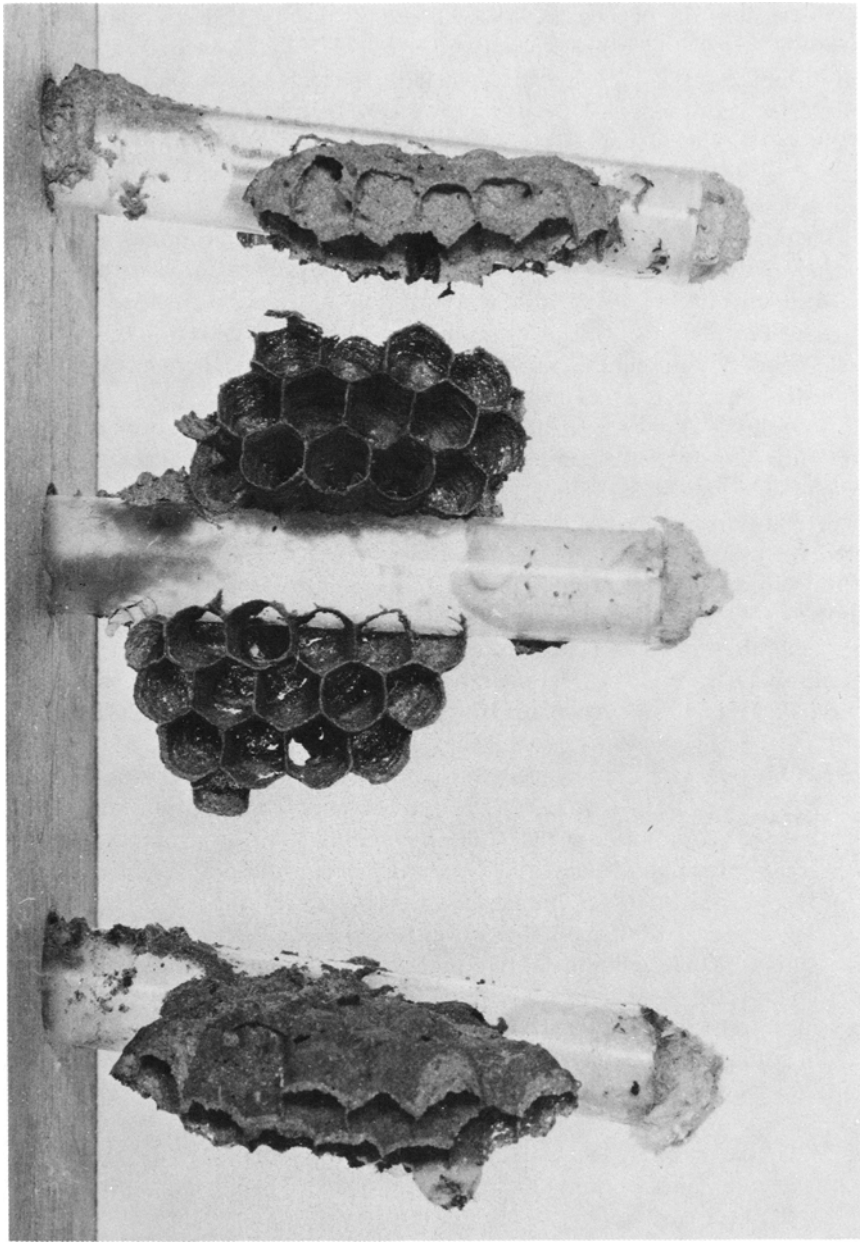

FIG. 1. Comb built by hornets (*Vespa orientalis*) on glass tubes. The central tube is turned by 90° in order to expose the built cells.

enhanced magnetic field, hypergravity, prolonged ether anesthesia, and overcrowding. Especially noticeable, however, is the fact that if wood or soil scrapings are taken from a previous resting spot within the ABB and placed elsewhere, the hornets will commence building in the new site. It should also be mentioned that hornets that had undergone prolonged anesthesia on the floor of the ABB will, upon waking, commence building not in the usual manner, i.e., from the roof down, but rather on the floor of the ABB—certainly an extraordinary phenomenon. Hornets that are kept in crowded conditions may build several combs, in fact, as many combs as there are resting places for the various overcrowded subgroups (Figure 2).

Males introduced into the ABB together with workers separate immediately into strictly male aggregates and do not participate in any of the building activities.

P. foederatus females, when transferred together with their embryonic comb into the laboratory, usually do not remain on their original comb but instead start building a new one within the breeding box. If the initial comb is removed but the pedicle connecting the comb to the substrate remains or even if only traces of the juncture of the pedicle to the substrate remain, the females commence building anew in the same spot a second or third time. When scrapings from the base of the embryonic nest are deposited in another place, the females start building there.

If by chance two or more females are kept together in the same container during the period of the collection from the field, upon release in the labora-

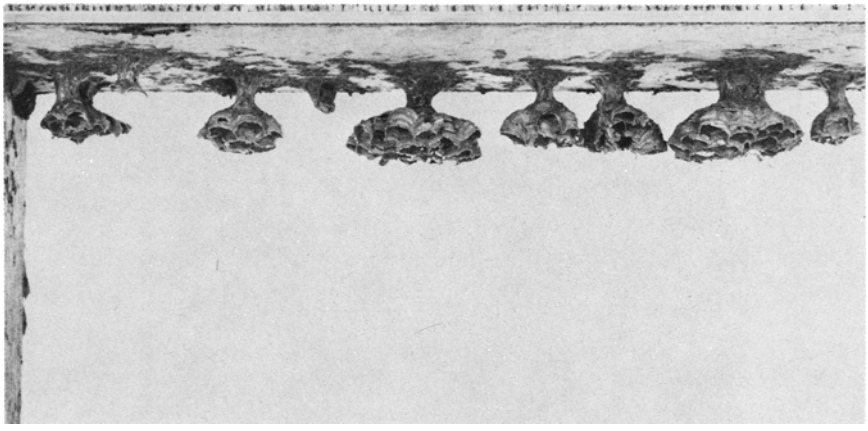

FIG. 2. Several combs built by hornet workers under the roof of the artificial breeding box. The hornets were kept crowded for several days.

tory, occasionally one of them—usually the α female—starts building cells on the thorax of the other (Figure 3). Shortly after she oviposits in the built cells and subsequently nurses the developing brood as if in the natural nest (Figure 4). Invariably before the α female starts to build on the thorax of the other female—the subordinate or β female—she first dominates her. This she does by beating with her antennae on the β female, while the latter assumes the subordinate position—bending the head down and remaining stationary (see Pardi, 1940, 1942, for details of dominancy behavior). The β female remains stationary till the α female completes the building, the egg laying or the brood inspection. Once the α female descends from her back, the β female commences using her forelegs to remove the cells built on her thorax. When ultimately the β female succeeds in removing every trace of building material from her body then a new comb is built somewhere else within the breeding

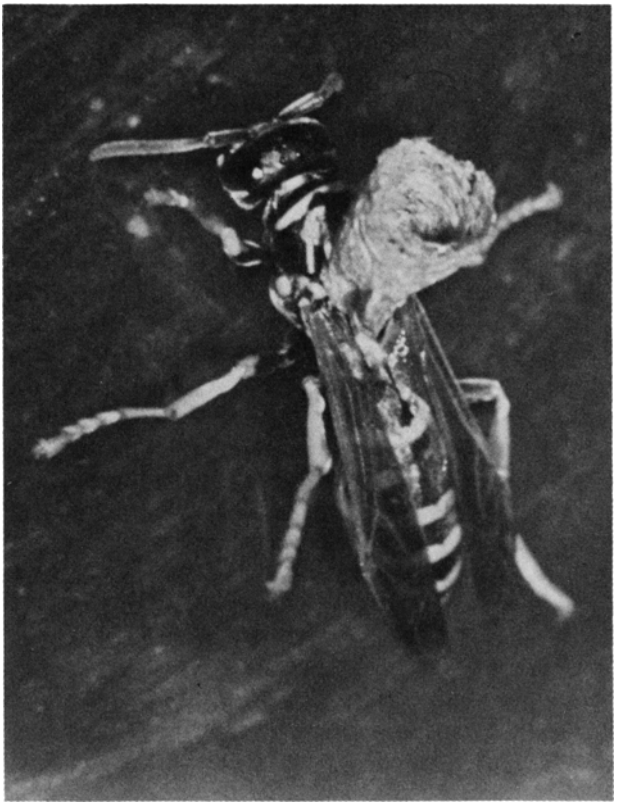

FIG. 3. Cell built by one α female of *Polistes foederatus* on the thorax of a β female of the same species.

FIG. 4. The α female inspects the cell laid in the cell previously built on the β female.

box. The same results were obtained with *V. orientalis* workers kept crowded together for several hours.

When workers eclode, they join the females in the care-taking of the comb and brood. Workers of *P. foederatus* never initiate building activities; however, after the α female initiates the building of new cells and oviposits in them, then the workers commence enlarging the cells to accommodate the developing brood.

DISCUSSION

The fact that wasps (and hornets) assemble into groups both in the queen-right, natural nest, as well as in the ABB containing workers only, suggests that we are dealing here with a substance(s) for which we propose the name wasp assembly pheromone(s) (WAP). Assembling pheromones have been reported for various groups of animals (Shorey, 1976). While the chemical composition of the substance(s) in hornets is as yet undetermined, it is clear that both the fertilized queens and the workers possess it and that it acts as a releaser pheromone. Work is now in progress to determine the chemical nature of such pheromone(s) in *V. orientalis*. It is not apparently the same as queen pheromone (Ishay et al., 1965; Ikan et al., 1969) since the queen phero-

mone produces inhibition of the oviposition. Meanwhile it may be noted that this suggested pheromone does not induce males to assemble together with the workers and that the former apparently possess their own pheromone which induces similar and separate male assembly. The males are thus insensitive to the substance(s) released either by the fertile queen or by the workers of their own nest. They are, however, sensitive to the presence of unfertilized young queens since they congregate around them outside the nest and copulate with them (Ishay, unpublished observation).

In all the examined species of Vespinae, building of the cells is relegated to the workers (only in the embryonic nest is the queen alone responsible for the building and after eclosion of the workers she rarely leaves the nest). Inasmuch as the workers build the foundation of a cell and, immediately afterwards, the queen oviposits into it, we believe that the phenomenon may be explained in the following manner: we postulate that the building capability of the workers, at least when they are numerous within the nest, is greater than the oviposition capability of the queen. Consequently the queen exerts a depressing effect on the building by the workers, modulating it to correspond to her own rhythm of oviposition.

In *P. foederatus* and in other *Polistes* species in which this phenomenon has been reported (Pardi, 1948; West-Eberhard, 1969) the process has simply been modified in the following respect: the α female inhibits both the subordinate female as well as the workers from starting to build new cells so that they can only continue to accrete onto and complete cells started by the α female. Here, too, it would seem that the amount of new building is geared to the oviposition capacity of the queen (= α female). Such inhibition of the initiation of undesirable building can probably be attained in various ways. It is therefore conceivable that in Vespinae the queen regulates the building (i.e., inhibits the building of new cells) by the release of a pheromone for which the name building depressor pheromone (BDP) is proposed, and that only when the release of such BDP is stopped occasionally can the workers build new cell. If this is true, then it seems reasonable to assume that as the queen oviposits, she ceases to release BDP and this allows the workers time to commence building new cells, i.e., to give expression to their *spontaneous* building capacity.

In *P. foederatus* the α female inhibits the start of building by other members of the nest. This inhibition can possibly be achieved by mechanical interference, i.e., the α female does not enable any other female to start building. (It is possible that workers of this species simply do not "know" how to start new cells.)

Hence, it is reasonable to assume that what is attained in Vespinae by way of pheromones is attained in Polistinae via behavior, mainly aggressive behavior on the part of the α female.

In any event, the completion of cells by the workers is performed in accordance with the needs of the developing brood and does not pose a challenge to the status of the queen. We are still in the dark as to why, upon death of the

fertile queen, we did not observe building of new cells in other species of Vespinae apart from *V. orientalis*. There may be various reasons for this failure to build, but the simplest explanation could be that the workers were not subjected to the optimal conditions necessary for eliciting building. Roland (1976) has in fact reported the building of new cells in the nest of *P. germanica* following death of the queen. In the case of *V. orientalis*, it is clear that cell building occurs both in queenless nests as well as by groups of workers maintained in the absence of a prior brood comb. Insofar as building commences at any spot at which the workers had assembled for several hours, it would seem that the start of building is triggered by the deposition, at the resting place, of a substance released by the workers and absorbed by the substrate at the point of contact of the workers' bodies with the substrate. We propose to call the material(s) building initiating pheromone(s) (BIP).

V. orientalis workers seem to prefer building on a glass sheet, perhaps because they are attracted to the electrostatic charge on the surface of the glass or, alternatively, because the substance(s) released from them as they choose to stand on the glass remains concentrated on the glass without being absorbed as by other substrates. Perhaps their BIP is more concentrated on the glass tubes also because they repeatedly visit the tubes in order to drink the sugar solution in them, and while walking on the tubes deposit BIP on them.

Instances where α females of *P. foederatus* build cells on the thorax of other females can be explained as follows: (1) the mere movement of the β female is probably not prohibitory to the start of building because under natural conditions as well, the α female commences building on the stems and branches of perennial shrubs that sway in the wind. The building proper occurs probably mainly while the branches or stems are stationary, and analogously in the case of β females—when the α female suppresses the movement of the β female for building purposes; (2) as for the actual reason why the one built on the back of the other, we suspect that in the course of their collection from the field the two females spent several hours together under crowded conditions within the collecting bag; they may have leaned one against the other and by chance the one female secreted building pheromones on the back of the other, so that several days later, she was stimulated to build at the site where the pheromone was deposited, namely, the back of the other. We offer in support of this concept the fact that even when the pedicle of a comb is removed in the early stages of building, the wasps continue to build anew in exactly the same spot and will do this again and again, which strongly suggests the deposition at the site of building of a substance triggering the initiation of building (BIP).

Building initiating pheromones belong, according to Wilson and Bosserts' (1963) classification, to the category of primers, and their effect is exerted usually several days after the assembly of workers in the prebuilding area.

Regarding our postulated building initiation pheromone(s), we have not

as yet determined the chemical composition but it is probably a substance of low volatility and fair thermostability because it continues to be active (i.e., it causes the hornets to start building where it is imbibed) even several weeks after its secretion and at a temperature of 28–30° C.

Butler et al. (1969) described a footprint pheromone released by *P. vulgaris* wasps and by bees at the site where they tread as they leave or enter the nest or hive. This substance is absorbed by the substrate and subsequently induces the insects to prefer walking over the impregnated spot rather than over any other spot. It does not, however, induce assembly of the insects as we have described herewith for hornets and wasps. Most likely, the role of the footprint pheromone is to guide the insects to a particular path or corridor, much the same as do the trail pheromones of ants (Wilson, 1971; Blum and Brand, 1972).

While we have not yet been able to locate the exact source of the substance(s) which we have named BIP, there are two possible origins for it: (1) it might be released from tarsal glands, impregnating the substrate as the wasps rest in a particular spot; (2) it might derive from the paired glands on the 6th gastral sternite near van der Vecht's organ for which Spradbery (1973) has proposed the name van derVecht's glands. These are composed of discrete cells which communicate with the outer surface of the sternite through long fine ducts. Conceivably the wasps rub their legs against these glands and thereby transfer their secretion to the substrate.

The use of a bodily secretion to mark the site where building should be initiated has been reported also for other wasps. Neuman (1975) described the swarming behavior of various species of neotropical social wasps (Polybiini), noting that as they landed at a site where a new nest was ultimately founded, "some wasps made straight, short runs while dragging the ventral surface of their gaster on the substrate, depositing a scent trail substance." An essentially similar account is given also by Jeanne (1975) on another species of the same group (*Stelopolybia areata*). Apart from the behavioral aspects, it would seem that such dragging of the ventral gaster is intended to effect deposition of a substance like our so-called building initiating pheromone. We suspect that we are dealing with a general phenomenon which probably occurs in many Vespidae and *Bombus* sp. (Röseler and Röseler, 1977), and this remains to be confirmed by further study.

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SIGNIFICANCE OF THE PYRROLIC NITROGEN ATOM IN RECEPTOR RECOGNITION OF *Atta texana* (BUCKLEY) (HYMENOPTERA:FORMICIDAE) TRAIL PHEROMONE AND PARAPHEROMONES[1]

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Abstract—Calculations of the charge of the pyrrolic nitrogen atom in the trail pheromone of *Atta texana*, methyl 4-methylpyrrole-2-carboxylate, as well as 17 analogs, showed that the most active compounds all had the same charge of −0.51 electrons on the nitrogen atom. It is suggested that the close value for this charge may be important in recognition by the receptor.

Key Words—*Atta texana*, trail pheromone, quantum calculations.

INTRODUCTION

The chemorecognition of a small molecule by a specific region of a large macromolecule is one of the most basic phenomena of living systems. This property has been demonstrated many times in both enzymology and the mechanism of drug action, but many examples are also available from studies of chemical communication in insects. Two of the main properties of both the small molecule and the receptor site are probably that their shapes and their electron densities must be complementary.

A major trail pheromone of the ant, *Atta texana* (Buckley), has been identified as the 2,4-disubstituted pyrrole, methyl 4-methylpyrrole-2-carboxylate (I) (Tumlinson et al., 1971), and trail-following activity studies of synthetic analogs of this compound have been conducted by Sonnet and Moser (1972, 1973). Their main conclusions concerning the stereochemical requirements for behavioral activity may be summarized as follows: (1) the pyrrole ring of the trail pheromone is planar; (2) the carbonyl carbon, both oxy-

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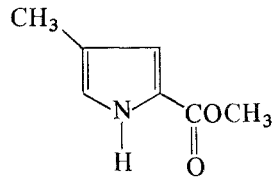


I

gen atoms, and the 4-methyl group, are coplanar with the pyrrole ring; (3) the pyrrole ring and the carbonyl group are conjugated; (4) the carbomethoxy group can have two conformations; (5) for activity to be retained, the substituent on position 2 cannot be enlarged but can be smaller with similar properties and the substituent on position 4 cannot be removed but can change its size and polarity; and (6) the receptor can distinguish the pyrrolic N from the ring carbons.

It was the last point mentioned above that attracted our attention. It seems likely that the pyrrolic N is important in the process of chemorecognition by the receptor site. We wondered whether the charge on this nitrogen in the most active compounds was the same, and distinct from that of the inactive compounds. By means of the extended Hückel molecular orbital procedure we calculated the charge on the nitrogen of many of the substituted pyrroles synthesized and assayed by Sonnet and Moser (1972, 1973) in the hope that this may add to the stereochemical conclusions they arrived at as requirements for activity. Our results show that the three most active compounds all have an identical charge on the pyrrolic nitrogen. It therefore seems possible that this parameter is important as an additional criterion of activity.

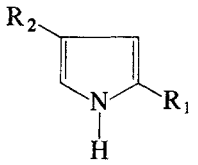
METHODS AND MATERIALS

The extended Hückel calculations on compounds I–XVIII were carried out according to standard methods (Hoffmann, 1963, 1964a,b,c) on an IBM 360 computer. The geometry of the pyrroles was generated from standard bond length and bond angle data, and the coulomb integrals (see Table 1) were obtained from valence orbital ionization potentials (Basch et al., 1965). The basis atomic orbitals were Slater-type atomic orbitals with the orbital exponents taken from optimization of the appropriate SCF calculations (Clementi and Raimondi, 1967).

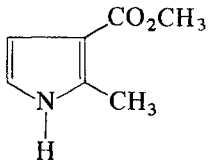
The reasonance integrals, which correspond to the off-diagonal elements of the Hamiltonian matrix, were computed from the Wolfsberg-Helmholz arithmetic mean formula (Wolfsberg et al., 1952)

$$H_{ij} = KS_{ij}(H_{ii} + H_{jj})/2$$

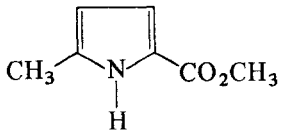
where K is 1.75 and H_{ii} and H_{jj} correspond to the coulomb integrals and S_{ij} is



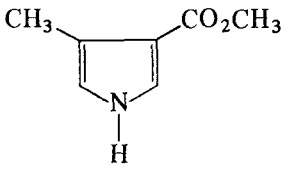
I $R_1 = CO_2CH_3$; $R_2 = CH_3$
II $R_1 = CO_2CH_3$; $R_2 = Cl$
III $R_1 = CO_2CH_3$; $R_2 = C_2H_5$
IV $R_1 = CHO$; $R_2 = CH_3$
V $R_1 = CHO$; $R_2 = Cl$
VI $R_1 = CO_2CH_3$; $R_2 = CHO$
VII $R_1 = COCH_3$; $R_2 = CH_3$
VIII $R_1 = CHO$; $R_2 = CHO$
IX $R_1 = CO_2CH_3$; $R_2 = CO_2CH_3$
X $R_1 = CHO$; $R_2 = CO_2CH_3$
XI $R_1 = CO_2CH_3$; $R_2 = COCH_3$
XII $R_1 = CO_2C_2H_5$; $R_2 = CH_3$
XIII $R_1 = CH_3$; $R_2 = CO_2CH_3$



XIV



XV



XVI

XVII $R_1 = CO_2CH_3$; $R_2 = H$
XVIII $R_1 = CHO$; $R_2 = H$

TABLE 1. VALUES OF COULOMB INTEGRALS FOR ATOMIC ORBITALS

Atomic orbital	Coulomb integral H_{ii} (eV)
Carbon 2*p*	−11.4
Carbon 2*s*	−21.4
Oxygen 2*p*	−17.9
Oxygen 2*s*	−35.30
Nitrogen 2*p*	−14.32
Nitrogen 2*s*	−27.50
Chlorine 2*p*	−15.8
Chlorine 2*s*	−24.00
Hydrogen 1*s*	−13.6

the overlap integral between orbitals i and j. With this format, both the total energy for each molecule and the charge on each atom were calculated. Charges were computed using a Mulliken population analysis (Mulliken, 1955, 1962).

The method of calculation does not permit us to calculate the nitrogen charge of the bromo derivative tested by Sonnet and Moser (1972, 1973).

RESULTS AND DISCUSSION

Payne (1974) has suggested that the more the structure of a parapheromone varies from the structure of the true pheromone, the greater the amount needed to elicit a significant electroantennogram. Furthermore, the EAG response is usually considered to reflect the behavioral response. Sonnet and Moser (1972) bioassayed the various pyrroles at three different concentrations. At the highest concentration some of the compounds, including the

TABLE 2. RELATIONSHIP BETWEEN CALCULATED CHARGE ON PYRROLE NITROGEN ATOM OF VARIOUS SUBSTITUTED PYRROLES AND THEIR OBSERVED BIOLOGICAL ACTIVITY AS TRAIL-FOLLOWING SUBSTANCES

Compound	Nitrogen charge (electrons)[a]	2,4-Disubstituted	Activity (0.4 ng/μl)[b]
I	−0.51	Yes	+++
II	−0.51	Yes	+++
III	−0.51	Yes	++
IV	−0.52	Yes	−
V	−0.52	Yes	−
VI	−0.48	Yes	+
VII	−0.52	Yes	+
VIII	−0.48	Yes	−
IX	−0.49	Yes	−
X	−0.49	Yes	−
XI	−0.49	Yes	−
XII	−0.51	Yes	−
XIII	−0.53	Yes	−
XIV	−0.57	No	−
XV	−0.59	No	−
XVI	−0.49	No	−
XVII	−0.51	No	−
XVIII	−0.51	No	−

[a]The relative precision of these values is ±0.001. They are rounded to the nearest 0.01 for convenience.
[b]Data from Sonnet and Moser (1972).

natural trail pheromone, were found to cause repulsion. It should be pointed out that the published bioassay results are only roughly quantitative and no corrections are possible for effects such as differences in volatility.

Our interest was concerned primarily with determining whether any calculable parameters of those compounds which mimicked the natural trail-following pheromone were unique. We therefore decided to consider only those compounds that showed high activity at the same concentration as the natural pheromone, rather than consider all the compounds that showed some activity, no matter what the concentration.

With this limitation imposed, only three compounds are considered highly active at 0.4 ng/μl (Sonnet and Moser, 1972; compounds I, II, and III in Table 2). Each of these three compounds has a charge of -0.51 electrons on the pyrrolic nitrogen atom. None of the compounds which showed good activity at 40 ng/μl (IV-VI) has this value. Of the other three compounds with a nitrogen charge of -0.51 electrons, compound XII has an ethyl group in position 2 which is apparently too large for activity (Sonnet and Moser, 1972) and neither XVIII or XIX has the necessary 2,4-disubstitution pattern.

We therefore suggest, although not without caution, that for optimal trail-following activity to be demonstrated, the receptor requires the charge on the pyrrolic nitrogen atom to be a very precise value in a compound with the correct steric properties. If good quantitative data on a series of compounds can be obtained with a bioassay, quantum calculations on the compounds may provide some insight into their mode of action and also have predictive value for compounds as yet untested.

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SYNTHESIS OF MULTISTRIATIN ENANTIOMERS AND THEIR ACTION ON *Scolytus multistriatus* (COLEOPTERA:SCOLYTIDAE)

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Abstract—A pheromone mixture containing enantiomerically pure (−)-α-multistriatin of known absolute configuration prepared by total synthesis was found to be as attractive as the natural pheromone to the smaller European elm bark beetle, *Scolytus multistriatus*, a vector of Dutch elm disease. Its (+)-enantiomer, on the other hand, was no more active than controls in both laboratory and field tests, and at high levels it appeared to inhibit the response to the (−)-enantiomer.

Key Words—Pheromone, enantiomers, α-multistriatin, synthesis, activity, *Scolytus multistriatus*, European elm bark beetle, Coleoptera, Scolytidae.

INTRODUCTION

One of the constituents of the aggregation attractant of the European elm bark beetle, *Scolytus multistriatus* (Marsham), is α-multistriatin (2-*endo*,-4-*endo*-dimethyl-5-ethyl-6,8-dioxabicyclo[3.2.1]octane, 1α). This molecule has been the subject of several studies since its discovery by Pearce et al. (1975) because of its interesting chemistry and its potential use (as part of an attractant blend) in controlling the beetle which transmits Dutch elm disease. The stereochemistry of α-multistriatin has been elegantly worked out by lanthanide-induced shifts in the NMR (Gore and Armitage, 1976) and two stereospecific syntheses (Gore et al., 1975; Elliott and Fried, 1976b). Attention has since been focused on the synthesis of optically active α-multistriatin, in order to es-

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tablish its absolute configuration and enantiomeric purity (Elliott and Fried, 1976a; Mori, 1976; Pearce et al., 1976; Cernigliaro and Kocienski, 1977).

The biological activity of enantiomers has recently been shown to be important in optimization of the activity and specificity of insect pheromones. Often, only one enantiomer is active (see Wood et al., 1976, and references therein). However, in the case of the ambrosia beetle, *Gnathotrichus sulcatus* (LeConte) (Borden et al., 1976) enantiomers act synergistically in attraction. Conversely, the unnatural enantiomer inhibits the response of the Japanese beetle, *Popillia japonica* Newman (Tumlinson et al., 1976), the 6-spined engraver beetle, *Ips calligraphus* (Germar); and the gypsy moth, *Lymantria dispar* (L.) (Vité et al., 1976). The synthetic racemic pheromones for these insects were not competitive with natural material. Since previous experiments provided a method for the synthesis of optically active α-multistriatin, we undertook a study of the biological activities of these molecules.

METHODS AND MATERIALS

Multistriatin was synthesized in both pure enantiomeric forms by the methods previously described (Elliott and Fried, 1976a,b), according to the outline shown in Figure 1. The multistriatin synthesized and used in the experiments described here was an 85:15 mixture of α and γ isomers that was pure insofar as it was free from contamination (>99%) with the other antipodes. It had previously been reported (Lanier et al., 1977) that the γ isomer is biologically inactive, so it was not deemed necessary to painstakingly separate the two isomers. The mixture still allows discrimination to be made between activity due to either enantiomer. Thus, whenever "multistriatin" or "M" is mentioned, it designates this 85:15 mixture of optically pure isomers.

The resolution of (5*RS*,6*SR*)-2,2,5-trimethyl-6-hydroxy-1,3-dioxepane (Figure 1) was performed with both (+)- and (−)-α-phenethyl isocyanate, and the twice recrystallized products were shown to be greater than 99% pure by the NMR method reported previously (Elliott and Fried, 1976a). The products, after reductive hydrolysis of the urethane grouping with $LiAlH_4$ and purification, had specific rotations of +45.3° and −45.6°, which compare well with those previously determined (+43.5°, −43.4°) (Elliott and Fried, 1976a). These materials were carried through the synthetic sequence (Figure 1) to provide an 85:15 mixture of (+)- and (−)-α:γ multistriatin in 28% overall yield. Minor modifications of the alkylation of diethyl ketone with the chiral iodide 6 were made in order to overcome the incomplete alkylation previously observed (Elliott and Fried, 1976b). Hexamethylphosphorus triamide (HMPA) was added simultaneously with the iodide 6 as cosolvent to provide two equivalents of HMPA to one of the preformed enolate. Successful hydrolysis of excess tosyl chloride in the conversion of 4 to 5 must be carried out by adding 5%

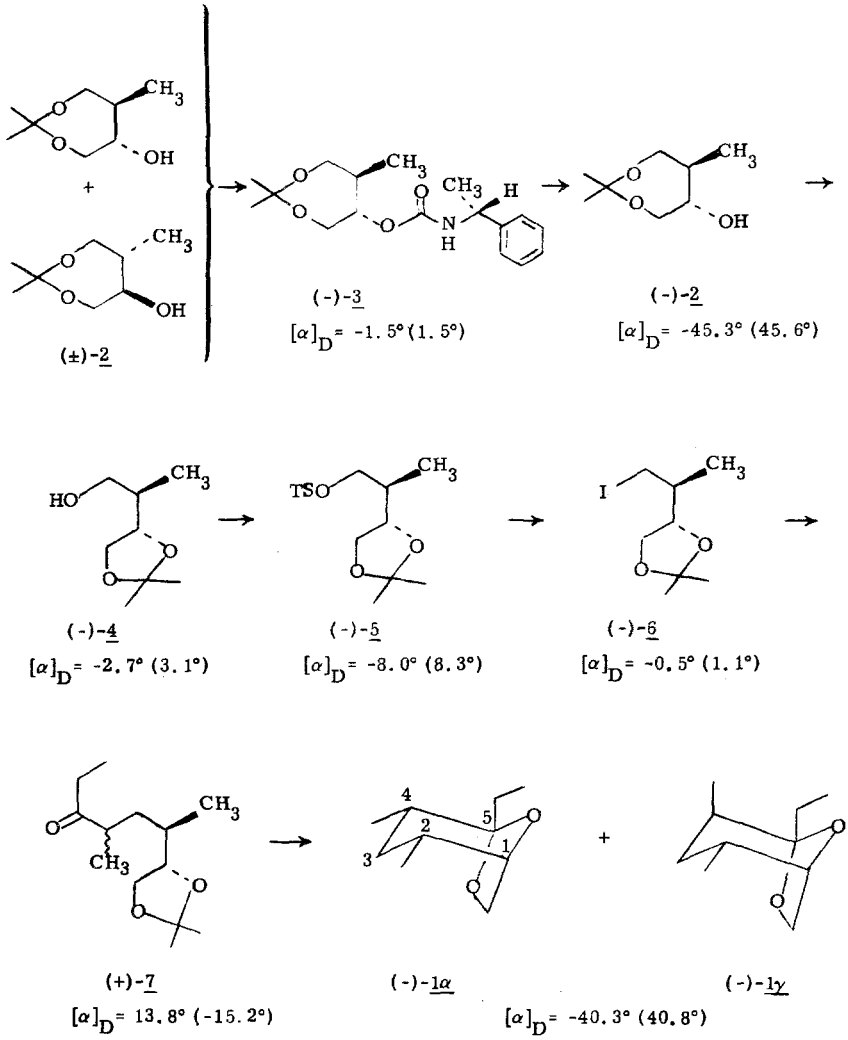

FIG. 1. Synthetic sequence leading to (−)-multistriatin. Specific rotations are given below the structure for each compound; those of their antipodes are given in parentheses.

(v/v) cold water to the pyridine solution, with stirring for 5 min before work-up, rather than by adding the pyridine solution to water.

All rotations and yields were determined on material purified by distillation or chromatography (tosylate 5) and shown to be at least 98% pure by 270 MHz NMR. The multistriatin isolated had specific rotations of +40.3° and −40.8° (c = 1.0, hexane), which are consistent with pure α-multristriatin having specific rotations of +46° and −46°. These data agree well with those of

Pearce et al. (1976), who calculated specific rotations for pure α-multistriatin of +44° and −47° (in hexane) from preparations which were 47% and 56% optically pure, respectively (Pearce et al., 1976).

It had previously been determined by the Horeau method that (−)-2 had the (5*R*,6*S*) configuration (Elliott and Fried, 1976a). By carrying this material through the synthetic sequence to multistriatin using reaction conditions involving no inversion of configuration at either chiral center, it can be concluded that the natural (−)-α-multistriatin (1α) has the (1*S*,2*R*,4*S*,5*R*) configuration. This assignment agrees with that made independently and by different methods by Pearce et al. (1976), Mori (1976), and Cernigliaro and Kocienski (1977).

The biological experiments designed to test the activity of pure multistriatin enantiomers were of two basic types: laboratory bioassays utilizing beetles from colonies, and field studies in which wild beetles were attracted to traps dispensing the pheromone. All biological studies were conducted in Syracuse in 1976 and 1977.

The laboratory bioassays utilized a Moeck (1970) olfactometer and the methods of testing and criteria for response described previously (Pearce et al., 1975; Lanier et al., 1977). Dosages of attractants were given in beetle-hour (BH) equivalents (Lanier et al., 1977): 4-methyl-3-heptanol (H, commercially available), 0.20 ng/BH; (+)- or (−)-multistriatin (M), 0.04 ng/BH; and α-cubebene (C) (distilled from cubeb oil and purified by GLC to 98%), 1 ng/BH. For testing, hexane dilutions of components were placed via 1-μl micropipets on strips of filter paper. Twenty-five male beetles were used in each test, which required 15 min. The insects were allowed to rest for 5 min between experiments; different beetles were used for each replicate. Responses were compared to a hexane extract of Porapak Q-collected volatiles emitted by virgin *S. multistriatus* females boring in elm logs (Peacock et al., 1975).

The field testing of the enantiomers was performed using the three-component attractant, including H (40 mg), (+)- or (−)-M (5 mg), and C (200 mg) (Lanier et al., 1977). A gross dosage of 10^6 BH of these substances dissolved in glass-distilled hexane was placed in a 7-ml polyethylene snap-top vial to be used as a bait. Release rates averaged 1000 female equivalents as monitored by weight loss, GLC, and attractiveness over time in the field. Baits were still active after termination of the experiment (30 days duration). Baits were placed in the center of a trap consisting of two adjacent sheets of polycoated white 46 × 66 cm cardboard treated with Stickem Special (Lanier et al., 1976). Trap panels were tacked around utility poles spaced 120 m apart with the long axis horizontal and the bottom of the trap approximately 3 m above the ground. Four traps were placed along each of four streets in Syracuse, New York, selected for their within-street uniformity in beetle catches in previous studies. Traps were rotated within each street so that each

treatment appeared once in each street replication and position. Captured beetles were removed from each trap at weekly intervals, counted, and sexed.

RESULTS AND DISCUSSION

In the initial series of laboratory bioassays (Table 1), (−)-multistriatin combined with 4-methyl-3-heptanol and α-cubebene [(−)-M + H + C] was as attractive as the natural attractant ($P < 0.05$). On the other hand, (+)-M + H + C was not significantly more attractive than the bipartite (H + C) control ($P > 0.4$). The (−)-M + H + C was significantly more attractive than H + C alone ($P < 0.02$), but the differences between (+)-M + H + C and (−)-M + H + C or the natural attractant were not quite significant ($P > 0.13$). These data indicate that the tripartite bouquet including (−)-M has full biological activity; (+)-M, on the other hand, adds little, if any, biological activity to the H + C mixture. This conclusion is consistent with the concept that (−)-M is the active enantiomer.

Figure 2 compares the dose-response relationships for (+)- and (−)-multistriatin. The maximum mean response difference is shown at 5.0 BH. At the 50 BH dosage, some beetles which appeared to be stimulated did not follow the air stream; at 500 BH, locomotion was inhibited and the few beetles which did meet the criteria for response proceeded toward the source very slowly and without klinotaxis. These data show that there is little difference between responses to (+)- and (−)-M at either very low or very high concentrations, presumably due to insufficient pheromone and adaptation of pher-

TABLE 1. RESPONSE OF *S. multistriatus* TO ATTRACTANT MIXTURES IN THE LABORATORY

Material[a]	Number replicates	Beetle response[b] (mean ± standard error)
Natural attractant	4	14.75 ± 0.75 a
(−)-M + H + C	5	14.60 ± 0.51 a
(+)-M + H + C	5	10.20 ± 2.40 a, b
(−)-M	2	11.00 ± 3.00 a, b
(+)-M	2	3.00 ± 0.71 c, d
H + C	5	7.80 ± 1.82 b, c
Air	5	1.00 ± 0.55 d

[a] Materials (M: α-multistriatin; H: 4-methyl-3-heptanol; C: α-cubebene) were as presented in 50 beetle-hours (BH) equivalents; see text for explanation of dosages.
[b] Number of beetles responding per 25 beetle replicate. Significant differences between responses ($P < 0.05$) are indicated where no letter is repeated in any two entries.

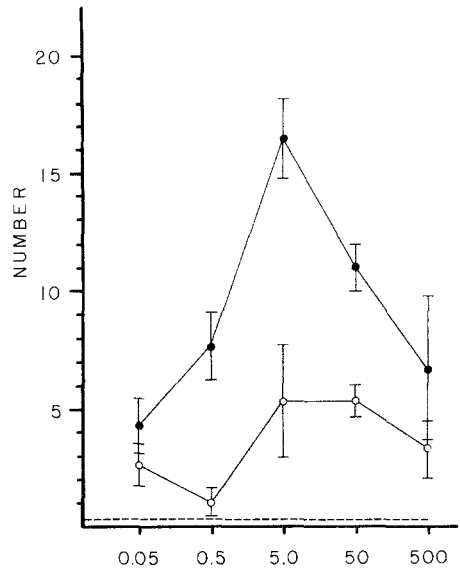

FIG. 2. Number of *S. multistriatus* responding in laboratory assays to enantiomers of multistriatin at concentrations from 0.05 to 500 BH while H and C were held at 12.5 BH. Closed and open circles indicate mean response (3 replicates of 25 male beetles each) for (−)- and (+)-multistriatin, respectively; vertical lines show standard errors.

omone acceptors, respectively. There appears to be no concentration of (+)-M that is as attractive as (−)-M at 5 BH; therefore the 5 BH dosage was used as standard in subsequent experiments.

Results from competition studies are shown in Table 2. These data indicate that (+)-M at 500 BH inhibits the biological response to (−)-M at 5 BH. Previous work (Pearce et al., 1975; Lanier et al., 1977) noted no difference between low concentrations of natural material and racemic multistriatin, but these comparisons involved only a twofold difference in concentration of (−)-M. As noted in the previous dosage-response tests, increasing the concentration of (−)-M from 5 to 50 or 500 BH also reduced response. It is clear that the declining response with increasing dosage of (−)-M in the previous test (Figure 2) is not attributable to changing the ratio of (−)-M and H + C; in fact, increasing dosages of H + C along with (−)-M may have added to the inhibitory effect shown in Table 2.

In the field tests (Table 3), the (−)-M + H + C treatment accounted for almost all of the beetles caught in each weekly collection and at each position. Of the 1662 beetles captured in the experiment, 98% were attracted to the (−)-M traps. It is evident that (+)-M at the concentration tested, is no more attractive to beetles in the field than either H + C or no pheromone at all. The

TABLE 2. RESPONSE IN THE LABORATORY OF MALE *S. multistriatus* TO VARYING CONCENTRATIONS AND RATIOS OF ATTRACTANTS

Material[a]	Number replicates	Beetle response[b] (mean ± standard error)
12.5 H + C	5	3.4 ± 0.75 c
5 (−)-M	12	12.0 ± 0.833 a
12.5 H + C		
5 (−)-M	5	5.6 ± 1.37 b, c
500 (+)-M		
12.5 H + C		
50 (−)-M	6	7.2 ± 0.90 b
50 H + C		
500 (−)-M	7	7.0 ± 1.45 b, c
500 H + C		

[a] Materials (M: α-multistriatin; H: 4-methyl-3-heptanol; C: α-cubebene) were as presented in 50 beetle-hours (BH) equivalents; see text for explanation of dosages.
[b] Number of beetles responding per 25 beetle replicate. Significant differences between responses ($P < 0.05$) are indicated where no letter is repeated in any two entries.

above data do not necessarily negate the apparent slight attractiveness for (+)-M in Table 1; insects frequently meet laboratory criteria for response to chemicals which do not attract them to field traps, especially when field tests include a very much stronger pheromone emitted simultaneously from a different trap.

Since response in lab bioassays was similarly depressed by high concentrations of (+)- or (−)-M, it seems possible that either molecule can occupy the (−)-M acceptors and that the mechanism of response inhibition is sensory adaptation rather than blocking of response behavior. Unlike the cases of the gypsy moth (Vité et al., 1976) and Japanese beetle (Tumlinson et al., 1977), the

TABLE 3. *S. multistriatus* TRAPPED IN FIELD TESTS, JULY 22, TO AUGUST 19, 1977

Treatment	Replicates 1	2	3	4	Total[a]
H + C	2	2	1	1	6
(+)-M + H + C	2	2	2	0	6
(−)-M + H + C	1071	61	85	418	1635
Blank	3	5	3	4	15

[a] Sex ratio of captured beetles approximately 1:1 in all treatments.

presence of the (+)-enantiomer in synthetic racemic material does not seriously affect trap catches in survey and control programs.

To summarize, it is clear from these studies that in the field (−)-multistriatin is a highly active substance, whereas its (+)-enantiomer could not be distinguished from controls under the test conditions. This represents another example of differential biological response to enantiomers.

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FEMALE SEX PHEROMONE OF THE GERMAN COCKROACH, *Blattella germanica* (L.) (ORTHOPTERA: BLATTELLIDAE), RESPONSIBLE FOR MALE WING-RAISING

IV. The Absolute Configuration of the Pheromone, 3,11-Dimethyl-2-nonacosanone

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Abstract—The component of the sex pheromone of the German cockroach, 3,11-dimethyl-2-nonacosanone (compound A), is a chiral compound. The chiral center at C-3 was assigned the *S*-configuration by ORD studies and PMR studies using a chiral shift reagent.

Key Words—German cockroach, *Blattella germanica*, sex pheromone, absolute configuration, (3*S*,11*S*)-3,11-dimethyl-2-nonacosanone.

INTRODUCTION

Two components of the female sex pheromone of the German cockroach, *Blattella germanica*, were isolated from cuticular wax of the females and characterized as 3,11-dimethyl-2-nonacosanone (Ia, compound A) and 29-hydroxy-3,11-dimethyl-2-nonacosanone (Ib, compound B), respectively (Nishida et al.,1974, 1975, 1976). These are chiral compounds showing small specific optical rotations; $[\alpha]_D^{22} + 5.1°$[2] (c = 3.54 in hexane) for compound

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[2]In earlier papers (Nishida et al., 1974, 1975) it was reported that the isolated compound exhibited no optical rotation in the range of 240–400 nm. The discrepancy was probably due to an acidic treatment of the sample prior to the ORD measurement, resulting in epimerization on the C-3 chiral center, as described below.

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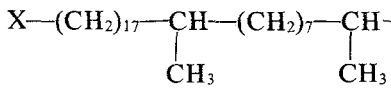
Ia and $[\alpha]_D^{18}$ + 7.1° (c = 0.35 in hexane) for compound Ib. We concluded that



Ia; X = H_3C—
Ib; X = $HOCH_2$—

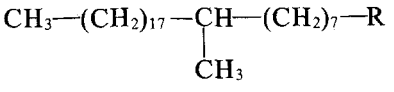
the C-3 chiral center of compound Ia is the *S* configuration from ORD studies and PMR studies using a chiral shift reagent.

METHODS AND MATERIALS

Spectral data were obtained with a Shimazu IR-400 spectrometer, Hitachi R-22 NMR spectrometer (90 MHz, TMS as a reference), Hitachi RMS-4 mass spectrometer, and JASCO ORD model J-5 spectropolarimeter. Melting points were uncorrected. The chiral shift reagent, tris[3-(trifluoromethylhydroxymethylene)-*d*-camphorato]europium (Eu-*d*-TFMC, Dojin-Kagaku Co.), was used without any purification.

(*S*)-(+)-2-Octyl acetate was prepared by acetylation of (*S*)-(+)-2-octanol (Nakarai-Kagaku Co.) with excess acetic anhydride in pyridine.

10-Methyl-2-octacosyl Acetate (IIa). *m*-Chloroperbenzoic acid (38 mg) dissolved in acetic acid (1 ml) was added to a carbon tetrachloride solution of natural compound Ia (Nishida et al., 1974, 1975) (45 mg), and the mixture was held at room temperature in the dark for 2 weeks. The mixture was treated with small



IIa; R = $CH(OCOCH_3)CH_3$
IIb; R = $CH(OH)CH_3$
IIc; R = $COCH_3$

crystals of sodium iodide to consume excess of the peracid and held at room temperature for several days. Then benzene was added and washed successively with 10% sodium thiosulfite, saturated sodium bicarbonate, dilute hydrochloric acid, and saline solution, and dried over anhydrous sodium sulfate. After removal of the solvent the product was placed on a column of silica gel (Wako-Gel C-200, 1.3 g) and eluted with benzene to give the acetate (IIa, 39 mg, 48%). The acetate was purified further by recrystallization from ethanol to give colorless needles melting at 26° C, IR (CCl_4): ν 1735, 1245 cm^{-1}; PMR (CCl_4): δ 4.76 (1H, sextet, J = 6.5 Hz), 1.91 (3H, singlet), 1.14 (3H, doublet, J = 6.5 Hz); $[\phi]_D^{21}$ + 6.1° (c = 3.0 in hexane).

Synthetic compound Ia (Nishida et al., 1975) was also oxidized by the procedure described above to produce a colorless oil which was identical with the optically active acetate obtained from natural compound Ia on TLC, GLC, and PMR analyses.

10-Methyl-2-octacosanol (IIb). The optically active acetate (IIa, 47 mg) was hydrolyzed with 5% potassium hydroxide in ethanol (5 ml) at room temperature overnight. The crude product was purified by column chromatography on silica gel (Wako-Gel C-200, 3 g). Recrystallization from ethanol gave colorless needles (28 mg); mp: 44–44.5° C; PMR ($CDCl_3$): δ 3.8 (1H, multiplet); $[\phi]_D^{25}$ + 34° (c = 2.8 in hexane).

The optically inactive secondary alcohol (IIb) was also obtained by hydrolysis of the acetate, derived from synthetic compound Ia, as colorless needles after recrystallization from ethanol; mp: 32–34° C.

10-Methyl-2-octacosanone (IIc). To the acetone solution (3 ml) of optically active 10-methyl-2-octacosanol (IIb, 43 mg), 5 ml of the oxidizing reagent, made from a mixture of chromic anhydride (5 g), water (10 ml), acetone (30 ml), and concentrated sulfuric acid (10 ml), was added, and the mixture was held at room temperature overnight. After isopropyl alcohol (1 ml) was added to consume the excess of oxidizing reagent, the reaction mixture was extracted with benzene, washed with saturated sodium bicarbonate, dilute hydrochloric acid, and saline solution to yield the methyl ketone (IIc). Recrystallization from ethanol gave colorless needles (19 mg); mp: 41.5–42° C; MS: *m/e* 422 (M^+), 58 (McLafferty rearrangement); IR (CCl_4): ν 1715 cm^{-1}; PMR ($CDCl_3$): δ 2.39 (2H, triplet, J = 7.0 Hz), 2.10 (3H, singlet), 0.84 (3H, triplet, J = 6.0 Hz), 0.82 (3H, doublet, J = 6.0 Hz); $[\phi]_D^{28}$ 0° (c = 0.48 in hexane); the 2,4-dinitrophenylhydrazone: mp, 67° C; $[\phi]_D^{17}$ 0° (c = 0.48 in hexane).

By the same oxidation procedure, the optically inactive alcohol (IIb) derived from synthetic Ia gave colorless fine needles of the racemic methyl ketone (IIc); mp: 40.5–42° C; its 2,4-dinitrophenylhydrazone: mp, 67° C.

Epimerization of Ia at the C-3 Chiral Center

Under Acidic Conditions. Natural compound Ia (30 mg) was dissolved in chloroform (2 ml) containing 5% hydrochloric acid in methanol (0.2 ml) and held at room temperature for a week. After evaporation under reduced pressure below 30° C, the resultant residue was crystallized repeatedly from ethanol to obtain colorless needles; mp: 36–37° C. This product and natural compound Ia (the starting material) were identical in their TLC, GLC, and PMR properties. But it showed no optical rotation. In its PMR spectrum with Eu-*d*-TFMC chiral shift reagent, the C-3 methyl doublet signal was separated into two doublets of equal intensity ($\Delta\Delta\delta$ = 0.07, [CSR]/[S] = 0.6, M = 0.17, in CCl_4).

Under Basic Conditions. A mixture of natural compound Ia (35 mg) in dioxane (5 ml) and 2% sodium hydroxide in ethanol (2 ml) was maintained at 55° C for 10 hr. Benzene was added to the mixture, which was then washed with saline solution and dried over anhydrous sodium sulfate. After removal

of the solvent, the resultant crystalline mass was crystallized repeatedly from ethanol to give the same epimerized product as above; mp: 36–37° C.

RESULTS AND DISCUSSION

Natural 3,11-dimethyl-2-nonacosanone (Ia), an active component of the female sex pheromone of the German cockroach, exhibited a positive Cotton effect as shown in Figure 1. The ORD curve is similar in both sign and the amplitude of molecular rotation to that of the (*S*)-enantiomer of 3-methyl-2-pentanone which was reported by Djerassi and Geller (1959). It was demonstrated (Crabbe et al., 1959) that the nature of the hydrocarbon substituent attached to the chiral center does not affect the optical rotatory dispersion. Accordingly the C-3 chiral center of natural Ia can be assigned the *S* configuration. More evidence for this assignment is provided by the following experimental results.

In the PMR spectrum of a mixture of natural and synthetic Ia (ratio 1:2) with Eu-*d*-TFMC shift reagent, the doublet signal arising from the methyl group attached to C-3 was shifted to a lower field and separated into two doublets with an intensity ratio of 1:2 as shown in Figure 2. This fact indicates that the C-3 carbon of natural Ia has one enantiomeric configuration.

Natural Ia was converted by the Baeyer-Villiger oxidation to (+)-10-methyl-2-octacosyl acetate (IIa). Since the Baeyer-Villiger reaction of ketones results in the insertion of an oxygen atom between the carbonyl and a highly substituted carbon with retention of absolute configuration (Hassal, 1957), the absolute configuration of the C-3 carbon of natural Ia should be retained

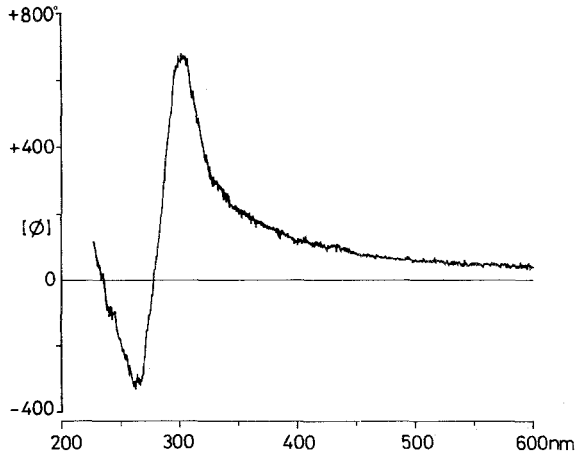


FIG. 1. The ORD curve of natural compound Ia (in hexane).

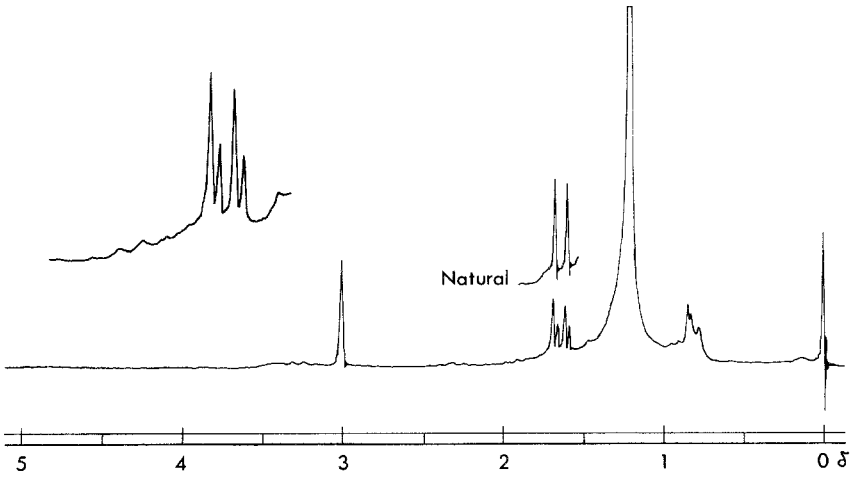


FIG. 2. The PMR spectrum of compound Ia (0.17M, [natural]/[synthetic] = 0.5) in CCl_4 in the presence of Eu-*d*-TFMC ([CSR]/[S] = 0.15).

at the C-2 carbon of the (+)-acetate obtained above, which could be compared with an authentic (*S*)- or (*R*)-2-alkyl acetate in PMR with a chiral shift reagent to assign the absolute configuration. The PMR spectrum with Ed-*d*-TFMC of a mixture of the (+)-acetate (IIa) and the optically inactive acetate (ratio 1:2) is shown in Figure 3a. The doublet signal arising from the terminal methyl (C-1), observed at δ 1.14 in the unshifted spectrum, is shifted to a lower field and separated into a pair of doublets. The upfield doublet is twice the intensity of the low field one, indicating that the doublet signal from the (+)-acetate is included in the upfield one. The same situation was observed in the PMR spectrum with Eu-*d*-TFMC of a mixture of (*S*)-(+)-2-octyl acetate and its racemate (ratio 1:2) as shown in Figure 3b, where it can be seen that the doublet signal arising from the terminal methyl (C-1) of the (*S*)-enantiomer is included in the upfield one of the two doublets observed. The nature of an alkyl substituent attached to the C-2 chiral center of these 2-alkyl acetates has no significant effect on the pseudo-contact shift resulting from an interaction between the C-1 protons and the shift reagent. Consequently the C-2 chiral center of the (+)-acetate (IIa) derived from natural Ia must have the (*S*)-configuration, which implies that the C-3 chiral center of natural Ia itself most probably has the *S* configuration as well.

This assignment is supported by the ORD data of the (+)-acetate (IIa) and its hydrolyzed (+)-alcohol (IIb) derived from natural Ia. As shown in Figure 4, the ORD curves of these compounds are quite similar to those of (*S*)-(+)-2-octyl acetate and (*S*)-(+)-2-octanol, respectively.

The positive Cotton effect exhibited by natural Ia (Figure 1) disappeared

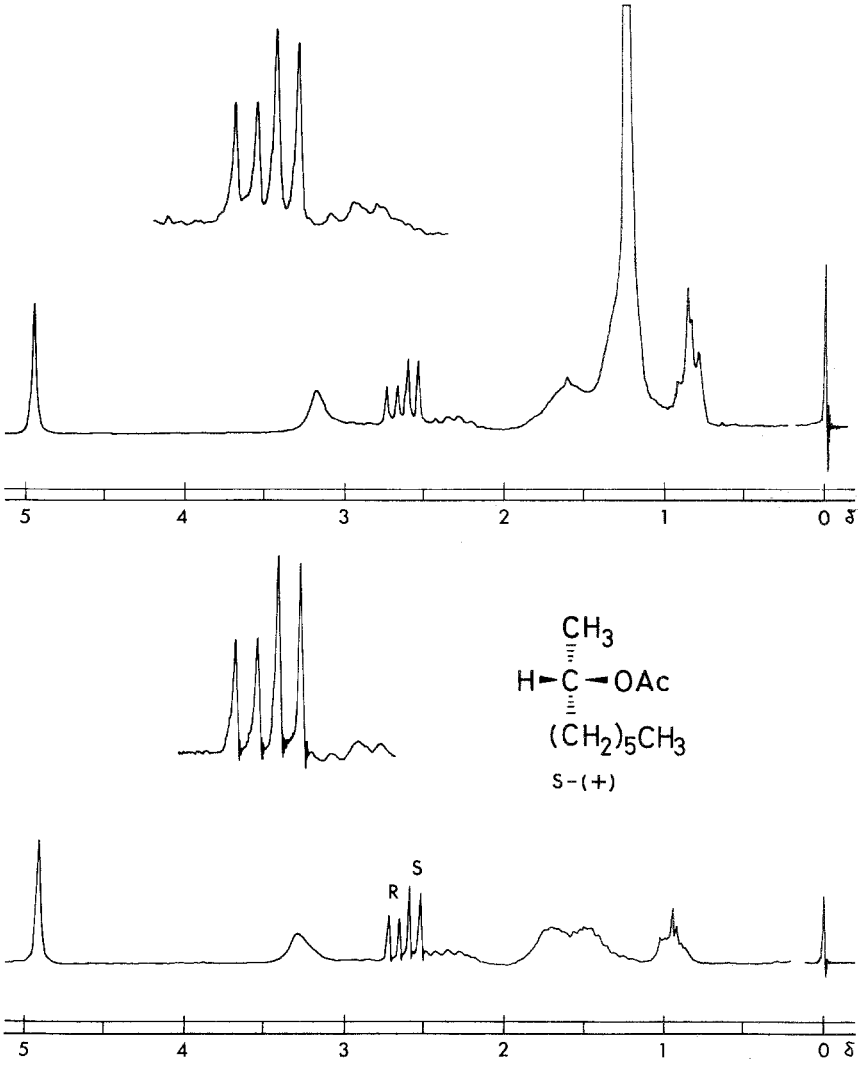


FIG. 3. PMR spectra with Eu-*d*-TFMC ([CSR]/[S] = 0.40) in CCl_4. (a) 10-Methyl-2-octacosyl acetate (IIa) (0.17M, [derived from natural Ia]/[synthetic] = 0.5). (b) 2-Octyl acetate (0.17M, [(*S*)-(+)]/[racemate] = 0.5).

on treatment with either acid or base. TLC, GLC, and PMR analyses indicated that the resultant product was identical to both the natural and synthetic compound Ia. However, its melting point (36–37°C) was intermediate (see Table 1). In the PMR spectrum of the product in the presence of Eu-*d*-TFMC, the C-3 methyl doublet was shifted to a lower field and separated into two doublets with equal intensity as was the case with the synthetic compound Ia.

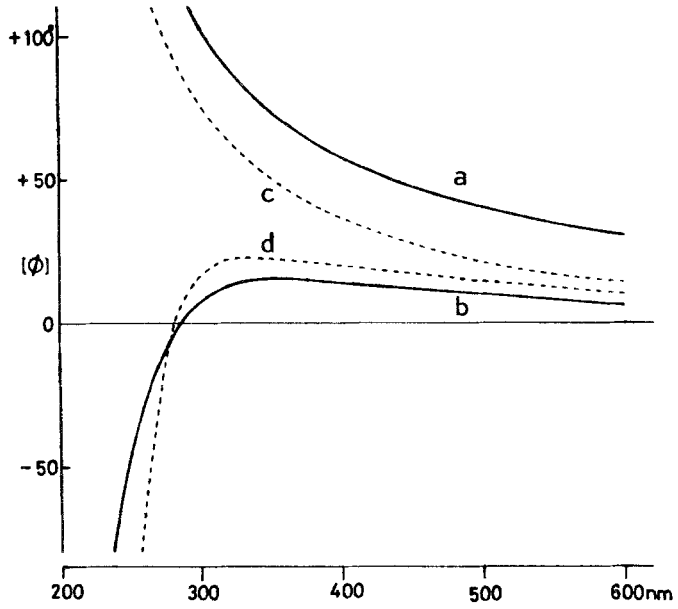


FIG. 4. ORD curves of (a) 10-methyl-2-octacosanol (IIb); (b) 10-methyl-2-octacosyl acetate (IIa); (c) (*S*)-(+)-2-octanol; and (d) (*S*)-(+)-2-octyl acetate (in hexane). (a) and (b) were derived from natural Ia.

These facts suggest that natural Ia was converted on treatment with either acid or base into a mixture of C-3 epimers, by enolization of the carbonyl group. This mixture of C-3 epimers, however, did not show any optical rotation which could be attributed to the C-11 chiral center.

Analyses of 10-methyl-2-octacosanone (IIc) derived from natural Ia *via* the corresponding secondary alcohol (IIb) might give some information about the configuration of the C-11 chiral center in natural Ia. However, neither the

TABLE 1. OPTICAL ROTATIONS AND MELTING POINTS OF NATURAL AND SYNTHETIC COMPOUND IA AND THEIR DERIVATIVES

Compound	Natural or its derivative		Synthetic
	$[\alpha]_D$	mp (°C)	mp (°C)
3,11-Dimethyl-2-nonacosanone (Ia)	+5.1°	45–46	29–31
10-Methyl-2-octacosyl acetate (IIa)	+1.3°	26	Oil
10-Methyl-2-octacosanol (IIb)	+7.9°	44–44.5	32–34
10-Methyl-2-octacosanone (IIc)	0°	41.5–42	40.5–42
2,4-Dinitrophenylhydrazone of IIc	0°	67	67

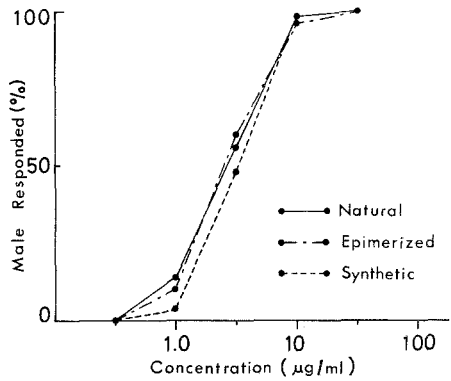


FIG. 5. Pheromone activities of natural C-3 epimerized mixture, and synthetic Ia.

methyl ketone (IIc) derived from natural Ia nor its 2,4-dinitrophenylhydrazone showed any optical rotation on their ORD measurement. Further, no difference in the melting points was observed between the methyl ketone (IIc) derived from natural Ia and from synthetic Ia or their 2,4-dinitrophenylhydrazone (see Table 1). Thus, these experimental results gave no significant information about the C-11 configuration of natural Ia. During the course of this study, however, Mori et al. (1978) were successful in syntheses of four diastereoisomers of compound Ia, which were kindly sent to us. The 3*S*,11*S*- and 3*S*,11*R* isomers showed positive optical rotation (see Table 2). Mixed melting point tests of natural compound Ia with them, as shown in Table 2, indicated unambiguously that it has the 3*S*,11*S* configuration.

Natural compound Ib, another component of the sex pheromone of the German cockroach, exhibited a similar Cotton effect ($[\phi]^{20}_{305}$ + 620°, c = 0.24 in ethanol) in both sign and amplitude to that of natural Ia. This fact suggests that natural Ib should also possess the 3*S*,11*S* configuration.

TABLE 2. OPTICAL ROTATIONS AND MELTING POINTS OF NATURAL AND SYNTHETIC DIASTEREOISOMERS Ia, AND THEIR MIXED MELTING POINTS WITH NATURAL Ia

Compound Ia	$[\alpha]_D$ in hexane	Melting point (°C)	Mixed melting point with natural Ia (°C)
Natural	+5.1° (c = 3.54)	45–46	
3*S*,11*S*[a]	+5.98° (c = 0.9)	44–44.5	44–45
3*S*,11*R*[a]	+5.73° (c = 2.04)	38–38.5	33.5–35
3*R*,11*R*[a]	−5.63° (c = 4.1)	44.5–45	35–37.5
3*R*,11*S*[a]	−5.68° (c = 4.0)	39–39.5	34.5–35.5

[a] Donated by Dr. K. Mori.

As shown in Figure 5, the mixture of C-3 epimers of natural Ia showed the same level of pheromone activity as those of natural and synthetic compounds when bioassayed with male German cockroaches according to the method reported previously (Nishida et al., 1974). Each of four synthetic diastereoisomers of Ia also showed the same level of the activity. It is interesting that the configurations of the C-3 and C-11 carbons do not contribute to the pheromone activity of compound Ia.

Acknowledgments—We thank Dr. K. Mori, University of Tokyo, for donating synthetic diastereoisomers of Ia, Drs. T. Ueno, H. Ohigashi and K. Koshimizu, and Miss S. Yamashita of Kyoto University for obtaining MS, ORD, and NMR data, respectively.

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Journal of Chemical Ecology, Vol. 5, No. 2, 1979

LETTERS TO THE EDITORS

IN DEFENSE OF THE TERM "PHEROMONE"

To the Editors:

Beauchamp et al. (1976) have challenged the appropriateness of the term "pheromone" when applied to mammals. They assert (incorrectly) that pheromones were originally defined for insects, and they question "how far theorists are willing to bastardize the pheromone concept . . . in describing chemically mediated social behavior of mammals." Karlson and Lüscher (1959) defined pheromones as "substances that are secreted to the outside by an individual and received by a second individual of the species, in which they release a specific reaction, for example, a definite behavior or a developmental process." Although a number of subsequent authors have experienced difficulty with some details of this definition, the term has received wide acceptance among students of chemical communication, including those who specialized in the chemical communication systems of mammals.

Beauchamp et al. (1976) present a list of five criteria that they regard as inherent in the concept of pheromones for insects but not for mammals. These criteria are listed and discussed below:

1. Relative Species Specificity. The degree of species specificity of mammalian pheromones has largely been untested. Among insects, some pheromones, especially those used in sexual communication, are highly species specific, whereas others, such as those used in the communication of alarm, may be very nonspecific, reaching across broad taxonomic groups. For nonspecies-specific insect pheromones, the environmental and/or physiological contexts in which the pheromone message is relayed often cause the message to be specific and appropriate for the prevailing situation (Blum, 1974; Shorey, 1976). Most importantly, species specificity is not inherent in the definition of a pheromone. Karlson and Lüscher (1959), in initially proposing the term, stated that "strict species-specific activity is not required; certain overlaps between closely related species may occur."

2. Genetic Programing, Minimizing Influences of Learning. We believe that the degree of genetic programing is irrelevant to the usefulness of the term "pheromone" and was not inherent in the initial definition. Mechanisms, such as genetic programing, which control the ability of an insect (or a mammal) to respond should not be elevated to the status of a defining criterion, especially

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since continua exist for both insects and mammals in the degree to which previous experience controls and modulates pheromone responses. For example, *Drosophila* mating preferences are pheromone mediated and highly dependent upon the age and previous experience of the females (Ehrman and Parsons, 1976; Leonard and Ehrman, 1976).

3. Made Up of One or a Few Compounds. Through the early years of insect pheromone experimentation, it was commonly believed that chemical simplicity was the general rule. However, as research continued, it became apparent that many insect pheromones are complex combinations of chemicals. For example, the mandibular gland of the weaver ant, *Oecophylla longinoda*, contains at least 33 chemicals in amounts greater than 5 ng and at least four of these are used in alarm communication behavior (Bradshaw et al., 1975). Some mammalian pheromones may consist of one or a few compounds and others, such as those used in individual recognition, may consist of a large number of compounds. In any case, the number of compounds that comprise a pheromone was not stated or implied as a criterion in the original definition.

4. Clear and Obvious Behavioral or Endocrine Function. The statement in Karlson and Lüscher's definition that a pheromone should "release a specific reaction" has caused difficulties for researchers on insects as well as mammals. For example, the female sex pheromone of the housefly may not cause males to make any noticeable response if they are not in a natural environment (if they are not next to a female) (Colwell et al., 1977). However, in a natural environment, the pheromone apparently lowers the threshold for the male to respond to the visual stimuli provided by the female and thus to mount the female and initiate courtship. Beauchamp et al. (1976) made the point that the response of a mammal to a pheromone often depends on the context in which the message is received. But the response of an insect to a pheromone also depends on the context, often (especially in social insects) to an extent much greater than that illustrated by the housefly example above. If the situation is contextually incorrect, neither insects nor mammals are likely to make natural responses to their species' pheromones. In many cases, in insects and mammals alike, no overt response to the pheromone may occur; instead, the animal's threshold of responsiveness to other, nonpheromonal stimuli may be altered.

5. Tested Against Control Odors (Other than Diluent or Alternative Fractions). The inference here is that one or a few chemicals uniquely stimulate a particular behavioral reaction. We believe that this inference is no more supportable for insects than for mammals. Certainly the fact that an investigator did not use adequate controls during his investigation in no way influences whether an odor (or a taste substance) should be called a pheromone.

We feel that Beauchamp et al. are needlessly confusing the field with their proposal that students of mammalian chemical communication might avoid the term "pheromone." Their criteria are illogical when applied to the definition, for both invertebrates and vertebrates, and were not originally associ-

ated with the definition. We fully acknowledge differences in behavioral complexity between the two groups, but Beauchamp et al. needlessly oversimplify the behavior of invertebrates as compared to that of mammals. The term "pheromone" has been of considerable value in the literature and has been adopted by most specialists of vertebrate as well as invertebrate chemical communication. The recent increase in interest in animal chemical communication has brought an increase in our awareness of the sophistication of the communication processes. However, the simplicity and usefulness of the original definition of a pheromone still stands. If we do not impose inconsistent and unnecessary restrictions, "pheromone" continues to serve as a useful shorthand notation for chemical substances that are released by an individual of a species and convey information to one or more receiving individuals of the same species.

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RESPONSE BY BEAUCHAMP ET AL.

To the Editors:

The term pheromone has been widely applied in the field of animal chemical communication. In a recent critique (Beauchamp et al., 1976), we discussed a number of conceptual problems in the use of this term (specifically in relation to mammalian chemical communication) and concluded that it has become

more misleading than useful. Katz and Shorey (1979) now dispute this conclusion. However, the major aspect of their rebuttal centers around a misconception of what we stated. In fact, using examples from insect species, Katz and Shorey amplify and dramatically illustrate many of the problems that we outlined in our chapter with regard to mammals.

We began our paper by listing a set of criteria which some investigators have implied or stated as being necessary for establishing whether or not a substance is a pheromone (relative species specificity; a well-defined behavioral and/or endocrinological function; a large degree of genetic programing; a limited number of chemical compounds; adequate controls to ensure the uniqueness of the isolated substances). These criteria were *not* our own; rather they were a distillation of criteria implicitly or explicitly held by a number of workers in the field of mammalian chemical communication (cf. Beauchamp et al., 1976, pp. 144–145). At no place did we state or wish to imply that these criteria were considered *the appropriate ones* for defining the term pheromone.

Following the listing, we showed that the majority of the reported mammalian "pheromones" did not meet these criteria. Likewise, we subsequently noted that if no operational criteria are established for determining whether or not a substance is a pheromone, then this term has little use as a meaningful scientific construct. We subsequently discussed, in the major portion of the paper, theoretical expectations concerning chemical complexity, the role of context, the role of experience, the central importance among mammals of individual recognition, and the question of the uniqueness of chemical stimuli (as compared with stimuli specific to the other senses) in affecting behavioral and endocrinological events. In these sections we, in effect, criticized most of the criteria listed earlier and pointed out that many of these criteria result in illogical distinctions.

The criticisms by Katz and Shorey of the criteria we listed in our chapter are, in fact, almost identical to our own criticisms presented in that publication. For example, consider the criterion of genetic programing. Katz and Shorey criticize this for three interrelated reasons: (1) it was not inherent in the initial definition; (2) such a mechanism should not be elevated to the status of a defining criterion; and (3) in fact, experience plays a role in insects as well as in mammals. We documented (p. 154) that this criterion was explicitly stated by some investigators as being required for a substance to be a pheromone, regardless of whether or not it was inherent in the original definition. We further argued that it is experimentally and conceptually difficult to base a definition on whether or not responses are unconditioned patterns of behavior. Finally, we too cited evidence (p. 154) that experience may modify the responses of insects (in addition to those of mammals) to conspecific chemical signals. Similarly, the points made by Katz and Shorey in regards to the criterion of chemical simplicity are nearly identical to our own, down to the inclusion of an

example from insects to show that here, too, expectations of chemical simplicity are incorrect (pp. 148–152).

There is, however, a fundamental difference in our position and that of Katz and Shorey regarding the use of the pheromone term for mammals. We recommend eliminating the term while Katz and Shorey recommend broadening its meaning. Thus, Katz and Shorey conclude their rebuttal with a characterization of pheromones as ". . . chemical substances that are released by an individual of a species and that convey information to one or more receiving individuals of the same species." The critical changes here from the original definition are "released" for "secreted", and "convey information" for "release a specific reaction." The movement to broaden the phenomena covered by the term is also seen in the proposal of Müller-Schwarze (1977) who, after listing a number of difficulties similar to those we initially described, coined a new term—the "informer pheromone." Thiessen (1977) has similarly sought to broaden the pheromone definition.

The argument in favor of defining all information-transmitting substances released by an animal as pheromones is that it provides a simple and useful shorthand notation. While this may be true, the presumed simplicity in its usage masks real difficulties if one wishes to make this term scientifically meaningful and unambiguous. For example, under appropriate circumstances most, if not all, chemicals that can be discriminated by mammals (and perhaps a number of other animals) may serve to transmit information (e.g., Mainardi et al., 1965; Marr and Gardener, 1965; Nyby et al., 1978). Furthermore, many information-transmitting substances appear to be derived from ingested food and/or by bacterial action on substrates provided by the organism (cf. Beauchamp, 1976; Leon, 1975; Porter and Doane, 1977; Skeen and Thiessen, 1977). Thus, the apparent simplicity of considering a pheromone as any substance "released by an individual" is illusory. To take another example, one is often asked whether or not there are human pheromones. Under the Katz and Shorey definition of the term, garlic breath odors and the stale urine odors found in subways are necessarily pheromones, as they are released chemical substances which transmit information to other members of the same species. But what is the usefulness in labeling these odors pheromones? Based on the past history of the construct, and the implications that are now frequently inherent in its use (cf. Beauchamp et al., 1976), it confuses rather than simplifies. Unfortunately, such implications are not easily removed from the scientific and public consciousness. Clearly, defining pheromones to include all released chemicals which transmit information will not help, regardless of how logical this appears to be. We reiterate here our view that all facets of mammalian chemical communication can be adequately and precisely described without resort to the use of a term with unclear meaning and the all-too-frequent underlying implication of externally secreted hormones.

While it is true that the original pheromone definition was not *specifi-*

cally restricted to insects (as we incorrectly implied), the context of the original publications (Karlson and Lüscher, 1959; Karlson and Butenandt, 1959) indicates that insect information formed the basis for the definition. Only one reference out of 12 in the original publication (Karlson and Lüscher, 1959) referred to vertebrates, in spite of the fact that there was considerable behavioral evidence that chemical substances play an important role in communicatory behavior of mammals (e.g., Darwin, 1871, pp. 842-844; Le Magnen, 1952). We emphasize that our criticisms of the term are based not on its original definition, but on how it has come to be used and on the confused meanings that have come to be attached to it. Further research will probably reveal wide variations in the characteristics of mammalian chemical communication systems. In some cases, the communicatory substances may closely resemble hormones, as was originally assumed by Karlson and Lüscher (1959, p. 55, paragraph 1), whereas in other cases similarities may be much less striking. Similarly, there may be chemical communication systems which fulfill most of the criteria listed in our critique; many others will not. In the face of this diversity, no clear-cut line of demarcation can be drawn between what is and what is not a pheromone. Arbitrarily dividing animal chemical communication into two classes—pheromonal and not pheromonal—could serve to fragment the field, while a completely inclusive definition has the danger of implying a similarity among phenomena that is illusory. As the complexity of chemoreception and mammalian behavior becomes more apparent, we believe the problems inherent in the use of the term pheromone will become even more evident.

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ANNOUNCEMENT

An international and interdisciplinary symposium, "Chemical Signals in Vertebrates and Aquatic Animals," will be held May 30 to June 3, 1979 at the College of Environmental Science and Forestry, Syracuse, New York.

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trans-PERILLENAL
A Furanoid Monoterpene from Pine Saw-Fly, *Neodiprion sertifer* (Hymenoptera: Diprionidae)[1]

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Abstract—Volatile constituents present in nanogram quantities in various body parts and glands of the males and females of the species *Neodiprion sertifer* Geoffr. (Hymenoptera: Diprionidae) have been investigated by GC-MS. A significant amount of a volatile constituent was detected in the lateral parts of the integument of abdominal segments II–III. The constituent was identified as the furanoid monoterpene *trans*-perillenal (I) previously not known to occur in nature. The identification and synthesis of this compound is described.

Key Words—*Neodiprion sertifer*, Pine saw-fly, Hymenoptera, Diprionidae, pheromone, *trans*-perillenal, monoterpene.

INTRODUCTION

There is a remarkable antenna dimorphism between the sexes of pine saw-flies of the genera *Diprion* and *Neodiprion*. The males have extremely enlarged, brushy antennae, which indicate a communication system with sex attractants released by the females. A very potent sex attractant was also shown by Coppel et al. (1960) to be present in the introduced saw-fly, *Diprion similis*. In field experiments virgin females attracted an average of 1000 males

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each, and an outstanding female attracted as many as 7000 males on her first day. Casida et al. (1963) tried to isolate the sex attractant from this species and found that amounts as small as 0.02 μg of a purified, but still unidentified material, attracted 500–1000 males at distances of 100–200 ft within 5 min in field experiments. Recently the major component of the sex attractant of *Neodiprion lecontei* and *N. sertifer* was identified as erythro-3,7-dimethylpentadecan-2-acetate, while the corresponding propionate was found to be the major component from *Diprion similis* (Jewett et al., 1976). Synthetic samples of these compounds, however, exhibited biological activity which was lower than the natural extracts. An explanation for these results was suggested to be that optically pure isomers were not used in the biological tests.

The aim of the present investigation was to study the sex attractants and other pheromones of *Neodiprion sertifier*. By means of GC and GC-MS techniques, we have investigated the volatile substances present in nanogram quantities in various parts of the body of the female and male of this species.

METHODS AND MATERIALS

Larvae were collected in their final stages in southern Sweden and were fed with needles of pine until pupation. The pupae were sorted into males and females according to the cocoon size. The sexes were kept separately. For chemical analysis newly emerged males and virgin females were used in most cases. Material was investigated from six separate populations, some of which were studied during two seasons.

The various body parts were dissected under a binocular microscope with cold light. The eggs and the digestive tract were always excluded from the abdominal samples.

The volatile compounds from the insects were analyzed by vaporizing the volatiles of various body parts on a precolumn connected with the GC glass capillary column, essentially according to a technique described by Ställberg-Stenhagen (1972). In some experiments the body parts were extracted with diethyl ether and the extracts analyzed using the precolumn technique. Adsorption of the volatiles on Porapak was also used in a few experiments. Capillary columns or a SCOT column with good separation efficiency were used for the GC analyses (Tables 1 and 2). The precolumn temperature was programed from 25 to 150°C over 7 min and then kept at 150°C for 3 min.

SOURCE OF ODORIFEROUS SUBSTANCES

Coppel et al. (1960) showed that the crushed heads and thoraces of females of the *Diprion similis* species had a very weak attractive effect on the males.

TABLE 1. BODY PARTS OF FEMALES OF *N. sertifer* ANALYZED BY GC

Head, cut into halves
Thorax, cut into halves
Legs
Anterior part of the abdominal integument (segments I–III)
Posterior part of the abdominal integument (segments IV–IX)
Dorsal part of the abdominal integument
Lateral parts of the abdominal integument of segments II–III
Dorsal and ventral parts of the abdominal integument of segments II–III
Apical part of the abdomen including the gland sebifique (segments VI–IX)
Hemolymph
Digestive tract

Crushed female abdomina, on the other hand, were more attractive to the males, indicating that the sex attractant emanated from that part of the body. A previously unknown paired gland was described by Mertins and Coppel (1972) from serial sections of the females of *Diprion similis*. Each gland is a small internal sac with a maximum size of about 200 μm, which opens on the lateral margin of the abdomen, in the intersegmental membrane between

TABLE 2. SUMMARY OF ANALYSES OF VOLATILE COMPOUNDS FROM *N. sertifer*

Year	Number of analyses: Females	Number of analyses: Males	Number of individuals	Column	Comments
Capillary gas chromatography					
1973	18		2–20, mostly 6	OV-1	Different parts of abdomen, precolumn
1974	19	1	1–19, mostly 10	OV 101	Different parts of body, precolumn, diethyl ether extract, adsorption on Porapak
1975	2		9–11	SF-96 (SCOT)	Abdominal integuments, precolumn
1976	2	6	6	SE 30	Abdominal integuments, precolumn
Capillary gas chromatography + mass spectrometry					
1974	45	2	3–50, mostly 6	OV 101	Different parts of body, precolumn diethyl ether extract, adsorption on Porapak
1975	4	2	9–15	SE 30 FFAP (SCOT)	Abdominal integuments, precolumn
1976	5		6	SE 30	Abdominal integuments, precolumn

tergites II and III. By serial sectioning of the whole insect, Mertins and Coppel (1972) found no other gland which could be the source of the sex attractant. They also tried in field experiments to show that the potent substances emanated from the paired gland which they had discovered. Their results from the field tests were, however, ambiguous, and they suggest that the gland might be the source of a repellant, the existence of which has earlier been inferred by Casida et al. (1963).

CHEMICAL ANALYSIS OF VOLATILE CONSTITUENTS

Some data on the analyses which were performed are given in Table 2. We were unable to detect by gas chromatography any significant amounts of volatile substances from any of the body parts (Table 1) of the males and females of *N. sertifer* except from the abdominal integuments. A significant amount of a compound with a relatively short gas chromatographic retention

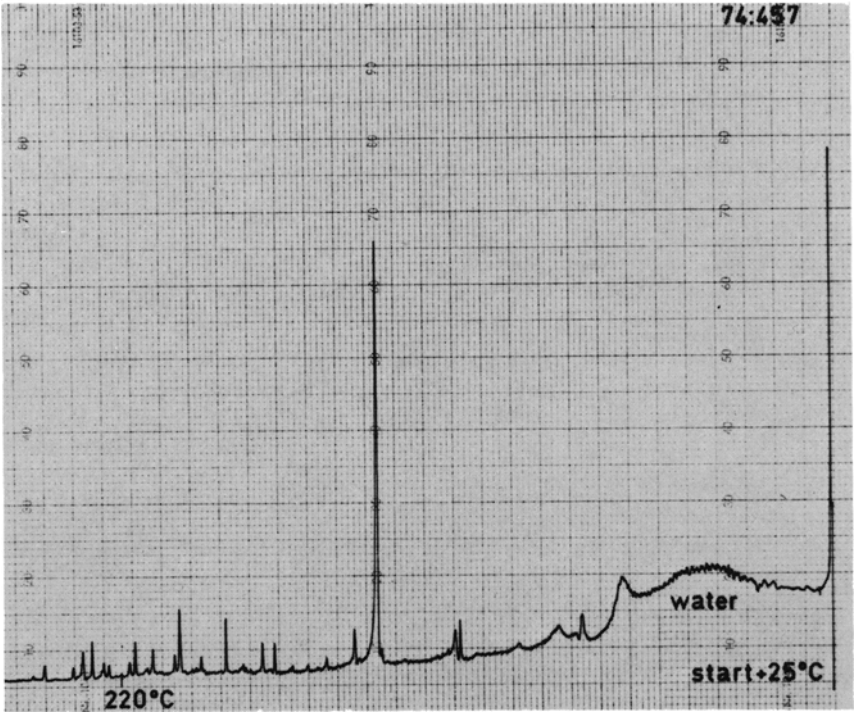


FIG. 1. Capillary gas chromatogram (stationary phase: silicone OV 101) of volatile material from the abdominal integument of segments II–III of 6 females of *N. sertifer*. Temperature programmed at 8° / min, from 25 to 220° C.

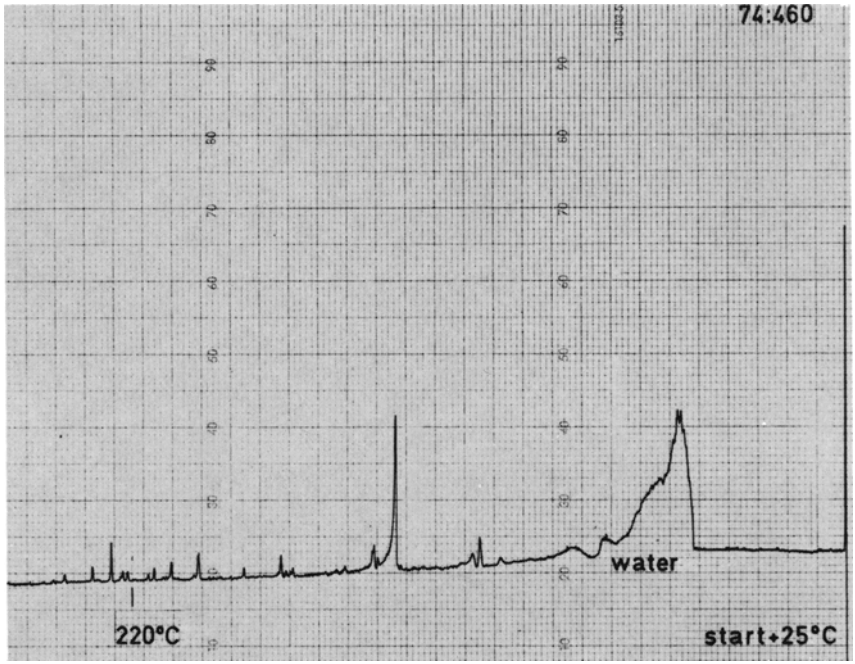


FIG. 2. Capillary gas chromatogram (same conditions as in Figure 1) of volatile material from the lateral parts of the abdominal integument of segments II–III of 6 females of *N. sertifer*.

time was present in preparations of the lateral anterior parts (segments II–III) of the abdomen. The capillary gas chromatograms given in Figures 1, 2, and 3 demonstrate this. Extraction and adsorption were abandoned as isolation methods in favor of precolumn vaporization.

Figure 1 shows the volatile material obtained from a preparation of the abdominal segments II–III of six females of *N. sertifer*. The temperature of the gas chromatographic oven was programed to rise from 25 to 220°C for this analysis. A nonpolar silicone capillary column (OV 101) was used. In a more elaborate preparation, the lateral parts of abdominal segments II–III were separated from both the dorsal and the ventral parts. The corresponding gas chromatogram thus obtained is given in Figure 2. Although there may have been some loss of volatile material due to the method of preparation, this analysis demonstrates that the major volatile compound emanates from the lateral parts of abdominal segments II–III. For further comparison, a capillary gas chromatogram of the medial parts of the abdominal sternites and tergites is shown in Figure 3. No trace of this major volatile compound was found in this preparation or in preparations

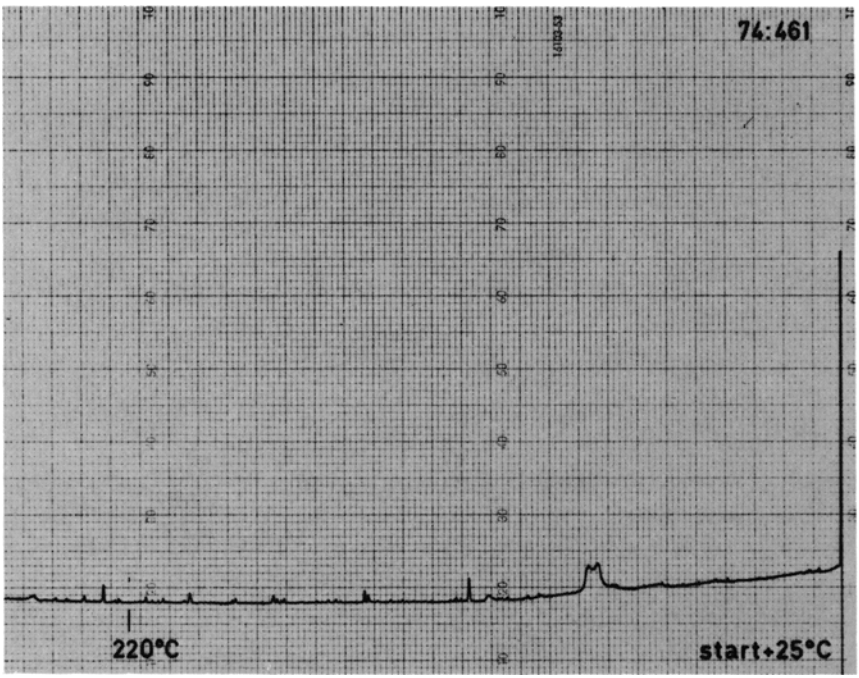


FIG. 3. Capillary gas chromatogram (same conditions as in Figure 1) of volatile material from parts of the sternites and tergites of the segments II–III of the abdomen of 6 females of *N. sertifer*.

from other parts of the body. A few chemical analyses were performed on newly emerged males, and the compound was shown to be present in the abdominal integument, which, however, was not further divided into smaller preparations.

The average amount of the major volatile compound present in females and males was somewhat different. On the average 1–3 ng were present in each individual. There may be a little more in the females, although no analyses were done on series of separate individuals.

The GC and GC-MS analyses of the abdominal segments also showed the presence of some minor volatile constituents, which have not yet been further investigated. No further substances were detected in the diethyl ether extract of 964 females.

Jewett et al. (1976) have reported the identification of the acetate of 3,7-dimethylpentadecan-2-ol in an ethyl ether extract obtained from 27,000 virgin females of *Neodiprion lecontei*. According to these authors, this compound is also present in extracts of *N. sertifer* and, as shown by electrophysiology, perceived by the chemoreceptors on the antennae. In our capillary gas chromatograms, we have looked for this compound and related

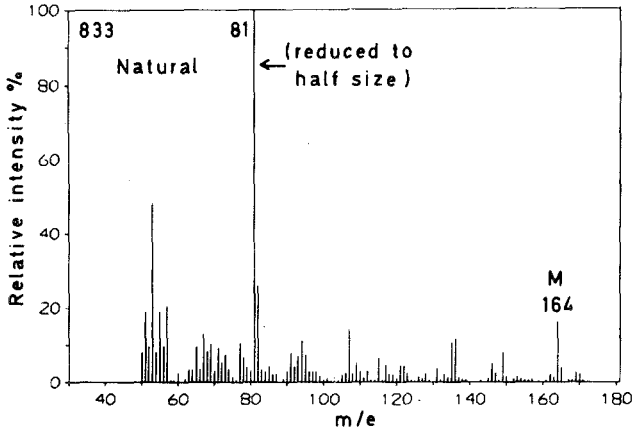


FIG. 4. Mass spectrum of the main volatile components from the abdominal segments of 6 females of *N. sertifer*.

ones, both from fine preparations and extracts of whole insects, but so far we have not found any GC or GC-MS data which indicate their presence.

IDENTIFICATION AND SYNTHESIS

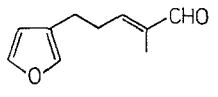
The main volatile constituent of the lateral anterior parts (segments II–III) of the abdomen exhibited a very characteristic mass spectrum (Figure 4) and indicated that the molecular weight of the compound is 164. Furthermore, there is a dominating fragment at *m/e* 81, which is a characteristic feature of substances containing a furylmethyl grouping, A similar fragmentation pattern is exhibited by the cis and trans isomers of 2-furylmethyl-1-propenyl ether, as shown in the Eight Peak Index of Mass Spectra.[5] The GC retention time of the compound is 1309 relative to *n*-C_{13}-ane (1300) measured on a nonpolar silicone (OV-101) column. The molecular weight of the compound indicated a chemical composition of $C_{10}H_{12}O_2$. Thus the compound was assumed to be of a monoterpenoid nature.

A few naturally occurring monoterpenes of furanoid nature are known, but only one, β-dehydroelzholtzione (II), has a chemical composition ($C_{10}H_{12}O_2$) corresponding to that of the compound isolated from *N. sertifer*. The mass spectrum of β-dehydroelzholtzione (II) and some related synthetic compounds became available upon a search in the mass spectral data bank of Firmenich S/A.[6] The mass spectra of the compounds (I, III, IV,

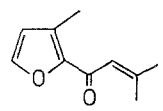
[5]Eight Peak Index of Mass Spectra (2nd ed.), Vol. 3, Part 1, Spectra M 3128 and M 3129, p. 1852; published and compiled by Mass Spectrometry Data Centre, Reading, 1974.

[6]Drs. G. Ohloff and B. Willhalm are gratefully acknowledged for putting the MS data at our disposal.

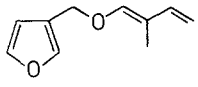
and V) exhibited a base peak at *m/e* 81 and showed similarities to that of the main volatile compound from *N. sertifer*. However, only the mass spectrum exhibited by a cis/trans mixture of the compound I was almost identical. We suggest the name perillenal for this compound since it is derived from the previously known furanoid monoterpene perillene.



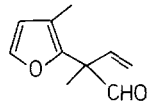
I
Perillenal



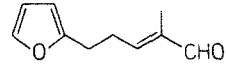
II
β-Dehydroelzholtzione



III

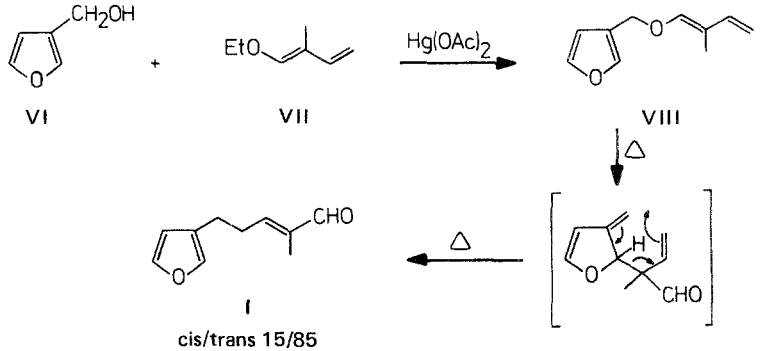


IV



V

The synthesis of perillenal, isolated as a mixture of cis and trans isomers, has previously been reported by Thomas (1968) and Thomas and Ozainne (1970). The synthesis starts from 3-furylmethanol (VI) which was allowed to react with 1-ethoxy-2-methylbuta-1,3-diene (VII) in the presence of mercuric acetate to yield the ether (VIII) as a mixture of cis and trans isomers. This isomeric mixture was isolated by a careful distillation under reduced pressure. The ether (VIII) was then allowed to undergo a thermal Claisen-Cope type of rearrangement (Scheme 1) to yield a mixture from which a cis/trans mixture of perillenal (I) was isolated as one of the main products by careful distillation. This synthesis was repeated by us, follow-



SCHEME 1. Synthesis of perillenal according to Thomas and Ozainne (1970).

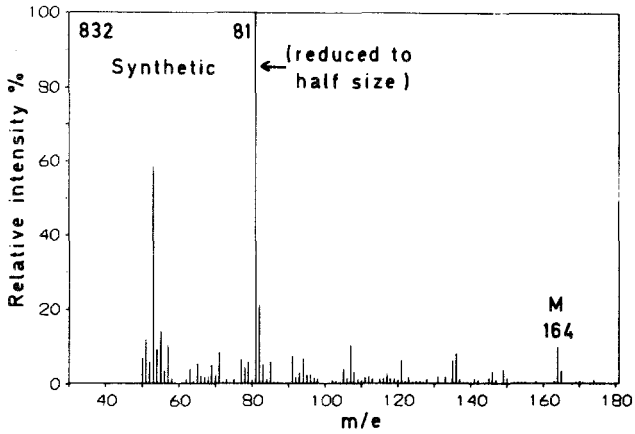
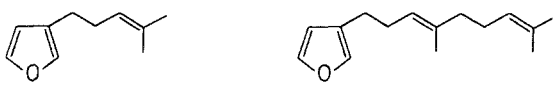


FIG. 5. Mass spectrum of synthetic *trans*-perillenal (I).

ing essentially the experimental details given by Thomas and Ozainne (1970)[7]. Pure *trans*-perillenal was obtained from the synthetic mixture of cis and trans isomers by preparative GC. A polyethylene glycol column (PEG 1500) operated at 140° was used (relative retention times: *trans*-perillenal, 1.0; *cis*-perillenal, 0.89). The mass and NMR spectra of *trans*-perillenal thus obtained were in accord with the literature data (Thomas et al., 1970).

The mass spectrum of synthetic *trans*-perillenal is given in Figure 5. It is identical with that of the major volatile compound from *N. sertifer*. The natural and the synthetic substances give identical capillary gas chromatographic retention times on two different columns (OV 1 and SE 30). Therefore, we conclude that the dominant volatile compound in our analyses of the lateral anterior abdominal segments of female and male *N. sertifer* is identical with *trans*-perillenal (I).

It is interesting to note that two structurally related furanoid terpenes, perillene (IX) and dendrolasin (X),



IX
Perillene

X
Dendrolasin

have been isolated from another Hymenoptera species, an ant, *Lasius* (*Dendrolasius*) *fuliginosus* Latr. (Bernardi et al., 1967).

[7]We thank Dr. A.F. Thomas of Firmenich S/A for advice and for a sample of compound VII.

DISCUSSION

Jewett et al. (1976) have recently identified erythro-3,7-dimethylpentadecan-2-acetate as the major sex attractant in *N. sertifer*. We have not been able to detect the acetate or any other similar substances from females of *N. sertifer*, but we have identified *trans*-perillenal, the biological function of which has not yet been determined. We have analyzed synthetic samples of the acetate (Magnusson, 1977) in the same way as that of our material from the insects. The gas chromatographic retention index of the synthetic acetate on a silicone OV-101 column was found to be 1915 relative to nonadecane (= 1900).

The difference between our results and those of Jewett et al. may be explained by the fact that different methods were used for isolation and identification of the constituents. The method used in this study favors volatile constituents, whereas the method of Jewett et al. (1976) should be better for compounds of less volatility.

Jewett et al. kept a great number of virgin females in glass jars with crumpled filter paper. The active principle was obtained from a diethylether extract of the filter paper. The glandular origin of the substance was not discussed.

trans-Perillenal has been isolated with a precolumn gas-chromatographic technique whereby the biological material was heated and the volatile constituents separated by gas chromatography. Most probably the aldehyde emanates from a small, lateral abdominal gland (cf., Mertins and Coppel, 1972) as indicated by gas chromatography of excised small parts of the abdominal wall consisting of the gland and the immediate cuticular region surrounding it. That we have not found the acetate in our analysis could be explained by the small amounts in which this compound occurs. Jewett et al. (1976) reported the isolation (by accumulation) of 4 μg of the acetate from 27,000 insects, *N. lecontei*, which means that the amount excreted by one insect will not be more than 0.15 ng.

A discussion of the biological function of *trans*-perillenal must await the results from investigations now in progress.

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VOLATILE COMPOUNDS FROM THE INTERDIGITAL GLAND OF REINDEER (*Rangifer t. tarandus* L.)

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Abstract—The isolation, identification, and synthesis of volatile compounds from the interdigital gland of reindeer (*Rangifer t. tarandus* L.) are reported. Two of the major constituents have been identified as 1-hydroxy-7-methyl-3-octanone and 7-methyl-1-octen-3-one.

Key Words—Gland secretion, hydroxy-ketone, ketones, mass spectrometry, pheromones, *Rangifer t. tarandus* L., reindeer, skin gland, volatile compounds, 1-hydroxy-7-methyl-3-octanone, 7-methyl-1-octen-3-one.

INTRODUCTION

External secretory glands occur in many groups of mammals, for example in rodents, ungulates, carnivores, and primates. Studies of mammalian chemical communication resulting from these glandular secretions have been largely constructed by the student of animal behavior and the reproductive physiologist. Little is known about the underlying chemistry, but in recent years there has been a growing interest in chemical investigations of mammalian chemical communication. A survey of such studies is given by Albone (1977).

In reindeer (*Rangifer t. tarandus* L.) three different skin glands have been known for a long time: the preorbital, tarsal, and interdigital glands (Schaffer, 1940; Quay, 1955). Recently a caudal gland was discovered by Müller-Schwarze et al. (1977).

The interdigital glands are located in the dorsal interdigital skin. The glands are more highly developed in the hindfoot than in the forefoot. The inside of the hindfoot interdigital gland is covered with stiff hairs which are

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coated with secretion. In the caribou (*Rangifer tarandus arcticus*) a behavior possibly involving the interdigital glands has been described by Pruitt (1960). It is the so-called "excitation jump," in which an animal rears up and leaves deep footprints. In order to establish the function of the interdigital gland in reindeer a bioassay was developed to assess the responses of reindeer to the secretion of the hindfoot interdigital glands of conspecifics (Müller-Schwarze et al., 1978). The main finding of these experiments was that reindeer do show interest in interdigital gland secretion, indicated by the more frequent sniffing of these secretions than sniffing of blank or control odors. A second result was sex-specific: males sniffed male interdigital secretion more often than that of females, and females responded more to female interdigital secretion than that of males. According to Müller-Schwarze et al. (1978), the third finding, that isolated individuals sniffed the ground more often, lent support to the theory that the gland function includes the aiding of single animals in finding their way back to the group.

In a previous paper the identification of short-chain aliphatic acids from the interdigital gland of reindeer, and their discrimination by reindeer, is discussed (Brundin et al., 1978). This paper deals with the isolation, identification and synthesis of some major constituents of the volatile fraction of the interdigital gland secretion of reindeer (*Rangifer t. tarandus* L.).

METHODS AND MATERIALS

The interdigital glands were collected at the annual slaughter at Gielas in northern Sweden in September 1977. The glands were excised within 30 min of death, frozen in liquid nitrogen, and then stored at −29°C in the dark until analyzed. Samples from eight living reindeer, kept as one group in a pen, were also taken. The secretion from the interdigital gland from the live group was collected with cotton swabs which were inserted into the hindfoot interdigital gland of the reindeer and rotated. Preliminary GC analysis of these samples showed no obvious sexual or individual variation and all available material was therefore pooled for analyses.

Volatile compounds from 52 interdigital glands were collected in dichloromethane by the method described by Andersson et al. (1975). The concentrated dichloromethane solution was fractionated by gas chromatography with a Pye Unicam GCD gas chromatograph equipped with a flame ionization detector (FID). It was fitted with the following glass columns: Column A, 5% Reoplex 400 on 100–120 mesh Chromosorb W-AW-DMCS, 1.5 m × 4 mm, 40 ml/min N_2 flow rate, held at 70°C for 10 min, then temperature programed at 4°/min to 140°C and held at 140°C for 20 min. Column B, 5% Carbowax 20M on 100–120 mesh Chromosorb W-AW-DMCS, 1.5 × 4 mm, 40 ml/min N_2 flow rate, held at 140°C for 25 min. Column C, 10% Reoplex 400 on 100–120 mesh Chromosorb W-AW-

DMCS, 1.5 × 4 mm, 40 ml/min N_2 flow rate, held at 90°C for 20 min. Column D, 0.2% SP-1000 (Supelco, Inc.) on 60–80 mesh Carbopack A-AW-DMCS, 2.0 m × 2 mm, 20 ml/min N_2 flow rate at 160°C.

Preparative gas chromatography was done with a Pye Unicam model 64 gas chromatograph equipped with an FID detector using the same columns and under the same conditions as in the GC analysis. The carrier gas was nitrogen and the effluent from the column was split so about 36 ml/min was collected in cooled glass capillaries (1.5 mm ID) and 4 ml/min was routed to the detector.

Mass spectra were obtained on a LKB 9000 mass spectrometer equipped with a Pye Unicam model 64 gas chromatograph. The carrier gas was helium at a rate of 30 ml/min. The columns and operating conditions for the gas chromatograph were as above. Operating conditions for the mass spectrometer were: separator temperature, 250°C; ion source temperature, 270°C; and electron energy, 70 eV.

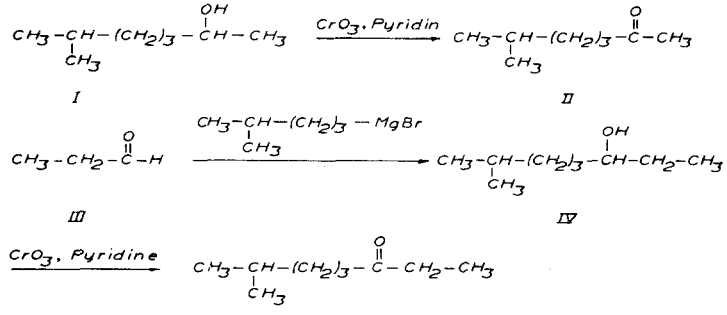
Nuclear magnetic resonance spectra were obtained using a JEOL PFT-60HL instrument. Chloroform-d (dried over molecular sieves) was used as solvent and for internal locking. Sample concentrations were in the range of 0.01 M.

Microozonolyses were carried out in the manner of Beroza and Bierl (1967).

Double bonds (C=C) were hydrogenated with hydrogen and neutral palladium catalyst in the inlet of the gas chromatograph as described by Beroza and Sarmiento (1966).

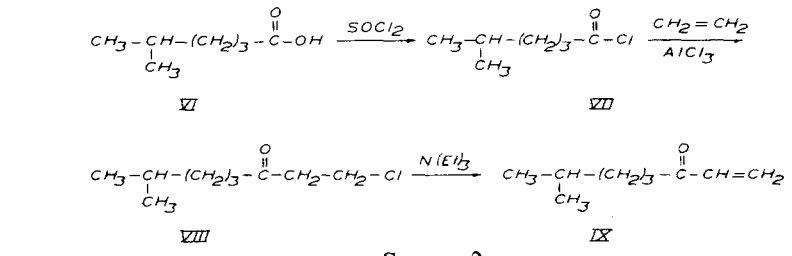
Synthesis

Synthesis of the major volatile compounds from the interdigital gland secretion were carried out according to the schemes (1–3). 6-Methyl-2-heptanone (II) and 7-methyl-3-octanone (V) were prepared by oxidation of the corresponding secondary alcohols with chromium trioxide–pyridine complex according to Ratcliffe and Rodehorst (1970) (Scheme 1).



I II III IV V

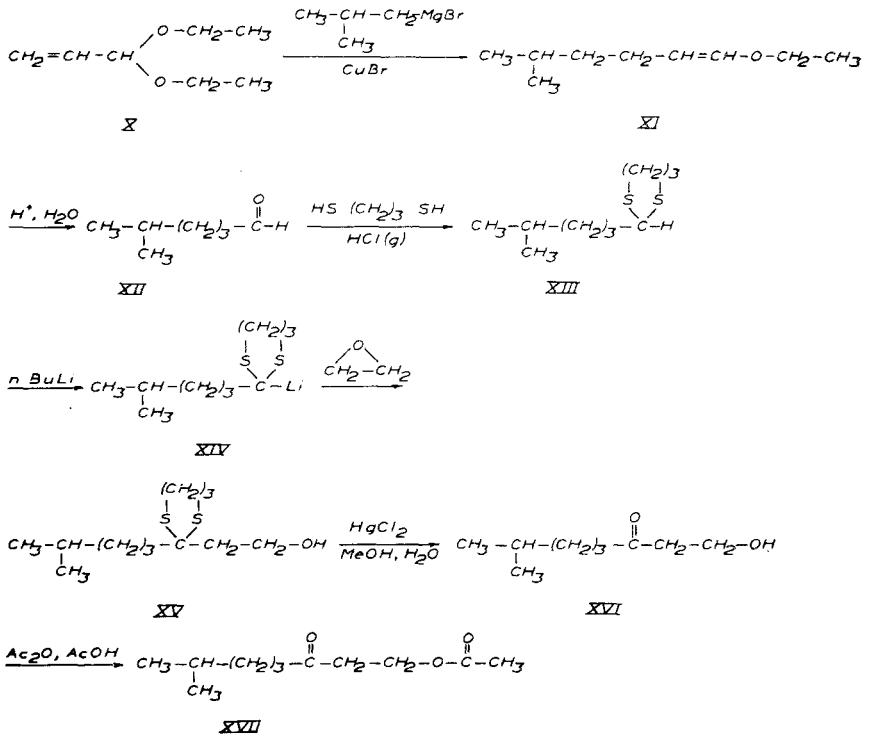
SCHEME 1



SCHEME 2

7-Methyl-1-octen-3-one (IX) was prepared by treating 5-methylhexanoyl chloride (VII) in dichloromethane solution with ethylene and aluminium trichloride as described by Thomas et al. (1964) (Scheme 2).

1-Hydroxy-7-methyl-3-octanone (XVI) was prepared from 5-methyl-1-hexanal (XII) via its 1,3-dithiane. The reaction or 2-(4-methylpentyl)-1,3-dithiane (XIII) with ethylene oxide in the presence of butyllithium gave 2-(β-hydroxyethyl)-2-(4-methylpentyl)-1,3-dithiane (XV). Hydrolysis of XV yielded 1-hydroxy-7-methyl-3-octanone (XVI) (Scheme 3).



SCHEME 3

The synthetic applications of 2-lithio-1,3-dithianes have been reviewed by Seebach (1969), Seebach and Corey (1975) and Gröbel and Seebach (1977).

7-Methyl-3-octanol (IV). Isopentyl magnesium bromide (0.1 mol) in ether was prepared in the usual manner.

The Grignard reagent was cooled to −10°C and 5.8 g (0.1 mol) of freshly distilled propanal (Fluka AG) in 100 ml of ether was added over a period of 2 hr. Hydrolysis (NH_4Cl) and isolation of the product in the usual manner followed by distillation gave 8.3 g (58%) of 7-methyl-3-octanol (IV); bp 85–86°C/12 mm.

7-Methyl-1-octen-3-one (IX). 5-Methylhexanoyl chloride (VII) (14.9 g, 0.1 mol) in 25 ml of methylene chloride was slowly added to a cold (−10°C) magnetically stirred suspension of 16 g (0.12 mol) aluminium trichloride in 25 ml of dichloromethane. Dry ethylene was passed through the mixture at such a rate that the temperature did not rise above 0°C. After 3 hr the mixture was poured into a mixture of crushed ice and dilute hydrochloric acid. The organic layer was separated, washed with water, dried, and distilled to remove solvent. The crude haloketone was dissolved in 125 ml of dry ether and triethylamine (0.1 mol) was added. The mixture was refluxed for 68 hr, after which the precipitated triethylamine hydrochloride was filtered off. The solvent was removed and the residue was distilled to give about 7 g (50%) of 7-methyl-1-octen-3-one (IX), bp 60–62°C/8 mm. When purified by distillation some polymerization of the vinyl ketone occurred. A pure product was obtained by liquid chromatography on Silica gel 60 (Merck) with CCl_4 as eluent. The purified product was stored over hydroquinone.

NMR δ (60 MHz, $CDCl_3$): 0.88 [d, 6H, $(CH_3)_2C<$]; 2.57 (t, 2H, —CH_2—CO—); 5.6–6.4 (m, 3H, CH_2=CH—CO—).

5-Methyl-1-hexanal (XII). This compound, prepared by the procedure of Normant et al. (1975), was obtained by treating acroleindiethylacetal (X) (Fluka AG) with isobutyl magnesium bromide in the presence of CuBr.

Isobutyl magnesium bromide (0.2 mol) in ether was prepared in the usual manner and added over a period of 30 min to a cold (−10°C) magnetically stirred solution of 26.0 g (0.2 mol) of acrolein diethylacetal and 1.0 g CuBr in 200 ml of THF. After an additional 2 hr the reaction mixture was hydrolyzed with saturated ammonium chloride and extracted with pentane. The organic layer was dried ($MgSO_4$) and the solvent removed by distillation. The residue was refluxed for 1 hr with 70 ml of 75% aqueous acetone and a few drops of concentrated hydrochloric acid. The acetone was removed by distillation, water was added, and the aqueous layer was extracted with ether. Drying ($MgSO_4$) of the organic phase followed by distillation gave 15.3 g (67%) of 5-methyl-1-hexanal (X); bp 140–142°C.

2-(4-Methylpentyl)-1,3-dithiane (XIII). To a magnetically stirred solution of 11.4 g (0.1 mol) 5-methyl-1-hexanal (XII), in 200 ml of chloroform

at −20°C, was added (in one portion) 10.8 g (0.1 mol) 1,3-propanedithiol (Fluka AG). Dry HCl gas was slowly bubbled through the solution for 10 min. The cold bath was removed and the reaction mixture was stirred at room temperature for 18 hr. Work-up was done by washing with water, 10% aqueous KOH, and again water, followed by drying (K_2CO_3). Evaporation of the solvent under reduced pressure and distillation gave 15.7 g (77%) of 2-(4-methylpentyl)-1,3-dithiane (XIII); bp 82–83°C/0.15 mm

NMR δ (60 MHz), $CDCl_3$): 0.92 [d, 6H, $(CH_3)_2C<$]; 2.7–3.0 [m, 4H, $>C(—S—CH_2—)_2$]; 4.02 t, 1H, $H—C<(—S—)_2$]. Mass spectrum (70 eV): *m/e* 204 (18%), 119(100).

2-(β-Hydroxyethyl)-2-(4-methylpentyl)-1,3-dithiane (XV). A solution of 2-(4-methylpentyl)-1,3-dithiane (XIII) (10.2 g, 0.05 mol) in 125 ml of dry THF (distilled from $LiAlH_4$) was magnetically stirred at −40°C in a dry nitrogen atmosphere. n-Butyllithium (Ventron) (0.05 mol, 2.17 M in hexane) was added over a period of 25 min. The reaction vessel was kept at −30° to −20°C under positive nitrogen pressure for 3 hr. The clear solution was cooled to −60°C and 2.5 ml (0.05 mol) ethylene oxide was added through a serum cap by syringe. After 1 hr the cold bath was removed, and the reaction mixture was stirred at room temperature for 18 hr. The solution was poured into 500 ml of ice and water and extracted with chloroform. The organic extracts were washed with 10% aqueous KOH, water, and dried (K_2CO_3). Evaporation of the solvent under reduced pressure followed by distillation gave 10.6 g (86%) of 2-(β-hydroxyethyl)-2-(4-methylpentyl)-1,3-dithiane (XV); bp 113–115°/0.03 mm.

NMR δ (60 MHz, $CDCl_3$): 0.89 [d, 6H, $(CH_3)_2C<$); 2.23 (t, 2H, $—CH_2—CH_2—OH$); 2.7–3.0 [m, 4H, $>C—(—S—CH_2—)_2$]; 3.10 (t, 1H, —OH); 3.80 (q, 2H, $—CH_2—O—$). Mass spectrum (70 eV): *m/e* 248 (35%), 230(4), 217(3), 203(30), 163(100).

1-Hydroxy-7-methyl-3-octanone (XVI). A vigorously stirred solution of 9.9 g (0.04 mol) 2-(β-hydroxyethyl)-2-(4-methylpentyl)-1,3-dithiane (XV) in 500 ml of 80% aqueous methanol was treated with $HgCl_2$ (0.08 mol) and $CaCO_3$ (0.04 mol). After refluxing for 5 hr, the mixture was cooled and filtered, and the methanol was removed by evaporation under reduced pressure. The residue was diluted with brine and extracted with ether. The combined ether extracts were washed with water, saturated aqueous ammonium chloride, and again with water, and then dried (K_2CO_3). Evaporation of the solvent and distillation gave 5.2 g (82%) of 1-hydroxy-7-methyl-3-octanone (XVI); bp 90–92°C/0.6 mm.

NMR δ (60 MHz, $CDCl_3$): 0.88 [d, 6H, $(CH_3)_2C<$); 2.3–2.8 (m, 4H, $—CH_2—CO—CH_2—$); 3.86 (q, 2H, $—CH_2—O—$).

7-Methyl-3-oxooctylacetate (XVII). A solution of 1-hydroxy-7-methyl-3-octanone (XVI) (1.6 g, 0.01 mol), 0.025 g sodium acetate, and acetic anhydride (1.1 g, 0.01 mol) was refluxed for 4 hr in 50 ml of benzene. The re-

action mixture was then cooled to room temperature, water was added, and the organic layer separated and washed twice with 5% aqueous sodium carbonate and finally with water. After drying ($MgSO_4$), the solvent was removed by distillation. The crude ester was purified by column chromatography on Silica gel 60 (Merck) with dichloromethane as eluent. The yield of 7-methyl-3-oxooctylacetate (XVII) was 1.6 g (80%).

NMR δ (60 MHz, $CDCl_3$): 0.85 [d, 6H, $(CH_3)_2C<$]; 1.95 (S, 3H, CH_3—COO—); 2.35 (t, 2H, —CO—$\underline{CH_2}$—CH_2—CH_2—); 2.65 (t, 2H, —O—CH_2—$\underline{CH_2}$—CO—); 4.25 (t, 2H, —COO—CH_2—). Mass spectrum (70 eV): *m/e* 130 (12%), 95(35), 70(78), 55(69), 43(100).

RESULTS

Gas chromatography (column A) of the volatile fraction of the interdigital gland secretion produced a large number of minor fractions and four major fractions (Figure 1). The latter fractions could be separated and isolated by preparative gas chromatography in sufficient quantities for recording of FT-NMR spectra.

The fraction eluting between 2 and 4 min on column B contained fractions 1, 2, and 3, and the fraction was therefore further purified on column C. Fraction 1, eluting between 5 and 6 min was collected in an amount of about 0.5 mg (from 52 interdigital glands).

The mass spectrum of fraction 1 [*m/e* 128(3.1), 110(7.1), 95(9.6), 71(13.5), 58(77.1), 55(8.1), 43(100), 41(18.5)] showed M^+ 128 and base peak at *m/e* 43. The intense peak at *m/e* 58 suggested a methyl ketone. Furthermore the prominent α-cleavage–H_2O peak at *m/e* 95 indicated a methyl substituent on the penultimate carbon, at least three carbon atoms removed from the carbonyl group (Eadon and Djerassi, 1970). The NMR spectrum provided confirmation with a singlet at 2.14 ppm (CH_3—CO—) and a doublet at 0.88 ppm [$(CH_3)_2C<$ H]. 6-Methyl-2-heptanone (II) was synthesized according to Scheme 1. The NMR and mass spectra of fraction 1 were identical with those of II.

Fraction 2, eluting between 7 and 8 min on column C, was collected in an amount of about 0.7 mg (from 52 interdigital glands). The mass spectrum of fraction 2 [*m/e* 142(5.6), 124(5.8), 113(13.2), 99(3.3), 95(68.9), 85(11.4), 72(61.4), 69(26.0), 57(100), 55(11.8), 43(95.5), 41(36.7)] showed M^+ 142, base peak at *m/e* 57, and an intense peak at *m/e* 72, which suggested an ethyl ketone. Again, a methyl substituent on the penultimate carbon, at least three carbon atoms away from the carbonyl group, was indicated by the intensity of the α-cleavage–H_2O peak at *m/e* 95. The NMR spectrum supported this with absorption at 0.8–1.2 ppm [$(CH_3)_2C<$ H, $\underline{CH_3}$—CH_2—) and 2.2–2.6 ppm (—CH_2—CO—CH_2—)]. Synthesis of 7-

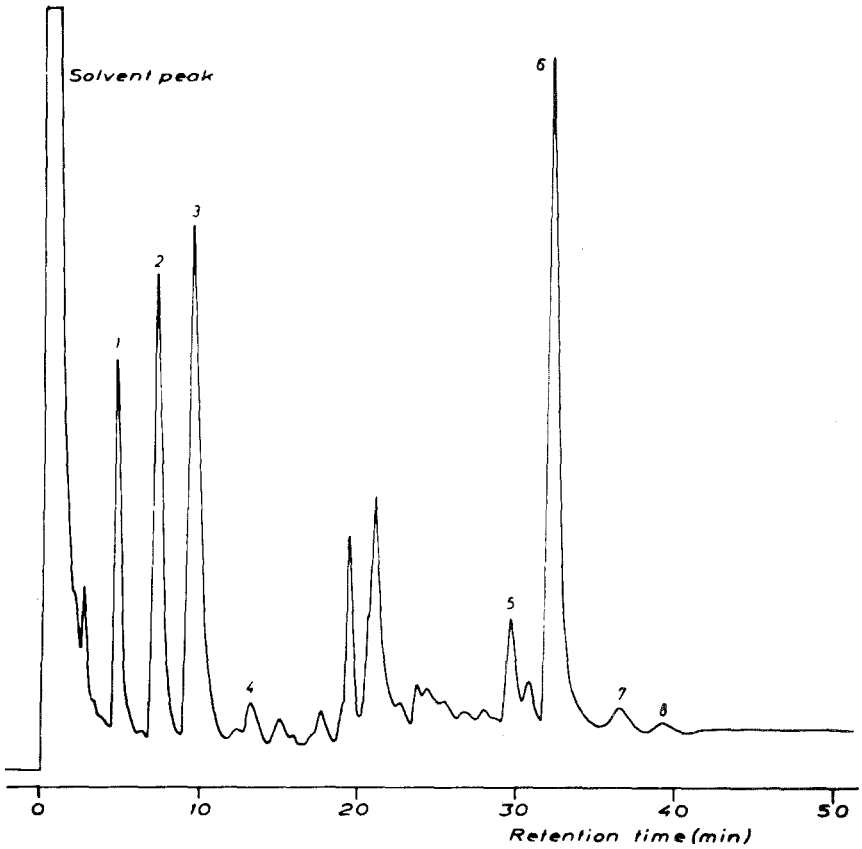
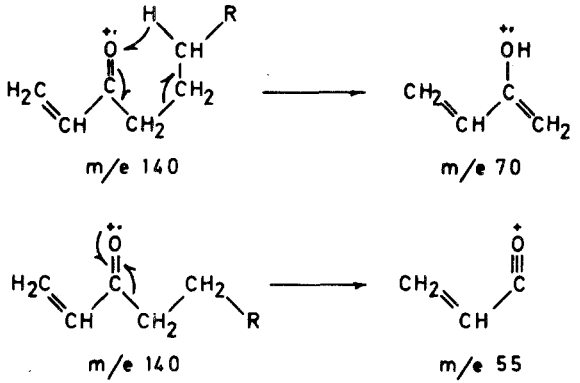


FIG. 1. Gas chromatogram of the volatile fraction of the interdigital gland secretion of reindeer (*Rangifer t. tarandus* L.).

methyl-3-octanone (V) was carried out according to Scheme 1. The NMR and mass spectra of fraction 2 and of V were identical.

Fraction 3, eluting between 7 and 8 min on column C, was collected in an amount of about 0.8 mg (from 52 interdigital glands). The mass spectrum (Figure 2) of fraction 3 showed a base peak at m/e 55 and an intense peak at m/e 70. The peak with the highest mass number, appearing at m/e 125, did not fit as a molecular ion, but might arise from loss of CH_3 ($M^+ -15$). Consequently a molecular weight of 140 was assumed, and the m/e 122 fragment was due to loss of H_2O ($M^+ -18$). These data suggest a molecular formula of $C_9H_{16}O$. The NMR spectrum (Figure 2) showed an ABC pattern at 5.5–6.4 ppm, which could be assigned to a vinyl group ($CH_2=CH-$). The doublet at 0.88 ppm was assigned to six protons [$(CH_3)_2C\leq H$]. In the mass spectrum the peaks at m/e 55 and m/e 70 could

be interpreted in terms of McLafferty rearrangement and α-cleavage of a vinyl ketone.



About 50 μg of the compound was hydrogenated with hydrogen as carrier gas in the inlet of the gas chromatograph equipped with the 5% Reoplex 400 column. The hydrogenation product was identified as 7-methyl-3-octanone (V) by combined gas chromatography–mass spectrometry.

About 100 μg of fraction 3 was ozonolyzed in CS_2 at $-65°C$, but the products could not be identified by gas chromatography. In fact the expected product, 6-methyl-2-oxo-1-heptanal, was synthesized, but it could not be found in the reaction mixture after ozonolyses of compound 3. 7-Methyl-1-octen-3-one (IX) was synthesized according to Scheme 2. Ozonolyses of IX gave the same result as with fraction 3. The NMR and mass spectra of fraction 3 and of IX were identical.

Fraction 6, eluting between 10 and 12 min on column B, was collected in an amount of about 1.1 mg (from 52 interdigital glands). The mass spectrum (Figure 3) showed a weak molecular peak at *m/e* 158 and a base peak at *m/e* 43. From the fragment series at *m/e* 158, *m/e* 140, *m/e* 122, and at *m/e* 143, *m/e* 125, and *m/e* 107, two water abstractions could be deduced. These data suggest a molecular formula of $C_9H_{18}O_2$. Since the NMR spectrum (Figure 3) did not indicate any C—C double bond, the compound was assumed to be a hydroxyketone. This assumption was strongly supported by diagnostic peaks in the mass spectrum at *m/e* 58($C_3H_6O^+$), 45($C_2H_5O^+$), 31(CH_3O^+). The NMR spectrum showed a quartet at 3.86 ppm (—O—CH_2—). The multiplet at 2.3–2.8 ppm was assigned to four protons (—CH_2—CO—CH_2—) and the doublet at 0.88 ppm to six protons [$(CH_3)_2C<H$]. Synthesis of 1-hydroxy-7-methyl-3-octanone (XVI) was carried out according to Scheme 3. The NMR and mass spectra of synthetic XVI were identical to those of the isolated compound.

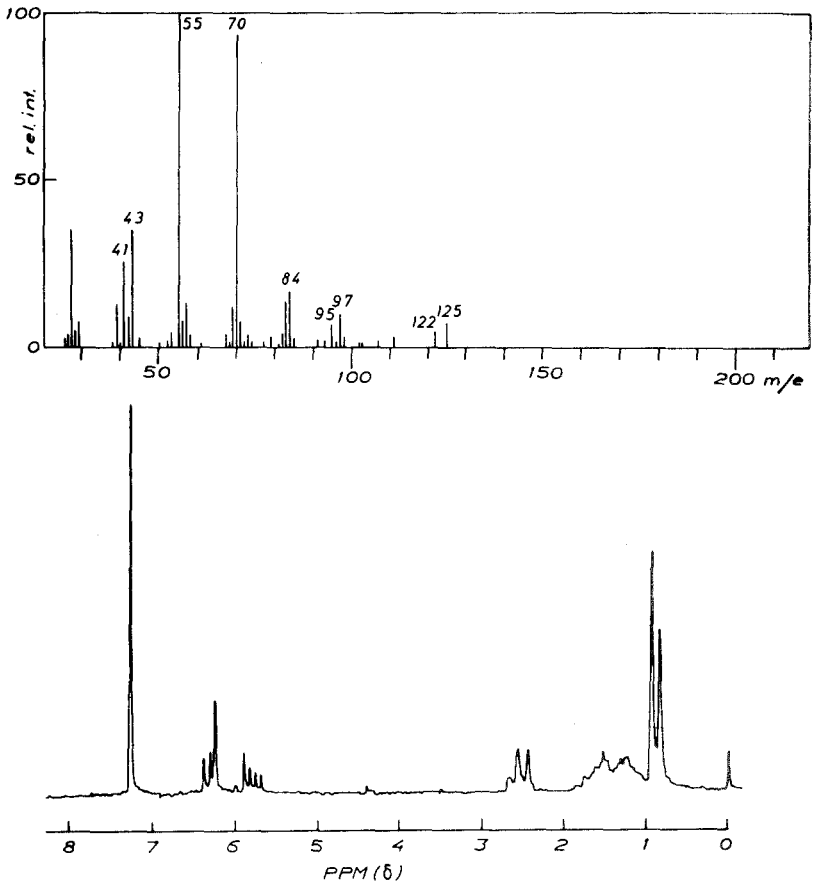


FIG. 2. Mass and nuclear magnetic resonance spectra for compound 3.

Fraction 4 was identified as 7-methyl-3-octanol (IV) by comparing its mass spectrum [*m/e* 126(1.3), 115(17.7), 97(39.6), 69(31.3), 59(100), 55(61.9), 43(30.4), 41(34.1), 31(21.7)] and GC retention time with those of a synthetic sample.

The five most abundant fragments in the mass spectrum of compound 7 were *m/e* 43, 70, 55, 95, and 130. Interpretation of the mass spectrum suggested that the compound was the acetate of XVI. Synthesis of 7-methyl-3-oxoctylacetate (XVII) and comparison of its mass spectrum and GC retention time with those of fraction 7 confirmed the structure.

Fractions 5 and 8 were identified as 2-phenylethanol and *p*-cresol by comparing their mass spectra and GC retention times on column D with those of authentic samples.

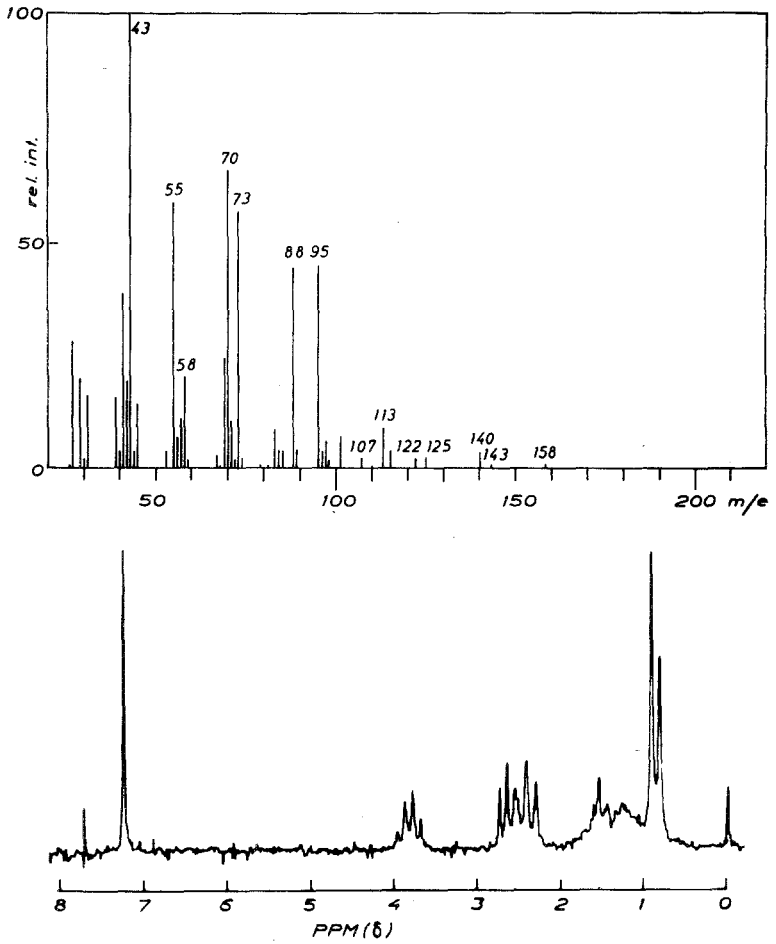


FIG. 3. Mass and nuclear magnetic resonance spectra for compound 6.

DISCUSSION

Except for 6-methyl-2-heptanone (II), all the aliphatic compounds from the interdigital gland secretion reported in this paper have the same branched-carbon skeleton containing different functional groups such as the ketone, alcohol, and ester functions. Control samples from the skin, taken from a region lacking specialized skin glands (the back), were also analyzed. None of the compounds mentioned above could be found in these samples. In addition, the interdigital gland secretion also contains a series of short-chain aliphatic acids (Brundin et al., 1978). However, the

amounts of the ketones far exceed those of the acids. By comparison, the tarsal and caudal glands have been found to produce volatile compounds other than those of the interdigital gland. The major volatile compounds from the tarsal gland have been identified as a series of straight-chain aldehydes and some fatty alcohols (Andersson et al., 1975). The aldehydes have also been found in the caudal gland, together with the acids mentioned above (Müller-Schwarze et al., 1977).

Correlation of the composition of the interdigital gland secretion with season-dependant behavior, such as sexual activity, antler growing, velvet shedding, antler casting, and calving, was considered when observing a yearly recurrent variation of the relative amounts of 6-methyl-2-heptanone, 7-methyl-3-octanone, and 7-methyl-1-octen-3-one in the interdigital gland secretion (Brundin and Anderson, 1978).

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EVIDENCE FOR DIGLYCERIDES AS ATTRACTANTS IN AN ANT-SEED INTERACTION

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

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

INTRODUCTION

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

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

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

METHODS AND MATERIALS

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

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

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

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

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

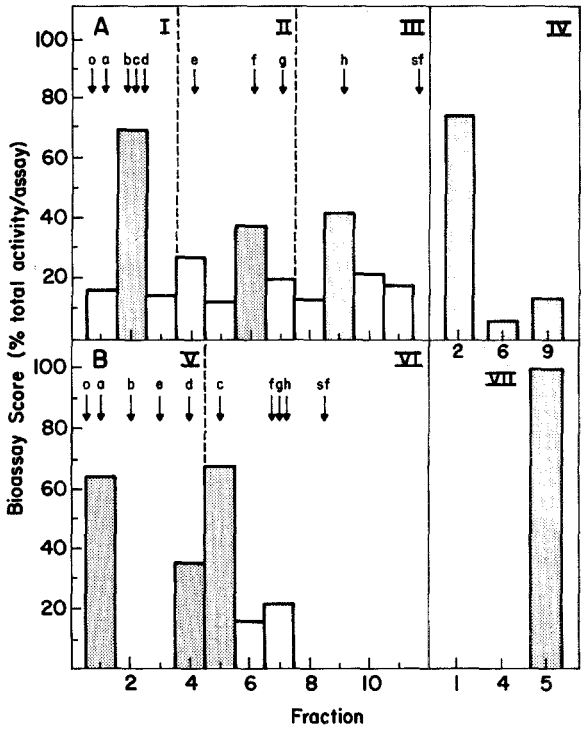

FIG. 1. Thin-layer chromatographic distribution of attractant activity in the nonpolar lipid extract of *V. odorata* elaiosomes separated with the following solvent systems: (A) hexane–diethyl ether–acetic acid, 80:20:2 (v/v/v); and (B) hexane–chloroform–ethanol, 10:10:1 (v/v/v). Fractions from each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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

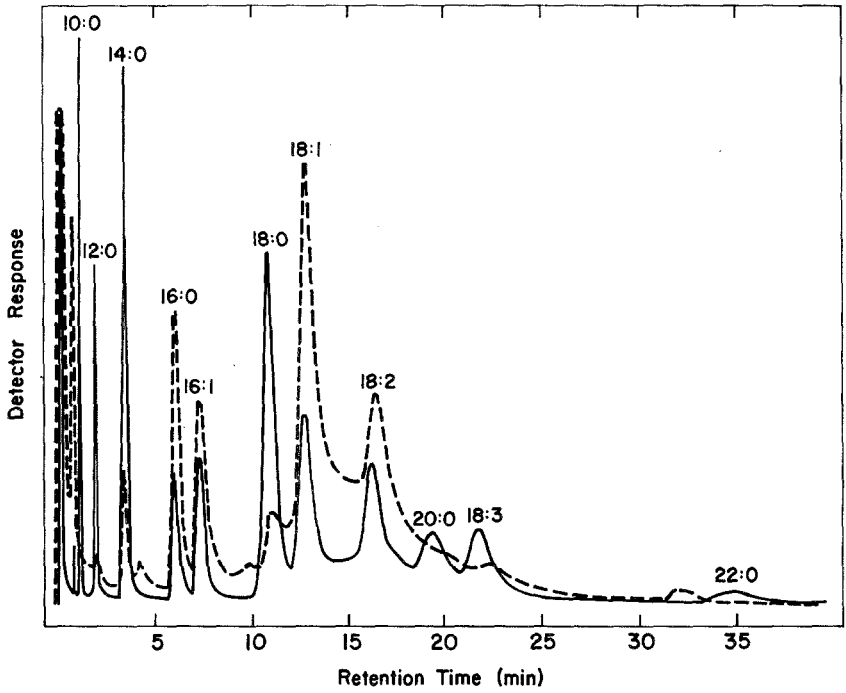

FIG. 2. Gas–liquid chromatography of methyl esters of fatty acids from the diglyceride fraction of elaiosomes (------) and of standards (———). The retention times for standards are denoted by their chain length and degree of unsaturation.

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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SUPPRESSION OF MALE EUROPEAN CORN BORER SEX ATTRACTION AND PRECOPULATORY REACTIONS WITH (*E*)-9-TETRADECENYL ACETATE[1]

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Abstract—(*E*)-9-Tetradecenyl acetate had previously been shown to be present in extracts of the female European corn borer, *Ostrinia nubilalis* (Hübner), along with (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate, and tetradecyl acetate. In field and laboratory assays (*E*)-9-tetradecenyl acetate suppressed male attraction and precopulatory behavior, but tetradecyl acetate had no influence. The bioassay data indicate that (*E*)-9-tetradecenyl acetate and the isomeric 11-tetradecenyl acetates are perceived through separate sensory channels. A blend of (*E*)-9-tetradecenyl acetate and the 11-tetradecenyl acetates might be useful as a disruptor of mating efficiency of this pest species in the field.

Key Words—Sex pheromones, European corn borer, *Ostrinia nubilalis*, insect sex behavior, inhibition of sexual behavior. (*E*)-9-tetradecenyl acetate, redbanded leafroller, *Argyrotaenia velutinana*.

INTRODUCTION

Klun et al. (1975) showed that (*E*)-11-tetradecenyl acetate could be used effectively to disrupt the sex attraction responses of European corn borer, *Ostrinia nubilalis* (Hübner), and redbanded leafroller, *Argyrotaenia velu-*

[1]Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the U.S. Department of Agriculture. The research work at UCLA was supported by a grant from the Mobil Foundation. The research is also a joint contribution: USDA and Journal Paper No. J-9103 of the Iowa Agricultural and Home Economics Experimental Station, Ames, Iowa 50011.

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tinana (Walker), in Iowa cornfields. For example, when the microencapsulated compound was applied in the field at 66 g/ha (hectare), male captures in pheromone traps were suppressed 90–100% for 2–3 weeks. Clearly, mechanisms regulating male flight-orientation responses of these moths to a source of sex attractant could be effectively counteracted. In the case of the European corn borer, however, it became apparent that the sexes could aggregate and copulate despite disruption of long-range pheromonal communications (Klun et al., 1975). The aggregation seems to be made possible by the mutual attraction of males and females to environmental stimuli such as headland vegetation of cornfields (Caffery and Worthley, 1927). Preventing copulation in this species, therefore, lies not in counteracting the mate-finding (long-range sex attraction) process but in severance of pheromonal communications between the sexes when they are only centimeters apart.

We initiated study of the chemical cues that stimulate close-range sexual behavior in the European corn borer and discovered that (*E*)-9-tetradecenyl acetate weakened male precopulatory reactions to (*Z*) and (*E*)-11-tetradecenyl acetate (compounds that are essential to elicitation of such male sexual behavior in the species).

In the laboratory and field bioassays reported here, (*E*)-9-tetradecenyl acetate suppressed male precopulatory reactions and attraction to the pheromonal stimuli known to be produced by the females of the species. Although we have not field-tested (*E*)-9-tetradecenyl acetate as a suppressant of European corn borer copulation, the compound might make an ideal pest-insect mating inhibitor: a chemical or blend of chemicals that decreases long-range communicative (sex attraction) efficiency and simultaneously interferes with male precopulatory (close-range) behavior.

METHODS AND MATERIALS

All field tests were conducted in the vicinity of Ankeny, Iowa. The traps, cylindrical ice cream cartons (20 × 9 cm diam.) coated on the inside with Tack-Trap, were deployed at the perimeter of cornfields and attached to wood stakes 1.5 m from the ground. The distance between traps within a replicate was 30 m, and all tests were conducted in a randomized complete-block design with 100–150 m between blocks. The chemicals used were prepared in our laboratories and were verified to be chemically and geometrically pure ($< 1\%$ impurities). The chemicals were evaporated from rubber septa that were placed on the Tack-Trap-coated interior of the ice cream cartons. Laboratory bioassays were conducted by the procedures described by Klun and Maini (1979), in which groups of 10 caged male moths were exposed for 30 sec to a given stimulus held upwind and the

number of males responding with wing vibration, extension of genitalia, and clasper responses during this time was recorded.

RESULTS AND DISCUSSION

(*E*)-9-Tetradecenyl acetate was identified in extracts of homogenates of female European corn borer (Iowa) abdomen tips (Klun and Junk, 1977). The extracts also contained (*Z*)- and (*E*)-11-tetradecenyl acetate and tetradecyl acetate. We anticipated that a combination of the four compounds might be more effective as a synthetic lure than previous lures that contained only the geometric isomers of 11-tetradecenyl acetate (Klun et al., 1973). The speculation was shown to be incorrect when we field-tested various combinations of the compounds in the proportions found in extracts of the female ECB. The results (Table 1) showed that tetradecyl acetate had no influence on male trap catches, the isomers of 11-tetradecenyl acetate were important attractants, and (*E*)-9-tetradecenyl acetate suppressed ECB male catch whenever it was deployed in combination with any of the compounds. In laboratory sex stimulation, assays of the compounds yielded

TABLE 1. SEX ATTRACTION RESPONSES OF EUROPEAN CORN BORER AND REDBANDED LEAFROLLER MALES IN THE FIELD TO TRAPS BAITED WITH COMBINATIONS OF (*Z*)- AND (*E*)-11-TETRADECENYL ACETATE (*Z*-11 AND *E*-11), TETRADECYL ACETATE (tdecyl ac), AND (*E*)-9-TETRADECENYL ACETATE (*E*-9)

Stimulus (μg)	$\bar{x}$ males/trap[a]	
	ECB	RBLR
84 *Z*-11 + 5 *E*-11 + 8 tdecyl ac	23.1 a	63.4 a
84 *Z*-11 + 5 *E*-11	19.0 ab	57.1 a
84 *Z*-11 + 5 *E*-11 + 3 *E*-9 + 8 tdecyl ac	13.4 bc	50.6 a
84 *Z*-11 + 5 *E*-11 + 3 *E*-9	10.0 cd	45.5 a
84 *Z*-11 + 8 tdecyl ac	7.9 d	3.9 b
84 *Z*-11	6.8 d	3.0 b
84 *Z*-11 + 3 *E*-9	2.5 e	1.4 b
84 *Z*-11 + 3 *E*-9 + 8 tdecyl ac	1.6 e	1.8 b
none	0.5 e	1.4 b

[a]Average trap catch in 8 replicates over 10 successive nights of trapping. Trap-catch data were transformed to square-root values for statistical analyses. Analysis of variance showed that treatment effects were highly significant. According to Duncan's multiple comparison test, means in each column that are followed by the same letter are not significantly different from each other.

TABLE 2. MALE PRECOPULATORY RESPONSE TO MIXTURES OF C_{14} ACETATE ESTERS IN PROPORTIONS THAT APPROXIMATE THE COMPOSITION OF EXTRACTS OF FEMALE EUROPEAN CORN BORER

Stimulus (ng)	$\bar{x}$ percentage response[a]
85 *Z*-11 + 3 *E*-11 + 9 tdecyl[b]	97 a
85 *Z*-11 + 3 *E*-11	97 a
85 *Z*-11	78 ab
85 *Z*-11 + 3 *E*-11 + 3 *E*-9 + 9 tdecyl ac	67 bc
85 *Z*-11 + 3 *E*-11 + 3 *E*-9	67 bc
85 *Z*-11 + 3 *E*-9	46 c
85 *Z*-11 + 3 *E*-9 + 9 tdecyl ac	41 c

[a]Mean percentage response of 7–10 males (15 replicates). Means followed by the same letter do not differ according to Duncan's multiple comparison test.
[b]*Z*-11 = (*Z*)-11-tetradecenyl acetate, *E*-11 = (*E*)-tetradecenyl acetate, *E*-9 = (*E*)-9-tetradecenyl acetate, and tdecyl ac = tetradecyl acetate.

TABLE 3. EFFECT OF INCREASING DOSES OF (*E*)-9-TETRADECENYL ACETATE (*E*-9-tda) ON TRAP CATCHES WHEN ADDED TO 84 μg 11-TETRADECENYL ACETATE (95:5, *Z*/*E*) COMBINED WITH 84 μg TETRADECYL ACETATE

	$\bar{x}$ males/trap[a]	
E-9-tda added to lure (μg)	ECB	RBLR
0	14.2	13.6
1	11.1	14.2
3	3.3	11.1
5	2.4	13.6
7	3.1	9.4
9	2.0	10.1

[a]$\bar{x}$ is the average of ten replicates (randomized complete-block design) over 18 consecutive nights. Analyses of variance showed highly significant treatment effects only for European corn borer (ECB). The mean square for dose was large compared with the error mean square. The dose sum of squares was partitioned into a linear component and a nonlinear component. The mean square of the linear component was large relative to the error mean square. Thus, there was an inverse linear relationship between ECB catch and *E*-9-tda dose. RBLR = redbanded leafroller.

results that were comparable to field-test results (Table 2). Notably, (*E*)-9-tetradecenyl acetate had no influence on attraction of redbanded leafroller males (Table 1), a species that also uses the isomeric 11-tetradecenyl acetates as part of its pheromone complement (Roelofs et al., 1975). Data in Table 3 show that increasing doses of (*E*)-9-tetradecenyl acetate (0–9 μg) added to the 11-tetradecenyl acetates in field traps also suppressed catches of male European corn borers, but did not suppress catches of redbanded leafroller males.

Additional results of laboratory sex stimulation bioassays are shown in Table 4. In these tests we used excess *E*-9-tetradecenyl acetate (32 ng) to enhance its effects in the assays. When males were exposed to the 11-tetradecenyl acetates, tetradecyl acetate, and (*E*)-9-tetradecenyl acetate simultaneously (stimulus B), precopulatory reactions to the stimulus were much weaker than reactions elicited by the 11-tetradecenyl acetates and tetradecyl acetate (stimulus A) alone.

When the males were exposed to (*E*)-9-tetradecenyl acetate for 30 sec and then exposed subsequently to stimulus B, male reactions were signifi-

TABLE 4. INFLUENCE OF EXPOSURES TO VARIOUS CHEMICAL STIMULI ON MALE EUROPEAN CORN BORER PRECOPULATORY RESPONSE

	Stimulus		$\bar{x}$ percentage response[a]	
Treatment	1st exposure	2nd exposure	First 30 sec.	Second 30 sec.
Experiment I				
1	none	*Z*-11 + *E*-11 + tdecyl (A)	—	89 a
2	none	*Z*-11 + *E*-11 + tdecyl + *E*-9 (B)	—	17 c
3	*E*-9	A	0	79 a
4	*E*-9	B	0	62 b
Experiment II				
5	A	A + A	78	72 a
6	B	B + A	16	35 b
7	B	B + B	11	11 c

[a]Average of 16 replicates. Means followed by the same letter in each experiment are not significantly different from each other according to Duncan's new multiple range test (5% level). Treatment 1: Male response to 84 ng (*Z*)-11-tetradecenyl acetate (*Z*-11) + 5 ng (*E*)-11-tetradecenyl acetate (*E*-11) + 8 ng tetradecyl acetate (tdecyl) = stimulus A in 30 sec of exposure time. Treatment 2: Male response to A + 32 ng (*E*)-9-tetradecenyl acetate (*E*-9) = stimulus B in 30 sec. Treatment 3: Male response to A in 30 sec following preexposure of males to 32 ng *E*-9-tda for 30 sec. Treatment 4: Male response to B in 30 sec following preexposure to 32 ng *E*-9-tda for 30 sec. Treatment 5: Male response to A in the first 30 sec and response in the second 30 sec to A with additional A. Treatment 6: Male response to stimulus B in 30 sec and response in the second 30 sec after A was added to B. Treatment 7: Male response to B in the first 30 sec and response in the second 30 sec to B with additional B.

cantly more intense than the responses that were elicited from males that were exposed to stimulus B without an (*E*)-9-tetradecenyl acetate preconditioning period. The result suggests that (*E*)-9-tetradecenyl acetate and (*E*)- and (*Z*)-11-tetradecenyl acetate might be perceived through separate sensory "channels" whose separate inputs are integrated centrally. Ostensibly, stimulation of male sexual behavior is greatly weakened when the (*E*)-9-tetradecenyl acetate and 11-tetradecenyl acetate channels make simultaneous inputs. Release or nonrelease of sexual behavior could be dependent upon mechanisms that judge the stimulative or inhibitory quality of the integrated sensory signal. Our bioassay results fit this model, since preexposure to (*E*)-9-tetradecenyl acetate caused males to perceive stimulus B as if the compound were not present. This concept agrees with the interpretations of Hirai et al. (1974) and Sower et al. (1974), who observed similar suppressive effects of certain olfactory stimuli on the sexual responses of tortricid and phycitid moths.

Results observed for treatments 1–4 in Table 4 indicate that (*E*)-9-tetradecenyl acetate would not effectively suppress ECB mating if it were used by itself in air-permeation field tests. On the other hand, stimulus B (a mixture of (*E*)-9-tetradecenyl acetate, tetradecyl acetate, and the 11-tetradecenyl acetate) could be useful. Data in Table 4 (treatment 6) show that when males were held in an atmosphere permeated with stimulus B and exposed to stimulus A, precopulatory reactions were significantly weaker than reactions in treatment 5, where the males were first exposed to an atmosphere permeated with stimulus A and then exposed to additional stimulus A. The results indicate that stimulus B would be a logical blend of compounds to deploy in the field as an antimating agent because the blend has the qualities necessary for both suppression of male attraction and weakening of male precopulatory reactions.

It is noteworthy that results obtained in treatments 6 and 7 showed the reaction of males preexposed to a *mixture* containing (*E*)-9-tetradecenyl acetate differed from the reaction of males preexposed to (*E*)-9-tetradecenyl acetate alone and subsequently exposed to a mixture containing the (*E*)-9-tetradecenyl acetate (treatment 4). From results obtained in treatment 4, one might have anticipated that preexposure of the males to a mixture containing (*E*)-9-tetradecenyl acetate would have resulted in habituation to the compound in the mixture and would have increased the response of the males when they were subsequently exposed to additional stimulus B (treatment 7) or stimulus A (treatment 6). This was not the case. Moreover, it appears as though the chemoreceptor system effectively measures temporal variation of odor stimuli.

The antisex activity of (*E*)-9-tetradecenyl acetate is puzzling, since it makes little biological sense for the European corn borer female to produce a sexual repellent. Furthermore, the compound does not deter attraction

of the redbanded leafroller, and therefore it does not cause the two species to be pheromone specific. Additional study will be required to resolve this enigma.

Populations of the European corn borer in different locales are known to have different blends of pheromone (Klun et al., 1975), and the genetic basis for this pheromonal polymorphism has been elucidated by Klun and Maini (1979). They showed that the 11-tetradecenyl acetate isomer composition of the female pheromone secretion is controlled by simple Mendelian inheritance. They also found that male sexual response preferences to isomer blends of 11-tetradecenyl acetate are also genetically determined and coupled to the communicative secretions generated by sibling females. The genetic study showed that three pheromonal genotypes (AA, Aa, and aa) of the insect occur in nature and that the *E*:*Z* 11-tetradecenyl acetate isomer blends secreted by the three genotypes are 97:3, 65:35, and 3:97, respectively. The strategies for efficient application of behavior-modifying chemicals for suppression of this pest species or use of synthetic pheromones in survey and population monitoring should take into account the elements of pheromonal polymorphism. For example, the genotypic profile of females at any given geographic location should be assessed in advance of the deployment of pheromone-baited traps to make certain that the most appropriate geometric blends(s) are used at the location and to ensure the highest level of sex attraction efficiency for the species in any given geo-

TABLE 5. COUNTERACTING EFFECT OF (*E*)-9-TETRADECENYL ACETATE (*E*-9-tda) ON EUROPEAN CORN BORER MALE PRECOPULATORY REACTIONS OF THREE PHEROMONAL GENOTYPES TO BLENDS OF 89 ng 11-TETRADECENYL ACETATE (11-tda) AND 8 ng TETRADECYL ACETATE

	$\bar{x}$ percentage male response				
	(*E*)-9-tetradecenyl acetate added (ng)				
Male genotype[a]	0	3	16	32	48
aa (IA)	91	78	37	22	14
Aa (F_1 hybrid)	87	63	37	24	13
AA (NY)	92	74	45	38	27

[a]The 11-tda isomer compositions used for the aa genotype, AA genotype, and Aa genotype were 95:5, 5:95, and 35:65 (*Z*/*E*), respectively. The *E*-9-tda was added in increasing amounts to the 11-tda + tetradecyl acetate stimulus. The experiment was conducted using a randomized complete block design with 16 replicates. Statistical analysis showed a highly significant *E*-9-tda dose–response interaction for all male types.

graphic location. Establishment of the profile of a parochial population can be accomplished via collection of samples of overwintering moths and subsequent chromatographic analysis of ovipositor washes from individual females by using the glass open tubular chromatography in a splitless sample-injection mode (Klun and Maini, 1979).

Data shown in Table 5 show that (*E*)-9-tetradecenyl acetate effectively suppresses the male precopulatory behavioral reactions to all three pheromonal types of European corn borer. Therefore, the compound could be applied in the field at any point where the pest is prevalent, and male sexual behavior should be disrupted no matter what the genotypic profile of the populations.

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SEX PHEROMONE OF THE STABLE FLY[1] Identification, Synthesis, and Evaluation of Alkenes from Female Stable Flies[2]

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Abstract—The cuticular alkenes of the female stable fly, *Stomoxys calcitrans* (L), which were responsible for inducing male fly copulatory behavior are (*Z*)-9-hentriacontene, (*Z*)-9-tritriacontene, 13-methyl-1-hentriacontene and 13-methyl-1-tritriacontene. The identifications of the branched alkenes and the synthesis of these four compounds are described. Bioassays indicate that these materials in combination with previously described methyl branched alkanes are more active than the individual components.

Key Words—Stable fly, *Stomoxys calcitrans* (L), pheromone, mating stimulants, alkenes, cuticular lipids, copulatory behavior.

INTRODUCTION

Since 1975 several reports (Carlson et al., 1975; Harris et al., 1976; Muhammed et al., 1975; Sonnet et al., 1977a,b; Uebel et al., 1975) have appeared that describe the chemical identity and biological activity of mating stimulants found in the cuticular lipids of male and female stable flies, *Stomoxys calcitrans* (L.). The female stable fly was shown to produce the normal C_{25}, C_{27}, and C_{29} alkanes and also two distinct series of branched alkanes with

[1] Diptera = Muscidae.
[2] Mention of proprietary or commercial products in this paper does not constitute an endorsement of this product by the U.S.D.A.

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odd-carbon backbones from C_{31} to C_{37} (Uebel et al., 1975). One group was singly methyl-branched; the other was doubly methyl-branched. The inference that these methyl groups were in a 1,5 or terpenoid relationship was derived from mass spectrometric (MS) fragmentation data. However, when the alkanes were subsequently synthesized (Sonnet 1976), the biological activity was found to be highest for the C_{31} and C_{33} alkanes bearing methyl groups on the 11 and/or 15 positions (Sonnet et al., 1977a).

Uebel et al. (1975) identified two of the major unsaturated materials obtained from stable flies as (*Z*)-9-hentriacontene and (*Z*)-9-tritriacontene. However, the gas–liquid chromatogram of the unsaturated hydrocarbons from the cuticular wash of 4-day-old females (Uebel et al., 1975) also indicated the presence of two more biologically active, unsaturated compounds. The present paper describes the identification of these two other unsaturated materials, their synthesis, and the evaluation of their activity. In addition, we investigated the activity of the saturates in conjunction with these unsaturates in the hope of paving the way for the field evaluations of these stimulants.

METHODS AND MATERIALS

Identification

Total stable fly hydrocarbons obtained as a hexane rinse were first fractionated on silver nitrated impregnated Florisil® to separate the saturated from the unsaturated hydrocarbons. The unsaturated hydrocarbons were then dissolved in 2,2,4-trimethylpentane, and a molecular sieve 5 Å was used to effect the separation of the straight-chain from the branched-chain hydrocarbons. The gross structures of the two major components were determined by comparing the gas chromatography retention times of the unsaturates and the hydrogenated mixture with appropriate standards (Table 1). Mass spectra were obtained with a Du Pont 491-B GC-MS equipped with a Du Pont 21-094 data system. Samples were introduced via a 1.2-m × 3-mm 3% Dexsil column programed from 200 to 300° C/min. The source temperature was 200°C for 70-eV electron impact spectra and 180°C for chemical ionization spectra. Isobutane was used as the chemical ionization reagent gas.

The structures of methylhentriacontene and methyltritriacontene, the two active materials whose structures we were investigating, were determined as follows: chemical ionization techniques yielded $M^+ = 448$, $C_{32}H_{64}$; and $M^+ = 476$, $C_{34}H_{68}$; the infrared spectrum of each component (trapped from GLC) indicated a terminal olefinic linkage (990 and 910 cm^{-1}). Reductive ozonolysis of each olefin and evaluation of the resulting aldehydes/ketones by GC provided only one peak from each ozonized alkene. The

Kovats indices (3261 for the product from the C_{32} compound, and 3462 for the product from the C_{34} compound) likewise suggested that the olefinic double bond being cleaved was at the end of the chain and the other product of cleavage was too volatile for our analysis. The alkenes were hydrogenated in hexane by using a platinum catalyst, and the resulting methylalkanes were examined by mass spectrometry. Again chemical ionization MS yielded $M^+ = 450$, $C_{32}H_{66}$; and $M^+ = 478$, $C_{34}H_{70}$. The electron impact (EI) spectra (Figures 1 and 2) indicated that the principal component of the material with the lower molecular weight was 13-methylhentriacontane; that of the other material was 13-methyltritriacontane. Each appears to contain a small amount of the 15-methyl isomer.

The problem of locating the methyl substituent relative to the double bond remained. Each olefin was trapped by GC and ozonized; the resulting ozonides were submitted to reaction gas chromatography (Beroza and Acree, 1964) which cleaved the oxygenated carbon atoms from the rest of the molecule. The principal product in each case was an 11-methylalkane with two carbons less than the original, indicating (1) that the double bond had indeed been terminal and (2) that the original olefins had been primarily 13-methyl-1-alkenes. The gas chromatogram of the ozonized/hydrogenolyzed materials also showed minor, but significant, amounts of methyl alkanes that were the result of the loss of four, rather than two, carbons. These were identified by EI-MS as 11-methylheptacosane and 11-methylnonacosane derived from the C_{31} and C_{33} chains, respectively. Thus some (5–10%) of the methyl-branched alkenes were $\Delta 3$ instead of $\Delta 1$. In addition, allylic fragmentation of the ozonides gave a few percent of the even-carbon-chain homologs.

Synthesis

The (*Z*)-9-hentriacontene and (*Z*)-9-tritriacontene were synthesized by a Wittig condensation by employing hexamethylphosphoric amide as a cosolvent so as to maximize the cis content (Figure 3). Because trans olefins had been observed from female stable flies (Muhammed et al., 1975) and had been reportedly active as pheromones, it seemed advisable to convert at least one of these cis alkenes into its trans isomer. This was accomplished by epoxidation of (*Z*)-9-hentriacontene and treatment of the resulting crude epoxide with triphenylphosphine dibromide in benzene; the resulting dibromide was then dehalogenated with powdered zinc in propionic acid. Column chromatography (silica gel, hexane eluant) produced (*E*)-9-hentriacontene (IR, 975 cm^{-1}). In similar cases, this sequence of reactions has been shown to invert olefins with complete stereospecificity as proven by capillary gas chromatography analysis of the olefin expoxides (Sonnet and Oliver, 1976).

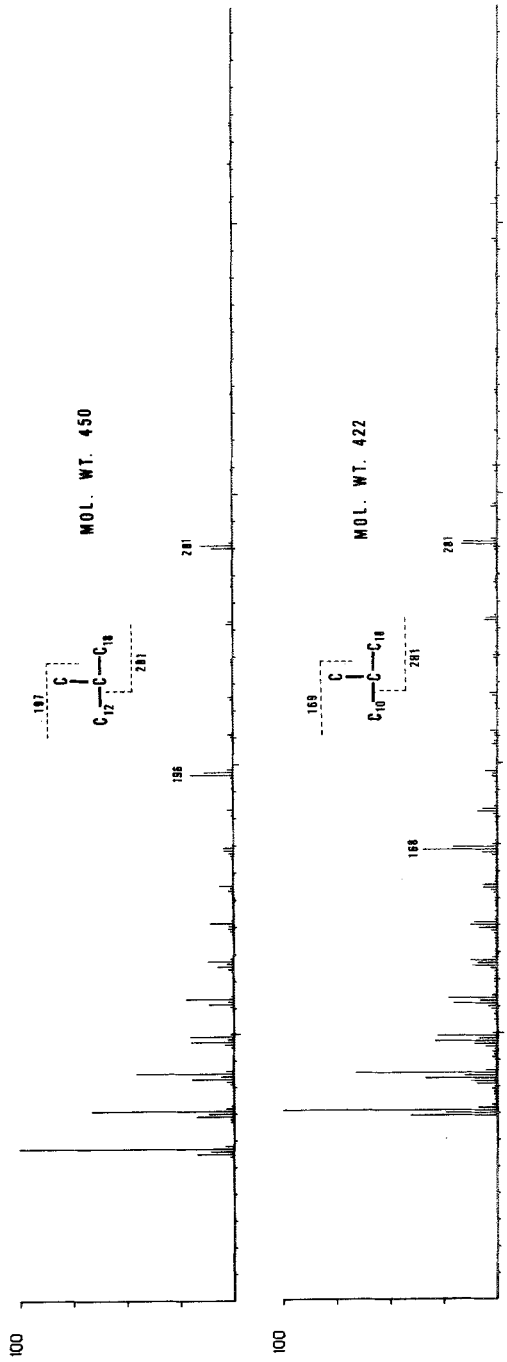

FIG. 1. Mass spectra of the methylalkane obtained by hydrogenation of the methylhentriacontene (above) and by ozonolysis/hydrogenolysis of that alkene (below).

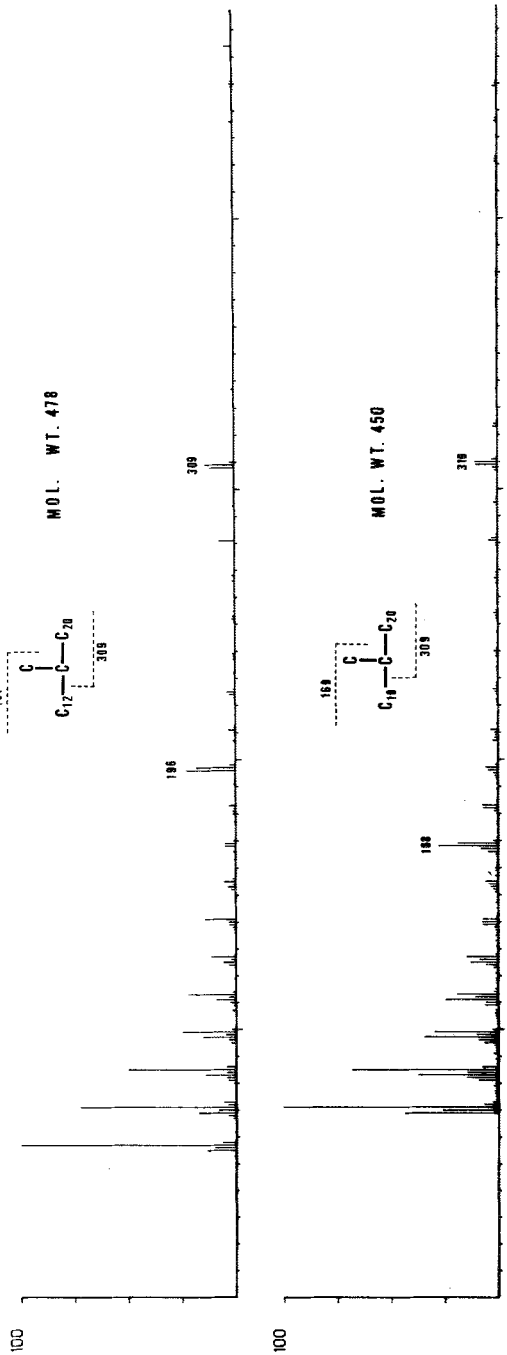

FIG. 2. Mass spectra of the methylalkane obtained by hydrogenation of the methyltritriacontene (above) and by ozonolysis/hydrogenolysis of that alkene (below).

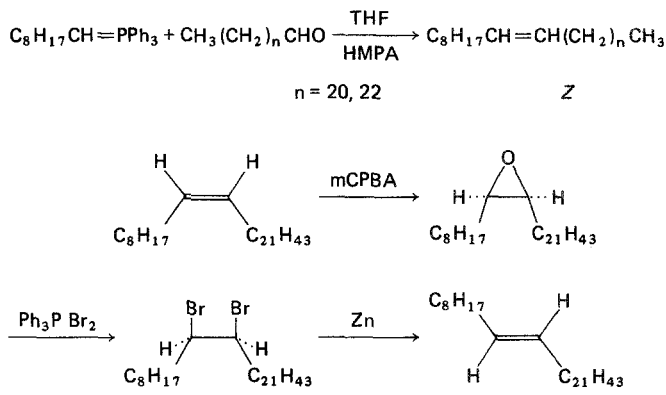

FIG. 3. Synthesis of (*Z*) and (*E*)-9-hentriacontene and (*Z*)-9-tritriacontene.

The methyl-branched 1-alkenes were synthesized as follows (Figure 4): ethylidenetriphenylphosphorane was allowed to react with 1-bromooctadecane (or 1-bromoeicosane) to produce an α-methyltriphenylphosphonium salt, I. The ylid of this salt was treated with the tetrahydropyranyl (THP) ether of 10-hydroxydecanal (synthesized from 1,10-decanediol). The resulting olefinic ether, II, was hydrolyzed to the alcohol hydrogenated over Adams catalyst (PtO_2), and converted to the bromide, III, by using triphenylphosphine dibromide. Lithium acetylide EDA complex in dimethyl sulfoxide–tetrahydrofuran provided the required two carbons bearing unsaturation; hydrogenation of the resulting acetylene over Lindlar's catalyst (Pd, $CaSO_4$ and quinoline-deactivated) yielded the desired 1-alkenes. The final

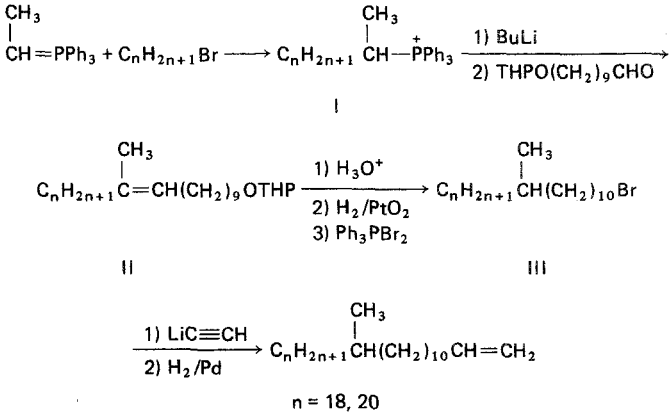

FIG. 4. Synthesis of the 13-methyl-1-alkenes.

TABLE 1. COMPOSITION OF UNSATURATED HYDROCARBONS OF 4-DAY-OLD FEMALE STABLE FLIES[a]

	% of total
Tetracosene	2.9
Heptacosene	9.0
Nonacosene	5.2
Methylnonacosene	4.9
Hentriacontene	18.9
Methylhentriacontene	25.7
Tritriacontene	10.3
Methyltritriacontene	16.0
Pentatriacontene	1.4
Methylpentatriacontene	5.0
Methylheptatriacontene	0.7

[a]Total hydrocarbon of the 4-day-old female is 14–17 μg.

products contained some alkane, so they were purified by fractionation on $AgNO_3$-impregnated Florisil for comparison with the natural products and for biologicasl testing. All the synthetic intermediates were identified completely by spectral data (IR, NMR), as well as by thin-layer and gas chromatography. The synthesized alkenes were identical in all respects to those of

TABLE 2. ACTIVITY QUOTIENTS (AQ)[a] MEASURED FOR SYNTHETIC ALKENES, TWO METHYL-BRANCHED ALKANES, AND SEVERAL PAIRS INVOLVING AN ALKENE WITH 13-METHYL-1-HENTRIACONTENE

	Average AQ ± SE[b]
1. 11-Methylhentriacontane	0.12 ± 0.056
2. 15-Methyltritriacontane	0.09 ± 0.032
3. (*Z*)-9-Hentriacontene	0.19 ± 0.102
4. (*Z*)-9-Tritriacontene	0.04 ± 0.021
5. (*E*)-9-Hentriacontene	0.05 ± 0.034
6. 13-Methyl-1-hentriacontene	0.34 ± 0.043
7. 13-Methyl-1-tritriacontene	0.16 ± 0.028
8. 6 + 1 (1 : 1 mixture)	1.10 ± 0.237
9. 6 + 2 (1 : 1 mixture)	0.40 ± 0.128
10. 6 + 3 (1 : 1 mixture)	0.66 ± 0.071
11. 6 + 4 (1 : 1 mixture)	0.58 ± 0.186

[a]Each compound or 1 : 1 mixture was bioassayed for 20 5-min periods each with 200 μg/male house fly. Different groups of 15 male stable flies are employed for each test. AQ = ratio of strikes on male house flies carrying the test material to strikes on female stable flies.
[b]SE = standard error of a mean.

the female stable fly, and the EI mass spectra of the 13-methyl-1-alkenes and their hydrogenated counterparts added to the corroboration.

Bioassay

Synthetic compounds were evaluated for their ability to induce copulatory responses in male stable flies by a bioassay similar to that described by Uebel et al. (1975). However, to ensure that the polyolefin borne by the male flies did not influence the results, we applied the test materials topically to male house flies (*Musca domestica* L.) rinsed with hexane, instead of to male stable flies. Each test sample (200 μg) was evaluated in the presence of 20 groups of 15 male stable flies during five 5-min observation periods. Although the female fly carries 14–17 μg of hydrocarbon when 4 days old, we did not obtain consistent assay results at other than the higher dose rate. Male fly responses to virgin female stable flies were also tabulated, and the ratio of strikes on male house flies to strikes on female stable flies was obtained (Table 2). This activity quotient then served as our measure of activity.

RESULTS AND DISCUSSION

A substantial number of compounds present in the cuticular lipids of female stable flies showed significant, but low, activity in our bioassay. For this reason several candidate synthetics were reexamined individually and in combination in an effort to maximize the responses. The data of Table 2 shows that a synergistic effect was obtained when these compounds were present in combination. The 13-methyl-1-hentriacontene was more active than either the branched alkanes or the normal alkanes, and when it was combined with 11-methylhentriacontane or (*Z*)-9-hentriacontene, its activity was significantly increased.

An exhaustive evaluation involving all possible combinations has not been made, but the most active pair so far was 11-methylhentriacontene and 13-methyl-1-hentriacontene. This pair, when tested at 200 μg/male house fly, was as active as a female stable fly in eliciting the male fly response. Again, we point out that the assay dose rate is quite high.

The principal components of the female stable fly sex pheromone consisted of (*Z*)-9-hentria- and tritriacontenes, 13-methyl-1-hentria- and tritriacontenes, and an array of methyl-branched hentria- and tritriacontanes in which 11- and 15-methyl-substituted alkenes were most active. However, none of these compounds were very effective by themselves in stimulating male stable flies to make mating strikes on the sample-bearing male house fly. Further testing designed to improve existing control measures for the stable fly with these materials should be taken into account.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS
VIII. Effect of Diet on the Kairomonal Activity of Frass from *Heliothis zea* (Boddie)[1] Larvae for *Microplitis croceipes*[2] (Cresson)[3-5]

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Abstract—*Heliothis zea* (Boddie) larvae were reared on fresh plant material (cowpea cotyledons) or on an artificial laboratory diet. Effect of these two diets on the kairomonal activity of the frass and cuticle from the larvae, as well as the kairomonal activity of the diets themselves, for *Microplitis croceipes* (Cresson) was determined. Diet was found to significantly affect the kairomonal activity of the frass although the diets themselves were not active.

Key Words—Kairomone, *Heliothis zea*, Lepidoptera: Noctuidae, *Microplitis croceipes*, Hymenoptera: Braconidae, diet.

INTRODUCTION

Lewis and Jones (1971) demonstrated that materials found in the frass, salivary secretions, and hemolymph of larvae of *Heliothis zea* (Boddie)

[1] Lepidoptera: Noctuidae.
[2] Hymenoptera: Braconidae.
[3] Part of the work reported here was done as a high school science project. This project was awarded the Grand Award in Life Sciences at the 29th and 30th Georgia Science and Engineering Fair, April 1977 and 1978, respectively; the Fourth Place Award in Zoology at the International Science and Engineering Fair held in Cleveland, Ohio, May 1977; the Second Place Award in Zoology at the International Science and Engineering Fair held in Anaheim, California, May 1978; and received other special awards.
[4] In cooperation with the University of Georgia College of Agriculture Experiment Stations, Coastal Plain Station, Tifton, Georgia 31794.
[5] Mention of a commercial or proprietary product in this paper does not constitute endorsement by the USDA.

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elicited a host-seeking response from female *Microplitis croceipes* (Cresson), a parasitoid of *Heliothis* spp. Jones et al. (1971) identified the most active compound as 13-methylhentriacontane, although numerous other compounds elicited lesser responses from *M. croceipes* females. They also found that hexane washes of *H. zea* cuticles were active.

The sequestering of chemicals from the food sources that serve as semiochemicals[6] of various types has been observed in some insect species. For example, larvae of the sawfly, *Neodiprion sertifer* (Geoffroy), sequester the terpenoid resin of its host plant (*Pinus sylvestris* L.) and use this resin as an allomone (Eisner et al., 1974). Also, kairomones important to the host-finding behavior of the parasitoids *Trichogramma evanescens* Westwood and *Orgilus lepidus* Muesebeck have been found in the food plants of their respective hosts, *H. zea* and *Phthorimaea operculella* (Zeller) (Hendry et al., 1976). The tachinid parasitoid *Lixophaga diatreae* (Townsend) is stimulated to larviposit by a kairomone found in the frass of its host *Diatraea saccharalis* (F.). This kairomone is sequestered from sugarcane, and larviposition is not stimulated by frass from larvae fed a soybean flour–wheat germ diet (Roth, King, and Thompson, personal communication). On the other hand, Hsiao et al. (1966) found no difference in the kairomonal activity of 70% ethanol extracts of frass from larvae of *Ostrinia nubilalis* (Hübner), which were reared on an artificial diet of corn, for the parasitoid *Lydella grisescens* Robineau-Desoosdy. In fact, some chemicals that function as hormones have been found in the food plants of various insects (DeSouza et al., 1969; Slama, 1969).

Therefore, diet may be an important factor in studies of semiochemicals in insects. The studies reported here were made to determine the importance of the diet of *H. zea* larvae to the production and/or sequestering of chemicals that are kairomones for the larval parasitoid, *M. croceipes*.

METHODS AND MATERIALS

The diets used in the study were fresh plant material and a modified pinto bean diet (Burton, 1969). Plant material used was pink-eyed, purple-hulled cowpea cotyledons grown in the greenhouse. *H. zea* larvae were reared from fresh eggs, obtained from a laboratory culture, in 1-oz plastic cups held at 26°C and 70% relative humidity. Frass was collected from larvae in the 3rd–5th instar. The *M. croceipes* used in the bioassay were obtained from a laboratory culture and had been reared according to the method of Lewis and Burton (1970).

[6] Semiochemical—a chemical involved in the chemical interaction between organisms.

Materials to be homogenized were lyophilized, weighed, and homogenized with distilled water (1 g/100 ml) in a blender.

The bioassay used to determine the kairomonal activity of the homogenates or extracts involved exposing individual 2- to 3-day-old mated *M. croceipes* females to a small drop of the test material, delivered with a Pasteur pipet to an area approximately 1 mm in diameter on a piece of Whatman No. 1 filter paper in the bottom of a petri dish. Because the parasitoids are negatively geotactic and positively phototactic, they could be guided to the treated sites by holding the dish in a vertical plane with the test side toward the light (Jones et al., 1971). Responses were scored on a 3-point scale. A score of 3 was given when a parasitoid stopped on the first pass over the sample, made an intense examination with her antennae, exhibited considerable excitement, and occasionally probed with her ovipositor (positive response). A score of 2 was given when a positive response occurred during the second pass. A positive response on the third pass was given a score of 1. When the parasitoid did not respond after three direct passes over the area, a score of 0 was given (Lewis and Jones, 1971). Each replication is the mean score for 8-10 female parasitoids.

PROCEDURES AND RESULTS

Experiment 1

Experiment 1 was conducted to determine the effect of diet on the kairomonal activity of *H. zea* frass. Some *H. zea* larvae were reared on cowpea cotyledons, while others were reared on the laboratory diet. Frass was collected daily from rearing cups and placed in a freezer. After a sufficient sample of frass from larvae reared on each diet was obtained, homogenates were prepared and bioassayed as previously described. This experiment was replicated 6 times.

Mean response to the frass homogenate from the larvae reared on plant material was 2.6, while mean response to the frass homogenate from larvae reared on laboratory diet was 0.3. Means were significantly different ($P < 0.0001$) by unpaired t test.

Experiment 2

Experiment 2 was conducted to examine the possibility that the diets themselves might have kairomonal activity. Samples of the cowpea cotyledons and laboratory diet were collected, freeze-dried, weighed, homogenized in distilled water, and bioassayed as previously described. This experiment was replicated 3 times.

Neither homogenate elicited any response from parasitoids.

Experiment 3

The possibility that the kairomonal concentration in the homogenate of cowpea cotyledon was too dilute to elicit a response was examined by reducing the volume of a 2-ml aliquot by half with a vacuum evaporator. These bioassays were conducted to establish the responses of female parasitoids to homogenates of the laboratory diet, the plant material, and the concentrated plant material homogenate. This experiment was replicated 3 times.

Again, none of the samples elicited any responses from the parasitoids.

Experiment 4

Experiment 4 was conducted to determine whether the activity of frass from larvae feeding on cowpea cotyledons was due to a combination of substances, some produced by the insect and some found in the plant material. The bioassays therefore compared the frass homogenate from larvae reared on laboratory diet, a mixture of the frass homogenate from larvae reared on laboratory diet, and the homogenate of the cowpea cotyledons (1 : 1 by volume), and the frass homogenate from larvae reared on plant material. This experiment was replicated 6 times.

The kairomonal activity of the mixture of homogenates was midway between the activities of the other homogenates (Table 1). Thus, unaltered substances in cowpea cotyledons may directly affect the kairomonal activity of frass of *H. zea* larvae.

Experiment 5

Experiment 5 was designed to examine other kairomone sources for differences in activity. Thus, *H. zea* larvae were again reared on laboratory diet or on fresh plant material. However, when these larvae were in the 4th

TABLE 1. RESPONSE OF *M. croceipes* TO A COMBINATION OF FRASS HOMOGENATE FROM LABORATORY DIET-REARED *H. zea* LARVAE AND HOMOGENATE OF PLANT MATERIAL[a,b]

Frass homogenate of laboratory diet-reared larvae	Mixture (1 : 1) of plant homogenate and frass homogenate of laboratory diet-reared larvae	Frass homogenate of plant-reared larvae
0.4a	1.0b	2.5c

[a] Data from 6 replications.
[b] Means followed by different letters are significantly different ($P<0.01$) as determined by Duncan's new multiple range analysis.

instar, 50 were placed in 50 ml of redistilled hexane and refrigerated for approximately 24 hr. The hexane was then decanted, diluted to 0.5×10^{-5} larval equivalents/ml, and bioassayed as previously described.

The mean responses to the cuticular wash from larvae reared on fresh plant material, as well as that from larvae reared on laboratory diet, were 1.7 and were not significantly different.

DISCUSSION

Experiments 1 and 3 demonstrated a significant difference in kairomonal activity of frass from *H. zea* larvae reared on laboratory diet vs. that from larvae reared on fresh cowpea cotyledons. Thus, chemicals from plants may directly contribute to the kairomonal activity of *H. zea* frass. Another possibility is that a chemical in the laboratory diet suppresses a positive response by the parasitoid. This seems unlikely since a homogenate of cowpea cotyledons alone elicited no response from *M. croceipes* females (Experiment 2). However, addition of this homogenate to the homogenate of frass from larvae reared on the laboratory diet increased the kairomonal activity of the combination (Experiment 4). This indicates that cowpea cotyledons possess a factor(s) that is at least synergistic to any chemical(s) that may be synthesized by the insect. Results of the bioassays of the cuticular wash (Experiment 5) also tend to support this point. The ecological significance of these data remains to be fully elucidated. They do, however, indicate the possibility that a mechanism does exist whereby a segment of a host population could escape attack by a parasitoid population by feeding on alternate food plants. Many of these chemicals may have a dual role in the host selection behavior of many parasitoids in that they may first be involved in the host habitat-finding behavior and then, after some processing by the host, mediate location of the host insect. Parasitoids are known to exhibit differential responses to various plants (Thorpe and Caudle, 1938; Monteith, 1958, 1967; Arthur, 1962; Streams et al., 1968; Herrebout and van der Veer, 1969; Read et al., 1970; Taylor and Stern, 1971; Lewis et al., 1972); for example, we know that *M. croceipes* females will search for hosts in cotton but not in corn or grain sorghum (Lewis, unpublished data). These differences may operate both at the host-habitat location and at the host-finding stages. These data also have important implications for evolutionary processes.

Extensive chemical analyses will be necessary to determine the chemical basis for differences demonstrated in this study. However, the data do demonstrate the importance of examining possible differences in the semiochemicals released by an insect species feeding on different diets and the possible ecological effect when feeding on different food plants. Also, the importance of considering these differences when studying semiochemi-

cals from insects reared on artificial diets is evident. For example, Vinson et al. (1976) found that the parasitoid *Bracon mellitor* Say would respond to methyl-*p*-hydroxybenzoate (methyl parasept) in the frass of boll weevil (*Anthonomus grandis* Boheman) larvae that were reared on a laboratory diet in which methyl parasept was used to prevent fungal growth. This response was apparently a case of associative learning.

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RESPONSE OF *Aedes triseriatus* LARVAE TO FATTY ACIDS OF *Cladophora*

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Abstract—The activity of a methanol extract residue from *Cladophora glomerata* against larvae of *Aedes triseriatus* is confirmed. Fractionation of this residue by solubility in solvents of varying polarity is monitored by bioassay with *A. triseriatus* larvae. The presence of fatty acids in the active hexane soluble fraction and saponified material obtained therefrom is indicated by ^{1}H NMR and IR. The identification of the individual fatty acids is achieved by co-injection and mass GLC of their methyl esters. Saturated and unsaturated fatty acids are screened against larvae and the LD_{50} values of the most active acids are determined. The LD_{50} of capric [10:0], lauric [12:0], myristic [14:0] and palmitoleic [16:1 (9*c*)] are 14, 7, 4 and 3ppm respectively. The most active acids 12:0, 14:0 and 16:1 (9*c*) are present in all the fractions examined and are released to water by the powdered alga at pH 8.5.

Key Words—Diptera, Culicidae, *Aëdes triseriatus*, Cladophorales, Cladophoraceae, *Cladophora glomerata*, fatty acid mosquito larvicides.

INTRODUCTION

The fresh water green alga *Cladophora glomerata* was implicated by Reeves (1970) as a naturally occurring control agent in mosquito breeding sites in southern California. *C. glomerata* in the form of a dehydrated powder, a crude methanol extract, and chromatographic fractions of the latter were larvicidal in bioassays employing various species of mosquito larvae as test organisms (Amonkar, 1969). However, the same study appears to lack clarity regarding active component homogeneity and stops short of identification.

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Cladophora appears in large wash-in crops on the shores of Lake Ontario. Because of its unpleasant features but contrasting beneficial possibilities in the control of water-bred pests, we have attempted the completion of the Reeves–Amonkar study. Our goals were: to confirm the larvicidal action of *Cladophora* preparations; isolate the active constituents; identify the latter and determine their larvicidal activity. Our results concerning methanol and aqueous basic extracts from *C. glomerata* are reported here and are concerned with the active acid components.

METHODS AND MATERIALS

Algal Extracts

Submersed *Cladophora* was harvested in August 1973 from the southern shore of Lake Ontario at the site of SUNY College at Oswego. It was identified as *C. glomerata*[3] according to the criteria of Prescott (1951). The alga was dislodged from rocks on which it was growing, washed with tap water, wrung manually, dehydrated in ovens under circulating air at 40–60° C, powdered to 40-mesh particle size in a Wiley mill, and stored at 4° C until use. A 5.12-kg quantity was extracted in a Soxhlet extractor with 11 liter of methanol[4] for 24 hr under N_2. The methanol was removed by vacuum evaporation at 4°C and thereby a 6.6% yield by weight of a dark green viscous residue was obtained.

A 128-g quantity of the dried, powdered alga was added to 1.5 liter of distilled water. Two grams of $NaHCO_3$ was added to raise the pH to 7 and a saturated aqueous Na_2CO_3 solution was added to raise the pH further to 8.5. The resulting mixture was kept at 25° C for 14 hr with occasional stirring. The alga was removed by centrifugation and the turbid aqueous solution, now at pH 7.2, was extracted continuously with a mixture of petroleum ether and ethyl ether for 24 hr. Twenty-eight milligrams of yellow oil was obtained by removing the solvent at reduced pressure from the organic layer. The aqueous layer was acidified with concentrated HCl to pH 1 and extracted continuously with a mixture of petroleum ether and ethyl ether for 24 hr. Removal of the solvent from the extract at reduced pressure left 63 mg of a dark brown oil consisting of the acids from the basic treatment of the powdered *Cladophora*.

Fractionations

The methanol extract residue was treated according to the fractionation scheme outlined in Figure 1. The acetone soluble fraction (A_s) was dis-

[3] Cladophorales: Cladophoraceae.
[4] All solvents were previously distilled.

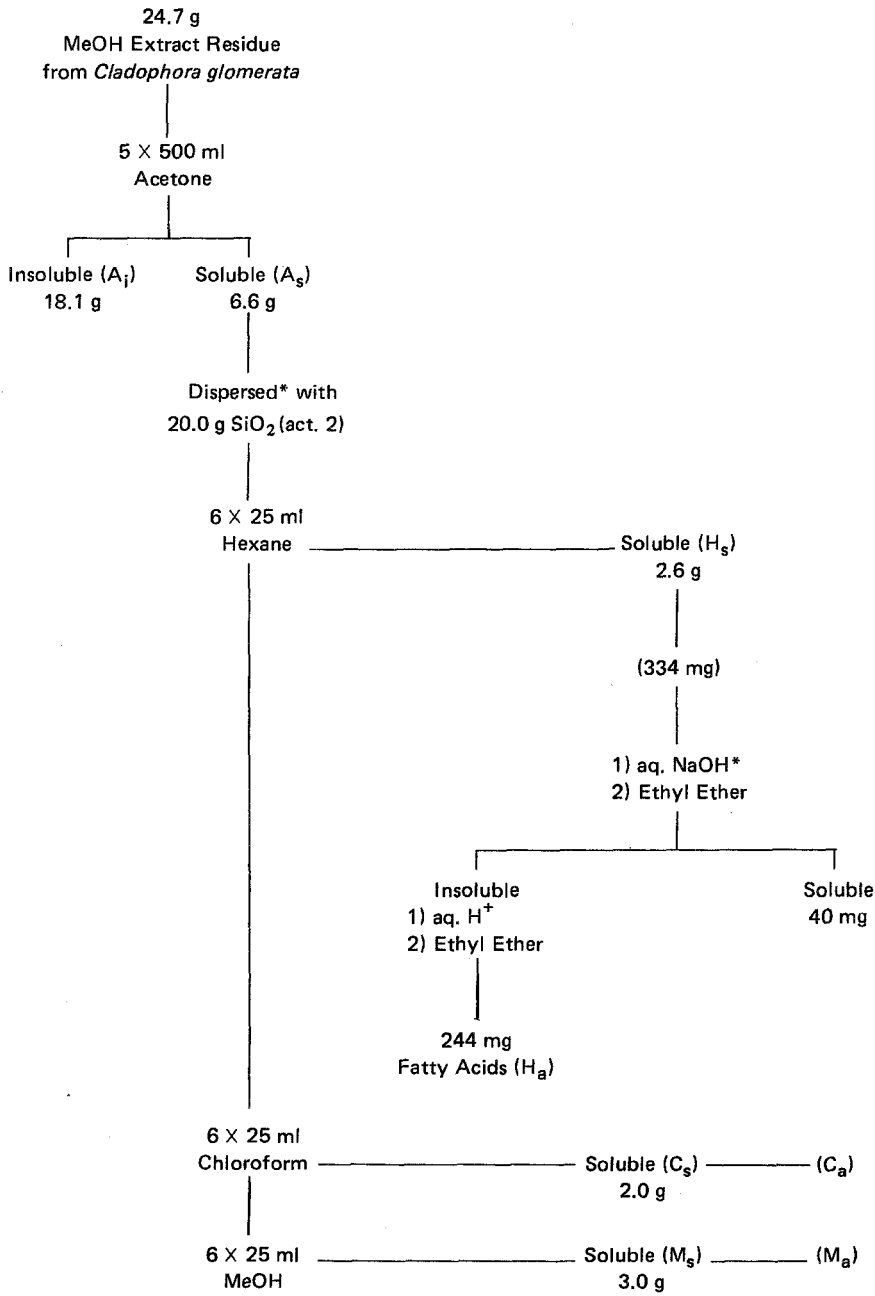

FIG. 1. Flow diagram indicating the fractionation procedure. The asterisk indicates further elaborations of the procedure in the Fractionations section of the text.

persed with silica gel, as indicated in Figure 1, and then treated sequentially with hexane, chloroform and methanol. Fraction M_s contained some silica gel and water. The fraction H_a, a mixture of fatty acids, was obtained from a 334-mg portion of H_s by the following procedure. The sample was treated with 90 mg of NaOH, 1 ml of water and sufficient methanol to give a homogeneous solution. The methanol was removed on the rotary evaporator at 30°C, the residue was treated with 4 ml of water, and the resulting mixture was extracted continuously for 2 hr with a mixture of ether and petroleum ether. After drying the ether extract and evaporating the solvent to dryness, 40 mg of glassy residue was obtained. The aqueous layer was acidified with 3 ml of 1 N HCl and extracted continuously three times, the first time for 1 hr, the second time for 2 hr, and the third for 3 hr, with fresh portions of ether–petroleum ether. From the combined ether extracts was obtained 244 mg of H_a. Fractions C_a and M_a were obtained in the same manner as H_a.

The mixture of fatty acids present in the methanol extract residue was isolated directly in the following fashion. A 490-mg sample of the residue was dissolved in 20 ml of methanol. Solid $NaHCO_3$ was added along with 20 ml of water. After solution, the methanol was removed on the rotary evaporator. The resulting mixture was extracted continuously with ether. The aqueous layer was acidified and extracted continuously with ether. The ether was removed on the rotary evaporator to obtain 70 mg of fatty acids.

Hydrogenation of H_a

A 90-mg sample of H_a in wet methanol was treated with hydrogen at atmospheric pressure and 25°C and with 10% Pd on charcoal until the uptake of hydrogen ceased. Thus a 66-mg sample of hydrogenated H_a was obtained.

Active Control

Ikeshoji and Mulla (1974) reported that 3-methyloctadecanoic acid (3-MODA) is a mosquito larvicide. 3-MODA was prepared by the method of Cason et al. (1949), recrystallized from acetone to a constant mp, 50.8–51.3°C, and employed at 75 ppm to monitor the sensitivity of the test organism in bioassay.

Test Sample Preparation and Bioassay

In testing samples at 700 ppm (700 μg/ml H_2O), a 70-mg quantity of the test substance was dissolved in 0.5 ml of acetone and the resulting solution was added to a 150-ml beaker containing 50, 24-hr-old *A. triseriatus*[5] larvae in

[5] Diptera: Culicidae.

100 ml of distilled water.[6,7] A 7.5-mg amount of 3-MODA was treated in a similar manner. The larvae were fed 8 mg of Tetramin every other day starting 24 hr from the time of hatching. The controls consisted of water, 0.5 ml of acetone in water, and the active substance control in acetone–water. The quantity of 3-MODA chosen for the active substance control was an amount necessary to kill approximately 50% of the larvae. In testing samples at 100 ppm, a 5-mg quantity of test substance was dissolved in 0.25 ml of acetone and the resulting solution was diluted to 50 ml with distilled water in a 150-ml beaker.

Bioassays were carried out in a constant temperature room at 27°C in triplicate. The test beakers were randomly placed in enamel pans partially filled with water. Surviving larvae were counted on day 6 from the day of hatching. The percent mortality was calculated on the basis of the total mortality per 150, and the 99% confidence levels for controls and test substances were determined.

Bioassays were carried out to screen those commercially available saturated and unsaturated fatty acids which also were present in *Cladophora*. These bioassays, routinely carried out on acids at 20 ppm, and those bioassays carried out to compare the activity of the *Cladophora* fatty acids (H_a) and hydrogenated fatty acids were performed in triplicate on 20 larvae each. The 24-hr-old larvae were placed in 50-ml beakers containing 20 ml of distilled water.[8] They were given the test substance in 0.1 ml of acetone and immediately were fed 4 mg of Tetramin. After a total of 48 hr from the time of hatching, the survivors were counted. Thus in time the period for all bioassays was shortened from 6 days to 2 days because ancillary experiments showed most mortality occurred within the first 24 hr of treatment with the active test substances and fractions. Bioassays to ascertain the LD_{50} values of the fatty acids determined most active at 20 ppm were carried out in triplicate on 50, 24-hr-old larvae in 50 ml of water in 150-ml beakers. The test substance was administered at 24 hr along with 8 mg of Tetramin, and the survivors were counted at 48 hr from the time of hatching. 3-MODA was not employed as an active control in the screening of commercially available fatty acids or the LD_{50} determinations. LD_{50} values were determined by the probit method from five different concentrations of test sample.

No difference in mortality was observed among blank solution bioassays carried out in 20, 50, or 100 ml of solution. The reductions in the volume of bioassay solutions was effected in order to conserve partially

[6] Chlorinated tap water is likely to be inappropriate for this kind of experiment since water containing more than 1 ppm of Cl_2, with or without traces of acetone, is a significantly active mosquito larvicide; chlorine water at concentrations of 5 and 1 ppm produced 100 and 19% mortality, respectively, while distilled water, used as the control, produced a mortality of 4%.

[7] Surface area/larva was 0.44 cm^2.

[8] Surface area/larva was 0.55 cm^2.

purified fractions for further separation and testing and to conserve active commercially available fatty acids for later LD_{50} determinations.

Identifications

Samples H_s and H_a were examined by infrared (IR), in $CHCl_3$ solution, and ^1H nuclear magnetic resonance (^1H NMR) spectrometry at 60 MHz in $CDCl_3$ solution using tetramethylsilane as the standard (δ0.00 ppm). The IR was determined on a Perkin-Elmer 137 spectrometer and the ^1H NMR on a Varian A60 spectrometer.

Authentic samples of saturated and unsaturated fatty acids were purchased from Applied Science Laboratories, Inc., State College, Pennsylvania, and were converted to their methyl esters with diazomethane in ether solution. Fractions H_s, H_a, that from the treatment of the methanol extract residue with sodium bicarbonate, and that from treating dried, powdered *Cladophora* with sodium carbonate–sodium bicarbonate were mixed with an etheral solution of diazomethane in order to identify by gas–liquid chromatography (GLC) the component fatty acids as their methyl esters. The GLC analyses were performed on an F and M 810 GLC equipped with a 3-m × 4-mm glass column containing 10% Silar 10C on 100/120 mesh Gas Chrom Q. The carrier gas was N_2. The temperature was programed from 140°C to 240°C at the rate of 4°/min. Initially, identification of the component fatty acid methyl esters was made by retention time comparison with authentic samples. Thereafter, the mixture of methyl esters from H_a was mixed with a single fatty acid methyl ester indicated present by retention time matching. Each authentic fatty acid methyl ester was mixed similarly in turn, and thereby the presence of these acids in a H_a was confirmed by coinjection GLC. Percentage amounts were approximated by cutting out the peaks from the chart paper and weighing them. An equal response of the flame ionization detector to each of the several fatty acid methyl esters was assumed in approximating the percentage composition. Mass chromatograms were obtained by GLC-EIMS (electron impact mass spectrometry) on a Finnigan Model 4021 operating at 17 eV with a filament current of 0.5 mA. GLC-CIMS (chemical ionization mass spectrometry) used methane as the reagent gas. The GLC column employed was the same type used for the coinjection GLC and was programed in the same manner.

The presence of potassium chloride was detected by an ETEC Autoscan electron microscope (model R1) equipped with a KEVEC detector and Finnigan analyzer.

RESULTS

The 6-day bioassay of the methanol extract residue indicated consistent mortality similar to that of 3-MODA at 75 ppm and above that of the con-

trols. These results are summarized as Series A in Table 1. The crude separation effected through acetone solubility resulted in a portion (A_s) which retained activity (Series B, Table 1). The acetone-insoluble portion (A_i) was not tested because of its insolubility in acetone–water, the test medium for all the other fractions. However, it appears unlikely that active components would be more concentrated in A_i than A_s because of the following two observations. Firstly, treatment of 18.1 g of A_i with chloroform and subsequent treatment of the resulting chloroform-insoluble residue (9.5 g) with methanol left a white residue (4.25 g) which was identified as potassium chloride by a scanning electron microscope equipped with an energy dispersive X-ray analyzer. Secondly, the methanol-soluble fraction (5.21 g) was tested at 100 ppm in the usual 6-day bioassay and showed no activity above control. Therefore no more than half the weight of A_i could be active, whereas more than two thirds the weight of A_s (fractions H_s and C_s) proved active. Moreover these results strongly suggested that the activity of A_s would be concentrated by treating A_s with solvents of low polarity (Figure 1). Results from Table 1, Series C indicate this to be the case. Of the fractions H_s, C_s, and M_s, the H_s fraction was slightly more active and consequently attention was focused on it.

Saponification of H_s yielded a mixture of fatty acids, H_a, which was also active (Table 1, Series C). The hydrogenated H_a appeared slightly less active than H_a (Table 1, Series D) and thus indicated that both saturated and unsaturated acid components of H_a were active.

The presence of fatty acids in both fractions H_s and H_a was consistent with the ^1H NMR and IR. Broad IR absorption at 2.9–4.1 μm and 5.8–6.0 μm indicated COOH presence. The ^1H NMR revealed: overlapping methyl multiplets at δ 0.97–1.05; strong CH_2 resonance at δ 1.28; a broad olefinic proton triplet ($J \simeq 4.5$ Hz) at δ 5.34; the carboxyl proton at δ 9.03.

The component fatty acids of H_a were revealed by the GLC analysis of the mixture of the derived methyl esters. Figure 2 reproduces a chromatogram in which the esters are identified and their approximate relative percentage amounts are given. The EI mass chromatograms of H_a indicated the presence of the eight parent ions corresponding to each of the peaks from C_{12} to C_{18} which are identified in Figure 2. In addition, a mass chromatographic peak for m/e 240, corresponding to the 14: 1 methyl ester, was found. In the case of four of the eight parent ions, only a single mass chromatographic peak was observed. In three other cases, the second peak had a longer R_t and represented a very much lower ion current value than the peak whose R_t corresponded to the conventional analytical GLC peak. Only in the case of m/e 214 were several peaks observed in addition to the mass chromatographic peak of the 12:0 methyl ester. Thus these mass chromatographic results support the identifications made by conventional coinjection GLC.

The GLC of H_s methyl esters was virtually identical, both qualitatively

TABLE 1. SUMMARY OF *Aedes triseriatus* MORTALITY RESULTING FROM A SIX-DAY TREATMENT WITH *Cladophora glomerata* METHANOL EXTRACT RESIDUE AND FRACTIONS THEREFROM

Sample	Concentration (ppm)	Mean mortality (%)	Range mortality (%)	Survivors in replicate of max. variation
Series A[a]				
MeOH extract residue	700	60.2	32.0–95.5	21, 15, 26
3-MODA[b]	75	41.9	23.4–54.0	15, 27, 27
Acetone control		8.2	1.3–22.0	43, 40, 35
Water		12.5	4.7–21.4	49, 31, 38
Series B[c]				
Acetone soluble (A_s)	700	71.7	68.0–75.4	0, 39, 9
3-MODA	75	58.0	53.3–62.7	37, 16, 17
Acetone control		4.0	2.0– 6.0	48, 48, 45
Water		5.0	4.7– 5.3	49, 47, 46
Series C[d]				
Hexane soluble (H_s)	100	97.4[e]		0, 0, 4
Chloroform soluble (C_s)	100	39.4[f]		31, 35, 25
Methanol soluble (M_s)	100	7.3[g]		47, 46, 46
Fatty acids (H_a)	100	44.5		24, 27, 32
3-MODA	75	39.6		32, 31, 35
Acetone control		3.3		49, 49, 47
Water		2.7		50, 47, 49
Series D[h]				
Hydrogenated H_a	40	81.7		5, 1, 5
	20	53.4		11, 5, 12
H_a	40	100		0, 0, 0
	20	90.0		2, 3, 1
Acetone control		13.3		20, 13, 19
Water		6.7		20, 18, 18

[a] Values in the mean mortality column represent the mean from four experiments each carried out in triplicate employing 50 larvae for a total of 150 larvae for each test substance or control.
[b] 3-Methyloctadecanoic acid as the active control substance.
[c] Values in the mean mortality column represent the mean from two experiments each carried out in triplicate employing 50 larvae for a total of 150 larvae for each test substance or control.
[d] Values in the mean mortality column represent results from a single determination.
[e] 92.6% mortality at 700 ppm; acetone control, 7.3% mortality.
[f] 100% mortality at 700 ppm; acetone control, 7.3% mortality.
[g] 82% mortality at 700 ppm; acetone control, 7.3% mortality.
[h] Employed 20 larvae in each replicate for a total of 60 and terminated the bioassay at 48 hr from the time of hatching.

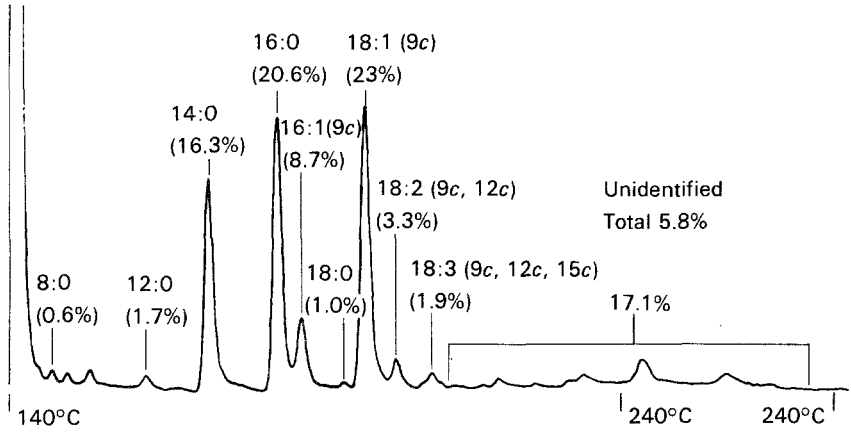

FIG. 2. The temperature programmed (4°/min) gas chromatogram of H_a performed on an F and M 810 using a 3-m × 4-mm glass column containing 10% Silar 10C on 100/120 mesh Gas Chrom Q.

and quantitatively, with that of H_a methyl esters. Qualitatively, the methyl esters from the acid mixture obtained by treating the methanol extract residue with aqueous bicarbonate were the same as those from H_s and H_a. But a relatively greater amount of the 12:0 acid was present in this third mixture of acids. Compared to the preceding three mixtures of fatty acids, the fourth mixture, obtained by treating the powdered alga with carbonate–bicarbonate at pH 8.5, showed the greater variation in the GLC of its methyl esters. All of the components of H_a were once again present according to coinjection GLC. But an additional major component having an R_t value between those of the 16:0 and 16:1 (9*c*) esters was observed. This peak is tentatively identified as the methyl ester of 4-ketononanoic acid by GLC-EIMS and GLC-CIMS. By the same methods of analysis, the presence of the dimethyl esters of C_9, C_{10}, and C_{11} dicarboxylic acids in the mixture is indicated. An account detailing these analyses and an indication of the larvicidal activity of dicarboxylic acids, a study now in progress, will appear in the future.

The data of Table 2 demonstrate that the larvicidal activity of saturated fatty acids appears within the narrow limits of the 10:0–14:0 even-numbered acids. Of these three, only 12:0 and 14:0, the two most active acids, were present in the four mixtures examined by GLC. Of the unsaturated acids, monoolefinic C_{14} and C_{16} acids and polyolefinic C_{18} acids were the most active. The most active unsaturated acid, 16:1 (9*c*) and the less active 14:1 (9*c*), 18:2 (9*c*, 12*c*), and 18:3 (9*c*, 12*c*, 15*c*) were detected also. The GLC analysis showed that each of the four acid mixtures examined contained the same active acids with only minor variations in the relative amounts. The only

TABLE 2. ACTIVITY, EXPRESSED AS LD_{50}, OF SATURATED AND UNSATURATED FATTY ACIDS AGAINST 24-HR-OLD LARVAE OF *Aedes triseriatus*

Sat'd acid	LD_{50} (ppm)	Unsat'd acid	LD_{50} (ppm)
6:0	>20	14:1 (9*c*)	$10 > LD_{50} > 5$
8:0	>20	16:1 (9*c*)	3
10:0	14	16:1 (6*t*)	3
12:0	7	18:1 (9*c*)	20
14:0	4	18:1 (9*t*)	20
16:0	>20	18:2 (9*c*,12*c*)	28
18:0	>20	18:2 (9*t*,12*t*)	$10 > LD_{50} > 5$
20:0	>20	18:3 (9*c*,12*c*,15*c*)	$10 > LD_{50} > 5$
22:0	>20	20:1 (5*c*)	>20
		22:1 (13*c*)	>20
		24:1 (15*t*)	>20

[a] Designated according to custom (Gunstone, 1967) by two numbers separated by a colon; the first number refers to the number of carbons in the chain and the second to the number of unsaturated centers. The position and configuration of the unsaturation is indicated by numbers followed by the letters *c* and *t*, for cis and trans, in parenthesis.

exception was the presence of the ketoacid and dicarboxylic acids in mixture 4 as already noted above.

DISCUSSION

At least some activity of *Cladophora glomerata* preparations against 24-hr-old larvae of *Aedes triseriatus* must stem from the presence of a small number of saturated and unsaturated fatty acids, identified as 12:0, 14:0, 14:1 (9*c*), 16:1 (9*c*), and 18:3 (9*c*, 12*c*, 15*c*), each possessing an LD_{50} less than 15 ppm. On a percentage basis, the sum of these five acids constitutes approximately one fourth of the total fatty acid content of *Cladophora*. Free active fatty acids are present not only in the methanol extract residue, as evidenced by their isolation with bicarbonate, but can be released from the powdered dry alga in aqueous suspension at pH 8.5, the pH of the Lake Ontario region, according to one of us (L.G.), and the St. Lawrence River region (Mills and Forney, 1977) where *Cladophora* abounds. The finding that *Cladophora*-produced fatty acids are toxic to *A. triseriatus* larvae serves as a launching point for the extended search for additional larvicides in other fractions of this alga as well as in other algae.

Maw and House (1971) have reported the potassium salt of the acid 10:0 gives 100% mortality of *Aedes aegypti* larvae at concentrations as low as 9×10^{-4} M (190 ppm). This is a more recent example of the long-known in-

secticidal effect of fatty acids and their salts that was pointed to by Puritch (1975) in connection with his observation that 1% solutions of the fatty acids 6 : 0, 8 : 0, and 18 : 1 (9*c*) were most effective in causing mortality to the aphid *Adelges piceae* at various life stages. Although Puritch also suggests possible modes of action, including an attractive one involving the uncoupling of oxidative phosphorylation, there is yet no firmly established explanation for the sensitivity of insects to a limited few free fatty acids.

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THE ATTRACTION OF FEMALE MOSQUITOES (*Anopheles quadrimaculatus* SAY) TO STORED HUMAN EMANATIONS IN CONJUNCTION WITH ADJUSTED LEVELS OF RELATIVE HUMIDITY, TEMPERATURE, AND CARBON DIOXIDE[1,2]

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Abstract—An apparatus has been assembled which permits storage and injection, at the same flow rate, of air containing human emanations into one port and air containing no emanations into the other port of a dual-port olfactometer, while monitoring the two flows for differences in temperature, relative humidity, and carbon dioxide level. Results of bioassays so conducted have led us to conclude that female mosquitoes (*Anopheles quadrimaculatus* Say) are attracted in significant numbers primarily by chemical emanations other than carbon dioxide and water.

Key Words—Mosquito, *Anopheles quadrimaculatus*, Diptera, Culicidae, attraction, bioassay, human skin emanations.

INTRODUCTION

Host attraction for mosquitoes, which has been explained on the basis of heat, moisture, carbon dioxide, odor, visual factors, and combinations thereof (Clements, 1963) is so complex that workers in the field are unable to embrace a unified theory of attraction. Indeed, it is impossible to reconcile the

[1] Diptera: Culicidae.

[2] The research reported in this manuscript was conducted in part with contract funds transferred from the Medical Research and Development Command, Office of the Surgeon General, U.S. Army. Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the USDA.

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views of Wright (1975) with those of Smith et al. (1970). Wright (1975) considered warmth and moisture as the only important attractants and therefore "saw no need for postulating a special skin odor," while Smith et al. (1970) postulated other chemicals, in addition to L(+)-lactic acid, as attractants, or at least synergists. Similarly conflicting views obtain in the case of heat, moisture, and carbon dioxide.

Acree et al. (1968) isolated L(+)-lactic acid from acetone washings of human skin. They found the isolate to be the major component in material which, added to an air stream containing 1000 ppm of excess carbon dioxide, attracted *Aedes aegypti* in the dual-port olfactometer of Schreck et al. (1967). Smith et al. (1970) continued the work and confirmed the importance of adding carbon dioxide to the lactic acid; in addition, they noted the conflicting results obtained by different workers in testing the effect of lactic acid upon *Ae. aegypti*. Consideration of these results caused Smith et al. (1970) to choose the testing technique of Acree et al. (1968) as most nearly approximating the normal response of mosquitoes to lactic acid emanations of human skin.

Using this technique, Carlson et al. (1973) screened chemicals structurally related to L(+)-lactic acid. Several α-hydroxy, -keto, -carboxy, -mercapto, -chloro, and -bromo short-chain aliphatic acids attracted *Ae. aegypti* at low dosages with added CO_2. The necessity for a free carboxyl group was shown by the inactivity of esters tested.

Most recently, Bar-Zeev et al. (1977) studied the attraction of *Ae. aegypti* to man. They tested mosquito response to human emanations, CO_2, H_2O, temperature, and various combinations thereof but did not reach any conclusions as to their order of importance.

Such results suggest that lactic acid, although perhaps the most important component in human emanations attracting mosquitoes, is not the only component. They also suggest a need for quantitating the effects of carbon dioxide and water on mosquito attraction, before attempting to isolate and identify any additional chemical(s).

We demonstrate the attraction of mosquitoes to human emanations, stored in gaseous form. The storage systems permit adjustment of humidity, temperature, and CO_2 before bioassays.

METHODS AND MATERIALS

Test Mosquitoes

Anopheles quadrimaculatus Say used in the bioassay came from a colony which has been maintained in the laboratory since 1943. The original stock for this colony came from the Malaria Control Branch, Division of

Health and Safety, Tennessee Valley Authority, Wilson Dam, Alabama. The mosquitoes were reared in trays until they reached the pupal stage, at which time they were transferred, in cups, into cages. After 24 hr, the cups were removed. Approximately 100 female mosquitoes, ranging in age from 7 to 10 days, were attracted into a trap by air which had passed over a hand and were transferred into an olfactometer without anesthesia (Smith et al., 1970).

Arm Box and Collection Systems

The apparatus devised for the bioassay comprises an arm box and three collection systems. The arm box (Figure 1) is a glass cone having dimensions which permit insertion of a human hand and forearm into a close-fitting rubber sleeve attached to the cone. A Cole-Parmer tube pump (model 7026-12, fitted with 1.2-cm ID silicone rubber tubing and driven by a variable speed motor) supplies olfactometer (OF) air to a circular copper tube, concentric about the arm and perforated to allow a 10 liter/min flow along the arm to transport any skin emanations. The OF air is held at 27 ± 0.5°C and maintained at the desired (60 ± 2%) relative humidity (RH) by a wet air plus dry air mixing system in conjunction with a Thunder Scientific Corporation humidity measurement system (Price et al., 1978). It is drawn from outside the building through an Absolute® filter which removes 99.97% of the particles as small as 0.3 μm and passed through an activated charcoal filter to remove organic molecules.

Each collection system consists of a pillow-shaped 90-cm × 150-cm Teflon® bag, containing two loose lengths of silicone tubing, two bulkhead connectors, two glass L's, two flow restrictors, a Lucite® Y, a plastic globe valve, and 1.2-cm ID silicone rubber tubing for connecting the parts shown in

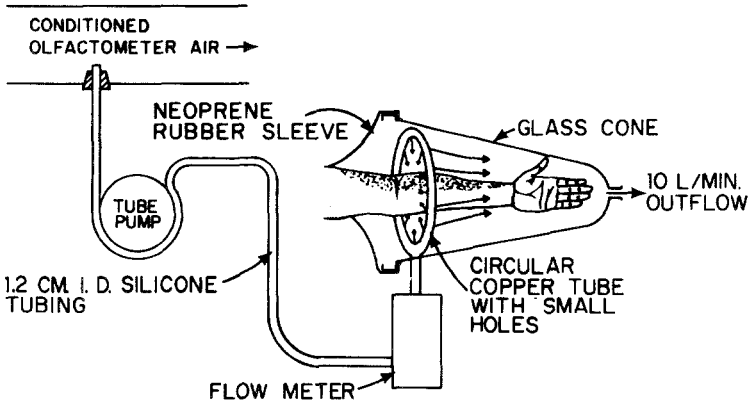

FIG. 1. Glass arm box for generating emanation-laden air.

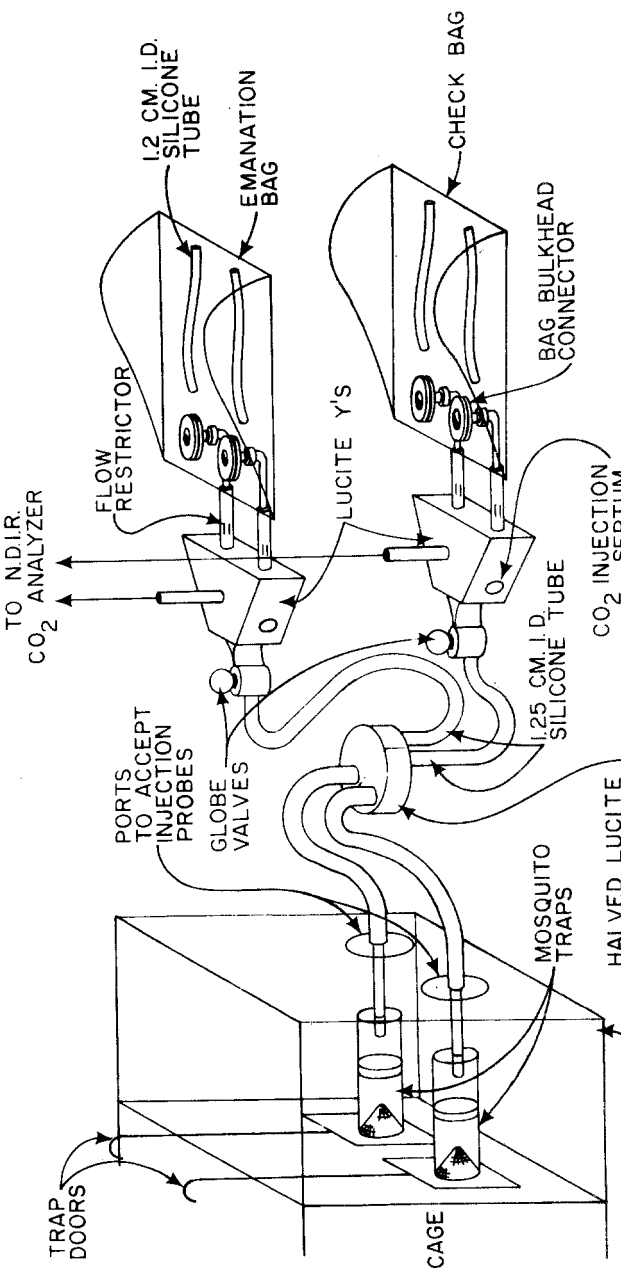

FIG. 2. Collection bags connected for bioassay.

Figure 2. The tubing within each bag is necessary because the bags tend to collapse first at the connector end when emptied. The bulkhead connectors are 25-mm Ultra-Torr® unions modified to permit hand-tightening. The flow restrictors are 2.5 cm long by 6 mm ID Teflon tubes which allow a bag connected to an OF for bioassay to empty for 8 min at 10 ± 0.1 liter/min without using a pump. Mounted on the front of each Lucite Y is a Nylon® garden hose-type globe valve; also, each Y is fitted with a septum for syringe injection of CO_2 and hose connections which permit removal of air for CO_2 analysis while a bioassay is in progress. To test the systems for leaks, each was filled with 100 liters of Freon® 12 and air mixture; subsequent "sniffing" with a refrigeration-type Freon leak detector found none.

Except for their globe valves, the three systems (only two of which are shown in Figure 2) are contained in a 70°C fan-circulated oven on 120-cm × 180-cm removable aluminum window screen shelves which are 20 cm apart. Thus, each bag is effectively immersed in oven air. The oven is mounted on a cart with casters to permit alignment of the bags with any one of eight olfactometers (Schreck et al. 1967).

Each bag can be connected to fill or to empty through the pillbox described below. The method of filling depends upon whether the bag is to hold emanations or check air. If emanations, the output of the arm box shown in Figure 1 flows through the pillbox and into a system. If check air, arm box is omitted. If a system is to empty, it is connected as shown in Figure 2. Two bags are used only for emanations; the third is reserved for check air.

Carbon Dioxide Level, Relative Humidity, and Temperature Determination

The carbon dioxide level in parts per million (ppm CO_2) was determined using a nondispersive infrared (NDIR) CO_2 analyzer (Mine Safety Apparatus Co., model 200 LIRA Analyzer). The analyzer sample cell is connected via the corresponding Lucite Y to one of two bags being emptied in a bioassay; the reference cell is connected to the other. A dual-head tube pump (Cole-Parmer catalog no. 7545 variable speed drive fitted with catalog nos. 7015 and 7015-20 heads) pulls air throuch each cell at 470 ml/min, resulting in continuous sampling of the two bags. If the CO_2 level in the sample cell exceeds that in the reference call, the difference is read out on a meter in ppm CO_2. Relative humidity (% RH) and temperature (T) are also determined during the bioassay by means of sensors located in the pillbox shown in Figure 2. The pillbox is a 9-cm-diameter × 4-cm-high Lucite cylinder divided into two halves; the emanation bag empties through one half, the check bag empties through the other. In each half are located % RH and T sensors. Humidity is monitored using two Thunder Scientific Corp. humidity measurement systems; temperature is monitored using an Atkins Technical, Inc., multiple

probe #2373810 temperature indicator. The humidity systems were periodically calibrated against an Atkins Technical, Inc., model #90023 portable psychrometer. The pillbox is located 15 cm from the OF front and 60 cm from the Lucite Y's. This arrangement permits the 70°C bag contents to cool to approx. 27°C as measured just before flowing into the OF.

Bioassays

Bioassays were run which quantitated the response of mosquitoes held in dual-port OFs (Schreck et al., 1967) to differentials between the two air streams entering each OF in % RH, ppm CO_2, and emanations. Before each bioassay, the OF doors were opened for 3 min to demonstrate no response to any contamination from handling. Plastic gloves were used when assembling OF parts.

Response to air with RH differentials was obtained by filling the check bag (oven off) with OF air raised to the desired % RH. After returning the OF air to the original 60% RH, the bag emptied through the check half of the pillbox into one of two open OF traps. The procedure was replicated at 13 different humidity levels. The same procedure, but with the oven on, made it possible to obtain 10 replications of mosquito response to warm, wet air.

Response to ppm CO_2 differentials was obtained by metering CO_2 into a 10 liter/min flow of air flowing into one of two open OF traps. The required CO_2 flow was set using the CO_2 analyzer. The procedure was replicated at three different CO_2 levels.

Response to emanation differentials was obtained by simultaneously emptying an emanation bag and a check bag into an OF. Adjustment of the % RH and ppm CO_2 in each bag made possible five replicates each of mosquito response to a constant amount of emanations vs. check air with: (1) a slight excess of H_2O and CO_2 in emanation bag, (2) the same amount of H_2O and CO_2 in both bags, (3) a slight excess of H_2O and CO_2 in the check bag, (4) a large excess of CO_2 in the emanation bag, and (5) a large excess of CO_2 in the check bag. In addition, response to warm, dry emanations vs. warm, wet air was obtained.

Response to reconstituted emanations was obtained as follows: a 5 liter/minute flow of nitrogen containing emanations was split and passed through two liquid nitrogen-cooled traps for 17.5 hr. After warming to room temperature, 2 ml of the resulting liquid (1.2 ml was equivalent to 120 liters of bagged emanations) was injected into a clean emanation bag containing 120 liters OF air. Two ml of distilled water was injected into the check bag also containing 120 liters OF air. After all liquid had evaporated, the bags were simultaneously emptied into an OF.

Finally, two sets of four replicates each were obtained in which the 10 liters/min output of an arm box was injected directly into one of two open

ports of an OF, first through a 30-cm length, then through a 360-cm length of 1.2-cm-ID tubing.

It should be noted (cf. Figure 2) that the 10 liters/min outflow from a bag was taken up in the 60 liters/min flow of OF air which passed through each trap of the OF used. Thus, any response was to a 1 : 7 dilution of the tested factor.

RESULTS AND DISCUSSION

Response to OF air at 13 elevated % RH levels (1–16.5) in competition with OF air at 60% RH is shown in Figure 3. Regression analysis of the data gave a curve with a slope of zero.

When the mosquitoes were presented with warm (32–35° C), wet (71–76% RH) streams of air in competition with relatively cool (26.4° C), dry (60% RH) OF air (Table 1), however, mosquito response to the warm stream was statistically higher (22% vs. 0%) at a 1% level of significance. Thus, the data suggest that a humidity differential alone is not enough; one more clue (a temperature differential) is required for mosquito response.

The response of mosquitoes presented with two streams of air differing only in CO_2 level is shown in Figure 4. Regression analysis of the data gave a

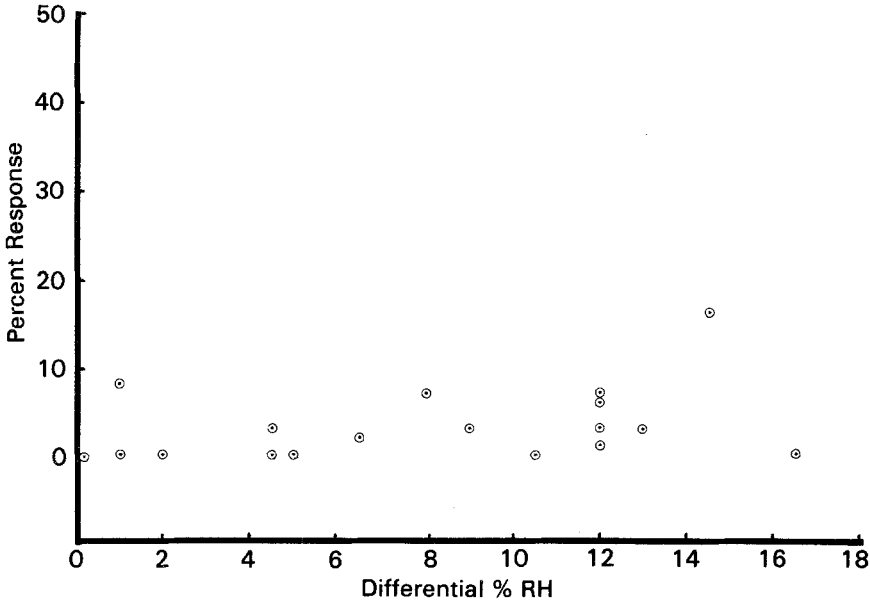

FIG. 3. Mosquito response to differential % RH.

TABLE 1. MOSQUITO RESPONSE TO WARM, HUMID AIR VS. OF AIR

Treatment[a]	Mean % response ± SD (6 min)	Humid air Avg. % RH ± SD	Temp. range
Air warmed, humidified	22.1 ± 10.0	74.2 ± 1.4	31.8–35
OF air	0	60	26.4

[a] 10 replications.

curve with a slope of 0.721, which was significantly different from zero at a 1% level of significance. It is apparent that response was not enhanced at a 10 ppm CO_2 differential, but was significantly enhanced at a 50 ppm differential.

Response to human emanations is shown in Table 2. In the first group of 5 tests, each check bag was filled with air adjusted, as closely as possible, to exceed the corresponding emanation bag by 16% RH and 10 ppm CO_2, values chosen with the results of the preceding % RH and ppm CO_2 experiments in mind. In the second group, each check bag was filled with air adjusted to have the same % RH and ppm CO_2 as the corresponding emanation bag. In the third group, each check bag was filled with air adjusted to be less than the corresponding emanation bag by 16% RH and 10 ppm CO_2. In all cases, the

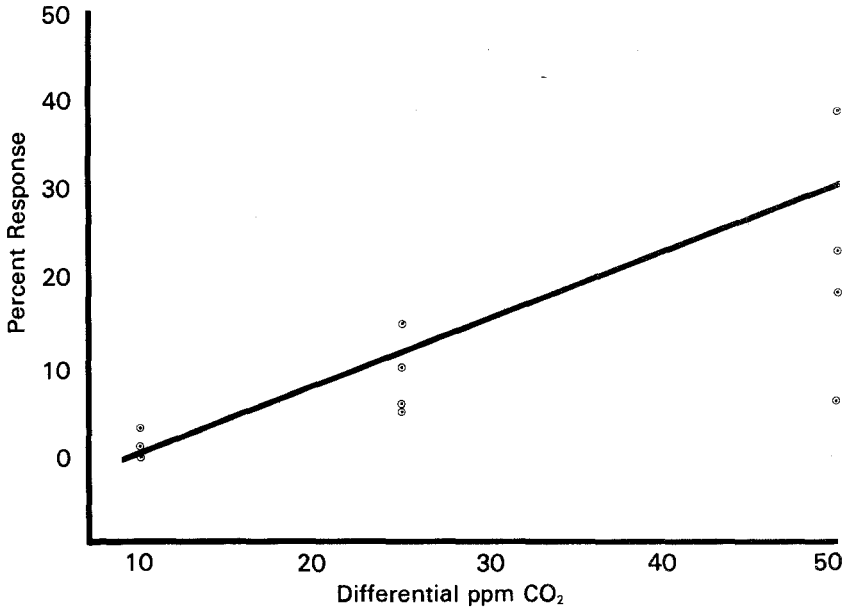

FIG. 4. Mosquito response to differential ppm CO_2.

TABLE 2. MOSQUITO RESPONSE TO HUMAN EMANATIONS WITH SMALL DIFFERENTIALS OF CO_2 AND H_2O

Mean % response ± SD (in 6 min)				
Emanations	Check	Mean Δ % RH ± SD[a]	Mean ΔT, °C ± SD[a]	Mean Δ ppm CO_2 ± SD[a]
Small excess of CO_2 and H_2O in check bag[b]				
30.0 ± 23.9	1.0 ± 1.4	6.5 ± 2.6	0.11 ± 0.08	7.0 ± 4.3
Balanced bags[b]				
26.0 ± 12.9	0.5 ± 1.0	1.2 ± 1.1	0.17 ± 0.17	1.0 ± 1.5
Small excess of CO_2 and H_2O in emanation bag[b]				
35.2 ± 20.1	0	10.4 ± 3.1	0.16 ± 0.06	8.0 ± 2.1

[a] Difference between average of 6 emanation readings and average of 6 check readings taken during test.
[b] Five replications.

TABLE 3. MOSQUITO RESPONSE TO HUMAN EMANATIONS WITH LARGE CO_2 DIFFERENTIALS

Mean % response ± SD (in 6 min)				
Emanations	Check	Mean Δ % RH ± SD[a]	Mean Δ T, °C ± SD[a]	Mean Δ ppm CO_2 ± SD[a]
CO_2 added to check bag[b]				
43.4 ± 10.2	5.6 ± 4.3	3.3 ± 2.5	0.09 ± 0.04	61.0 ± 13.5
CO_2 added to emanation bag[b]				
56.4 ± 13.8	0	2.8 ± 1.2	0.08 ± 0.04	61.7 ± 7.9

[a] Difference between average of 6 emanation readings and average of 6 check readings.
[b] Five replications.

TABLE 4. MOSQUITO RESPONSE TO WARM, DRY EMANATIONS VS. WARM, MOIST AIR

Treatment	Mean % response ± SD (in 6 min)	Mean % RH ± SD	Temp. range (°C)
Dry emanations[a]	48.6 ± 20.4	59.4 ± 3.5	29.0–37.4
Moist OF air[a]	3.3 ± 1.2	72.8 ± 2.2	28.8–37.3

[a] Five replications.

temperature differential was minimized. While trends are apparent, an analysis of variance for the data showed no statistical difference in the three groups. Thus, it appears impossible to explain mosquito attraction on the basis of the % RH and ppm CO_2 levels which exist in air passed over the human hand and forearm, levels which, in our tests, have always been lower than those chosen for the above series. Air from the OF increased about 15% in RH and less than 1 ppm CO_2 after passing over an arm in the arm box.

In an effort further to elucidate the action of CO_2 in attraction, human emanations were collected and 50–100 ppm CO_2 was added to the emanation bag or to the corresponding check bag. The data, shown in Table 3, were subjected to an analysis of variance. There was no statistical difference in the rates of attraction of the two groups at a 5% level of significance. Thus, CO_2, at a level exceeding that shown to attract mosquitoes in the absence of other attractants (Figure 4), shows little, if any, effect in competition with human emanations. We conclude that those emanations are a more powerful attractant than CO_2 at the 50–100 ppm level and thus the primary attractant at the CO_2 levels ordinarily associated with human skin.

The response of mosquitoes to warm, dry emanations in competition with warm, wet air is shown in Table 4. The higher response to emanations (statistically significant at a 0.05% level of significance) leads us to conclude that chemical emanations are a more satisfactory explanation for mosquito response than warmth and moisture.

To determine the activity of cold-trapped emanations, two replicates of mosquito response to reconstituted emanations were run. The results (Table 5) suggest that cold-trapping would be an acceptable method of separating the emanations from the air which transports them. Also, condensed, regenerated, or synthetic emanations may be presented to the mosquitoes from the bags at a constant concentration, which is not possible when air streams are passed over treated filter paper.

A final experiment was performed in an effort to explain the decreased response to bagged emanations compared to emanations injected directly from the arm box: as described above, emanations were passed through a 30-

TABLE 5. MOSQUITO RESPONSE TO RECONSTITUTED EMANATIONS VS. OF AIR

Treatment[a]	Mean % response ± SD (in 6 min)
Emanations, 2 ml	35 ± 1.41
Water, 2 ml	0.5 ± 0.7

[a] Two replications.

TABLE 6. MOSQUITO RESPONSE TO EMANATIONS VS. OF AIR: EMANATIONS PASSED THROUGH LONG OR SHORT TUBE CONNECTING ARM BOX AND OF

Treatment[a]	Mean % response ± SD (in 6 min)
Long tube	45.5 ± 16.35
Check	0
Short tube	84.9 ± 1.78
Check	0

[a] Four replications each treatment.

cm length or through a 360-cm length of tubing into an OF. The results, shown in Table 6, were subjected to an analysis of variance; the emanations passed through the short tube elicited a statistically higher response at a 1% level of significance. We believe the decreased response to emanations passed through a long tube to be explained by absorption, diffusion, and/or solution effects. Any one or all would serve to remove attractants from the air stream, where they must remain to be sensed by the mosquitoes.

Biological organisms, specifically mosquitoes, respond to a hierarchy of stimuli having elements which probably, even necessarily, overlap. While recognizing the futility of attempting to dissociate one element from all others, we believe that we have at least simplified the mosquito attraction hierarchy. The collection systems, the olfactometers, and the instrumentation made possible presentation to the mosquitoes of two streams of air, each at a desired level of % RH (±2%), ppm CO_2 (±2 ppm), temperature (±0.02° C), and flow rate (±0.1 liter/min). In addition, it was possible to include human emanations in one stream. Without exception, the mosquitoes responded to the emanation air, even when a biologically realistic excess of CO_2 and/or H_2O had been added to the check air. We believe the experiments clearly demonstrate the preeminence of human produced chemicals other than CO_2 and H_2O in attracting female mosquitoes in an olfactometer from more than 1 m to less than 1 cm away.

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QUANTITATIVE ESTIMATES OF THE WESTERN PINE BEETLE[1] ATTRACTIVE PHEROMONE COMPONENTS, *exo*-BREVICOMIN, FRONTALIN, AND MYRCENE IN NATURE[2]

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Abstract—Three parameters are described for estimating the natural occurrence of chemically defined insect pheromones: (1) the rate and duration of release by the insect, (2) the density of the pheromone-emitting insect population in both time and space, and (3) dispersal and degradation rates of the chemicals. Each of these parameters, except dispersal, was estimated for a population of *Dendroctonus brevicomis* LeC, and its three component attractive pheromones. A single generation of 610,000 beetles, believed to comprise the entire population in a 65-km^2 forest, was estimated to have released 0.78, 3.7, and 370.5 g of frontalin, *exo*-brevicomin, and myrcene, respectively, within a 30-day period.

Key Words—*Dendroctonus brevicomis*, *exo*-brevicomin, frontalin, myrcene, attractant, pheromone, pheromone production, pest management, bark beetle.

[1]*Dendroctonus brevicomis* Le Conte (Coleoptera: Scolytidae).

[2]Parts of this report were prepared for the U.S. Forest Service to accompany a future application to the Environmental Protection Agency for registration of these attractants for use in the suppression of *D. brevicomis* populations. The research was supported by grants to the University of California (D.L. Wood) from the National Science Foundation/Environmental Protection Agency (NSF-6B-34718/BMS75-04223), Forest Service, U.S. Department of Agriculture and Rockefeller Foundation. The findings, opinions, and recommendations are those of the authors and not necessarily those of these institutions.

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INTRODUCTION

Several insect pheromones have been isolated and synthesized during the past decade and are now being tested for possible use in various pest management programs. Risks of environmental disruptions caused by possible widespread use of pheromones should be much less than for conventional pesticides because pheromones appear to be highly specific, usually affecting one or only a few species, and probably will not be noxious. We believe that the application of synthetic pheromones in complex forest ecosystems could cause the following behavioral effects: (1) concentrate the target or related pests within, around, or away from the treated area; (2) change the dispersal and searching behavior of predators, parasites, and competitors; and (3) change the behavior of arthropods not associated with the target species, e.g., in soil and water. These potential behavior effects could result in changes in species abundance, which could cause environmental disruption. The above effects become more probable as the release rate, total quantity, and site of application of synthetic pheromones deviate from the natural occurrence. Therefore, qualitative and quantitative data on the occurrence of pheromones in nature should be of interest to both those responsible for the selection and design of control strategies using synthetic pheromones and for regulation of their use.

Questions about the natural occurrence of pheromones require that the exact chemical definition, including the absolute structural configuration, of these compounds be known before the rate and duration of pheromone release by the insect, the density of the pheromone-emitting insects in both time and space, and dispersal and degradation of the pheromone can be determined.

The complete chemical definition of an insect pheromone is, in most cases, a complex procedure because: (1) the insect produces only a minute amount, (2) there are problems associated with interpretation and quantification of pheromone-mediated behavioral responses, (3) compounds occur together in specific relative and absolute amounts, (4) pheromones often occur in mixtures of several compounds, and (5) chirality is difficult to elucidate.

Many insects appear to discriminate between subtle differences in molecular configurations of their pheromones and/or closely related isomers (Payne, 1974). Silverstein and Young (1976) discuss recent research demonstrating the ability of several insects to discriminate between geometric isomers and between enantiomers.

The western pine beetle, *Dendroctonus brevicomis* LeC. responded to a mixture of the (−) enantiomer of frontalin [(1*S*,5*R*)-1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane], the (+) enantiomer of *exo*-brevicomin [(1*R*,5*S*,7*R*)-*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane], and myr-

cene (7-methyl-3-methylene-1,6-octadiene),[6] while the other enantiomers of both *exo*-brevicomin and frontalin appeared to be inactive in all test combinations (Wood et al., 1976). *D. brevicomis* produced only the active enantiomers in nature (Stewart et al., 1977). However, until Mori (1974, 1975a,b) synthesized the enantiomers with known configurations for both species and made them available to biologists, all studies had been done with racemic mixtures (Bedard et al., 1970; Bellas et al., 1969; Kinzer et al., 1969; Byrne et al., 1974). Racemic mixtures of *exo*-brevicomin and frontalin were used as attractants in two large-scale population suppression studies (Roettgering, 1973; Wood and Bedard, 1974). Because *D. brevicomis* responds essentially to only one enantiomer of *exo*-brevicomin and frontalin, the effective dose of the racemate used in those studies was one-half the actual dose evaporated.

Rate and Duration of Pheromone Release

For most insects, little or no quantitative information pertaining to the rate and duration (including periodicity) of pheromone release is available. This situation is changing as technology has been developed that facilitates the measurement of pheromone production rates. This technology permits the efficient extraction of pheromones directly from air at the extremely low concentrations in which they naturally occur. At present there are two methods of air extraction: (1) the volatiles are absorbed onto Porapak Q (Byrne et al., 1975); (2) the pheromone-laden airstream passes through a cryogenic trap where the air is liquefied and subsequently distilled (Browne et al., 1974). Both methods appear to collect volatiles efficiently, especially compounds with molecular weights in excess of 50 (Byrne et al., 1975; Browne, unpublished). With either method it is possible to measure the rate of pheromone production by *D. brevicomis*. Recently, Gore et al. (1977) used Porapak Q to measure the amount of individual components of the attractive pheromone released by the female European elm bark beetle, *Scolytus multistriatus* (Marsham), boring into elm bolts over a 3-day period.

Among Coleoptera, daily calling behavior coincident with pheromone production has been observed in several species of dermestids (Hammack et al., 1976; Barak and Burkholder, 1977; Ma and Burkholder, 1978), and daily rhythmic pheromone release in the frass of the male boll weevil, *Anthonomus grandis* Boheman, has been measured (Gueldner and Wiygul, 1978). Such rhythms of pheromone production have not been demonstrated

[6]We prefer to consider myrcene a pheromone component rather than a kairomone, since its essential function is to synergize the intraspecific aggregation response to beetle-produced components. There seems to be no direct beetle/tree interaction mediated by myrcene. We see no need to burden the definition of a pheromone with the requirement that all components be biosynthesized by the emitting organism. In the present case, the beetle can be considered to "release," "emit," or "produce" myrcene by its boring activity in the host plant.

for Scolytidae (Borden, 1977). However, since calling and mating behavior of most Scolytidae is cryptic, direct observation is difficult.

The number of beetles trapped on trees during host colonization (Stephen and Dahlsten, 1976) may indicate the period of pheromone release. Host colonization is a dynamic process, in which the recruitment of new beetles is concurrent with a cessation of pheromone production or with production of antiattractants or repellents among the earlier arrivals (Wood, 1972; Wood and Bedard, 1977). The average duration of pheromone production for individual beetles is probably more accurately assessed by the length of time that tree parts, infested with known numbers of beetles, remained attractive under field conditions (Bedard, unpublished). In these studies, recruited beetles were prevented by a screen from boring and producing attractants. Thus the attraction was caused only by the previously attacking beetles.

Insect Densities

The second parameter in determining the natural occurrence of pheromones, i.e., the density and distribution of pheromone-emitting beetles both in time and space, expands the production of the individual to area-wide production through time. Thus, an estimate of the absolute population per area is required. For this report, we made pheromone production estimates based on population data obtained at Bass Lake, Madera County, California, where the number and distribution of *D. brevicomis*-killed trees and the within-tree beetle densities were estimated over a 65-km^2 area. These data were obtained in an attempt to estimate population changes caused by an experimental deployment of pheromone-baited traps (Bedard and Wood, 1970, 1974; Browne, 1978; Gustafson, 1972).

Dispersal and Degradation

Questions about either pheromone dispersal or degradation in nature require models of air movements for specific temporal and spatial limits. During the Bass Lake experiment, insufficient meteorological data were collected to construct any meaningful model of atmospheric transport and diffusion. Such models, whether dealing with short distances and a few cubic meters of air (a scale of interest to the insect behaviorist) or vast distances and many thousands of cubic kilometers of air (a scale of interest to the pollution scientist), require large amounts of highly specific meteorological and geographical data and thus are difficult and expensive to construct. While turbulent mixing is clearly the most prominent factor contributing to the diffusion of airborne pheromones in open environments, Aylor (1976), in a review of diffusion within the forest stem space, remarked that "few generalities can yet be made about turbulence in the forest."

Pheromones, like other natural product vapors in the environment, should eventually degrade. Perhaps situations exist where the rate of rapid depletion of an active component(s) could have behavioral significance. This apparently is not the case with the two known beetle-produced pheromone components of *D. brevicomis*. Our test indicates that frontalin and *exo*-brevicomin appear to be quite stable in the presence of air and sunlight. Small amounts of these compounds were sealed individually in large quartz tubes containing air and placed in full sunlight in Gainesville, Florida, for a period of three weeks during February and March. GLC tracings for both compounds were essentially identical before and after exposure. Myrcene oxidizes and polymerizes but can be stabilized for long term trapping experiments.

In this report we estimate the total amount of the three pheromone compounds released by *D. brevicomis* both in the laboratory and field.

PHEROMONE RELEASE RATES

A. Rates of Pheromone Release from Cut Bolts in the Laboratory

Methods. Eight recently cut ponderosa pine bolts, each approximately 75 cm long and 25 cm diameter, were infested with newly emerged female *D. brevicomis*. Beetles were placed individually into the phloem tissue through holes drilled in the bark on a grid pattern approximately 7.5 cm apart. Four bolts infested with a total of 360 females were placed into each of two 55-gal steel drums equipped with ring-lock lids. The infestation process took less than 4 hr, and the drums were closed immediately. Both the steel drums and the bolts within were positioned horizontally. Air flowed into one end of the drum, passed around and along the bolts and out the opposite end, where it condensed in a 750-ml glass conical flask immersed in liquid nitrogen (Browne et al., 1974). Air flowed through the system and liquefied in the flasks at an initial rate of 2.25 liter/min, which gradually decreased to 1.3 liter/min at the end of 4 hr. The flasks were replaced on a 4-hr schedule. The liquid air was distilled slowly from flasks by placing them in a Dewar flask stored at −50°C. After 24 hr, the lids of both drums were removed for a period of 30 min and 728 male *D. brevicomis* were added to the upperside of the bolts in one of the drums (drum 2). Both drums were resealed and the collection procedure was resumed for an additional 24 hr. After 48 hr of pheromone collection, the bolts were removed from the drums and debarked to determine mating success and extent of gallery excavation. Air collection from both drums began at 7:00 PM on February 13, 1973.

The pheromone collected in the flasks were extracted from the trapped water by thawing the flask contents at room temperature, saturating the organic-water mixture with NaCl and then transferring it to a liquid–liquid

TABLE 1. RELEASE RATES OF SIX COMPOUNDS BY *D. brevicomis* BORING IN ARTIFICALLY INFESTED BOLTS[a] IN THE LABORATORY, FEBRUARY 1973, BERKELEY, CALIFORNIA

Collection schedule		Compounds measured (ng/beetle/hr)					
Day	Hr	Myrcene	*exo*-Brevicomin	*endo*-Brevicomin	*trans*-Verbenol	Verbenone	Frontalin
Drum 1, Females only							
13	7:00–11:00 PM	12088	22.9	3.2	67.6	0.02	
14	11:00–3:00 AM	971	9.2	2.0	47.2	0.07	
	3:00–7:00[b]						
	7:00–11:00	1676	1.4	2.4	43.1	0.07	
	11:00–3:00 PM	3938	46.7	12.0	146.5	0.15	
	3:00–7:00	3077	44.5	44.9	199.6	1.84	
	drum opened						
	7:30–11:30	2024	74.4	12.8	137.4	0.23	
	11:30–3:00 AM	7070	6.7	9.5	132.8	0.19	
	3:30–7:30[b]						
	7:30–11:30	2784	93.4	13.7	145.6	0.21	
	11:30–3:30 PM	458	43.0	4.5	68.7	0.11	
	3:30–7:30	2198	94.3	11.2	130.0	0.21	

Drum 2, Females plus males on second day							
13	7:00-11:00 PM	940	7.1	1.1	31.0	0.08	
14	11:00-3:00 AM	2394	21.1	12.8	176.4	2.22	
	3:00-7:00[c]	11170	122.3	45.8	571.8	1.42	
	7:00-11:00 PM	1746	36.3	7.0	133.9	0.81	
	3:00-7:00	135	4.3	1.6	6.5	0.01	
	drum opened ♂♂ added						
	7:30-11:30	780	30.1	4.5	71.8	0.06	
	11:30-3:30 AM	1986	40.8	5.7	55.0	0.09	
	3:30-7:30[b]						
	7:30-11:30	1135	34.6	6.8	72.7	0.12	1.69
	11:30-3:30 PM	1844	49.6	70.0	85.1	0.15	3.13
	3:30-7:30	3333	87.8	5.1	167.6	0.40	8.98
Average both drums		2820	43.5	13.5	126.1	0.41	4.6[d]
ANOVA mean (SE)		2403 (586)	39.5 (5.1)	12.2 (4.2)	104.6 (14.9)	0.36 (0.15)	0.69 (.45)

[a]273 and 282 ♀♀ excavated galleries in drums 1 and 2, respectively. 192 ♂♂ joined females in drum 2. These numbers were used to calculate the average per beetle.
[b]Sample destroyed.
[c]Sample not included in ANOVA as the paired sample in drum 1 was destroyed. It is included in the average for both drums.
[d]This average was based on the last three sample periods only. We do not know when the male actually starts to release frontalin as the third sample was lost.

extractor along with the diethyl ether used to rinse the flask. After extraction in diethyl ether for 8–10 hr, the ether extract was dried over Na_2SO_4 and concentrated to a 500- to 1000-μl volume under a 25-cm × 1-cm column packed with glass beads. These concentrates were analyzed for the presence and amount of myrcene, frontalin, *exo*-brevicomin, *endo*-brevicomin, *trans*-verbenol, and verbenone by comparison with authentic samples on GLC. Although the behavior mediated in *D. brevicomis* by *endo*-brevicomin, *trans*-verbenol, and verbenone is not well understood (Wood, 1972; Wood and Bedard, 1977), we included them because several investigators have shown that they cause behavioral changes. The six compounds were fractionated and recollected using a 6-m × 6-mm glass column packed with Carbowax 20M (4%) on Chrom G (60–80 mesh), He flow 60 cc/min, 100° isothermal for 42 min, then to 175° at 6°/min. Frontalin was further purified on a 5% PDEAS 6-m × 6-mm column. All compounds except *trans*-verbenol were reinjected onto a 5% Apiezon L 6-m × 6-mm column: at 130° isothermal for myrcene, *exo*-brevicomin, and *endo*-brevicomin; at 100° for frontalin; and at 150° for verbenone. *trans*-Verbenol was verified by coinjection with a standard onto the Carbowax 20M (4%) column. Amounts of each compound were estimated by comparing peak area with known amounts of the standards. Three of the 24 samples were not analyzed—the 3:00–7:00 AM sample from February 14 "female only" drum (no. 1) and both drum samples from February 15, 3:30–7:30 AM were lost.

Quantities of each compound produced per insect in both drums and in each sample period were compared in a 2-way analysis of variance (ANOVA). For these comparisons the 3:00–7:00 AM drum 2 sample was omitted, as its paired sample from drum 1 was lost.

Results. The GLC quantification of compounds produced during each 4-hr collection period for both drums were averaged together to obtain an overall mean for each compound. These means are slightly different from the ANOVA means because sample 3:00–7:00 AM drum 2 is included (Table 1). The large standard error of the mean reflects possible errors both in sample preparation and measurement as well as changes in production levels. None of the ANOVA f ratios were significant at the 0.05 level for comparisons between drums and between collections. Visual inspection of the signs of the residuals indicated no obvious trend (diel or otherwise) through time, except for frontalin following the addition of males. However, light was excluded from these drums. From these data, it is estimated that each female released 4.3×10^{-8} g of *exo*-brevicomin and each male released 4.6×10^{-9} g of frontalin per hour. These estimates are based on the number of beetles found under the bark at the end of the trapping period (Table 1). The estimated 2.8×10^{-6} g of myrcene released per female per hour included myrcene that evaporated from the cut ends of the infested bolts, through the bark, and from the entrance tunnels.

B. Rates of Pheromone Release from Trees during Colonization

Methods. A series of experiments was conducted to quantify the release rates of the three pheromone components from living trees being colonized by natural population of *D. brevicomis*. All air passing around the colonized portions of two such trees was liquefied and distilled. The residues obtained were analyzed quantitatively for *exo*-brevicomin, frontalin, and mycrene. In these experiments, beetle attacks were initiated on two trees, designated trees A and B, by releasing each of these three compounds at approximately 1×10^{-5} g/hr from a point located at approximately midbole on each tree for a period of 12–24 hr. Boring dust or stridulation sounds from males were used to indicate when the concentration phase of host colonization had been initiated. At this point, the pheromone bait was removed and the colonization process allowed to continue. The attractiveness of the tree to *D. brevicomis* was monitored by observing the catch on sticky traps placed along the bole. When it was estimated that the tree had several hundred beetles involved in the colonization process, the traps were removed and the colonized portion of the tree bole was wrapped with an airtight aluminum sheet to form a cylinder, which was then sealed to the tree bole at each end. Two holes, one near each end, were cut through the aluminum sheet. One hole held a flowmeter that monitored the inlet air flow rate. The other opening was connected to a cryogenic trap (Browne et al., 1974) into which the air from around the colonized bole was continuously liquefied in one part of the trap and subsequently distilled into the atmosphere from another part, leaving behind all less volatile materials. Thus continuous air flow rates of 10–12 liter/min were maintained over the bole. Organic materials condensed out of the air from around both trees were separated from the condensed water by salting, solvent extracting, drying, and concentrating as described above. Fifty to sixty μl of these concentrated extracts were repeatedly fractionated by GLC using Carbowax 20M, Apiezon L, and FFAP 6-m $\times$ 6-mm glass columns. Mass spectra were obtained to verify structures. Air from tree A was trapped for 3 days, and the residues were removed at approximately 12-hr intervals. Residue from only one of these intervals (overnight 9:00 PM–7:00 AM) was analyzed to determine the amounts of the three pheromone compounds present. On tree A, 5.25 m of the bole (318-dm^2 bark area) was wrapped. After the third day of air trapping, the tree was felled and cut into eight bolts of equal length. Three randomly selected bolts were dissected to estimate the number of beetles colonizing the tree.

Tree B was baited to initiate the colonization process in the same manner as above. However, only a 3-m section of the bole that came under attack was wrapped with the aluminum cylinder. Air passing through the cylinder was trapped continuously for 22 hr at 7–11 liter/min. The resulting extract

was analyzed with GLC. This tree was not dissected, nor was the bark surface area measured; however, the relative attractiveness of the extract was compared with that of tree A in the laboratory olfactometer. In this assay, 40 male beetles were released, in groups of ten in an open arena, and those walking 10 cm upwind to the odor source were scored positive (Wood and Bushing, 1963). All dilutions were made in pentane, and the solutions were injected into the assay airstream at 2 μl/min with a motor-driven syringe. Extract dilutions were expressed as "tree equivalents," which referred to the proportion of the extract volume, collected per minute from the tree, that was injected per minute into the assay airstream. Laboratory responses to synthetic pheromones were evaluated in August 1969 with 50 male *D. brevicomis* exposed to each of two concentrations of a mixture of racemic frontalin, racemic *exo*-brevicomin, and isolated myrcene.

Results. The length of the bole from which air was trapped was not the same for both trees; thus the amount of pheromone compounds collected from each was expressed as grams/meter of bole/hour (Table 2). The magnitude of each compound released was similar for both trees. Tree A produced 88% more frontalin, 10% more *exo*-brevicomin, and 65% less myrcene per meter length of bole than did tree B. Pheromone production from the two trees is compared further by a series of laboratory assay presented in Tables 3–5.

First, laboratory assays of a series of dilutions of extracts from each tree gave similar responses at each dilution (Table 3). The Mantel-Haenszel procedure was used to analyze these data. The chi-square test for difference between extracts was not significant ($P = 0.371$). Furthermore the chi-square test for homogeneity rejected the hypothesis that responses to extracts of trees A and B were consistently different at all three dosages (0.025). Second, the similar amounts of frontalin, *exo*-brevicomin, and myrcene, es-

TABLE 2. RELEASE RATES OF *D. brevicomis* PHEROMONE COMPONENTS FROM TREES UNDER ATTACK IN NATURE, JUNE 1973, BASE LAKE, CALIFORNIA

Tree	Compound	Production rate (g/m of bole/hr)
A	Frontalin	3.3×10^{-6}
	exo-Brevicomin	2.0×10^{-5}
	Myrcene	2.1×10^{-3}
B	Frontalin	4.0×10^{-7}
	exo-Brevicomin	1.8×10^{-5}
	Mycrene	4.7×10^{-3}

TABLE 3. LABORATORY RESPONSE OF *D. brevicomis* TO COLD-TRAP CONDENSATES COLLECTED FROM TWO TREES UNDER ATTACK, AUGUST 1973, BERKELEY, CALIFORNIA

Injection rate[a]	% Responding (40 males tested per concentration) Tree A	Tree B
2×10^{-4} tree equivalents	82.5%	70.0%
2×10^{-5}	62.5	42.5
2×10^{-6}	32.5	14.0
control: 2 μl/min of pentane	3.3[b]	7.5

[a]A tree equivalent = the volume of condensate collected from around the tree per minute. Aliquots of this volume were diluted in pentane to obtain the desired tree equivalents, and injected into the assay airstream at 2 μl/min.
[b]30 males tested.

timated to have been injected into the assay airstream at the 2×10^{-4} tree equivalent rate for both A and B (Table 4), stimulated 82.5% and 70%, respectively, of the male *D. brevicomis* to respond (Table 3). At this rate these extracts appear to be at least as attractive as the mixture of synthetic frontalin and *exo*-brevicomin and isolated myrcene when assayed at slightly higher concentrations (Table 5). These comparisons indicate that the amount of the three compounds estimated to be in the extracts (Table 4) probably were not overestimated by the GLC analysis. In fact, the lower response (52%, Table 5) obtained with the synthetic materials at approximately the

TABLE 4. ESTIMATED ELUTION RATE OF *D. brevicomis* PHEROMONE COMPONENTS IN THE LABORATORY OLFACTOMETER AT 2×10^{-4} TREE EQUIVALENTS,[a] AUGUST, 1973, BERKELEY, CALIFORNIA

Tree	Myrcene (g/min)	*exo*-Brevicomin (g/min)	Frontalin (g/min)
A	3.7×10^{-9}	3.5×10^{-10}	3.6×10^{-11}
B	4.4×10^{-8}	1.6×10^{-10}	3.6×10^{-12}

[a]Tree equivalent = volume of condensate collected from around the tree per minute.

TABLE 5. LABORATORY RESPONSE OF *D. brevicomis* TO MIXTURES OF MYRCENE, RACEMIC *exo*-BREVICOMIN, AND FRONTALIN, AUGUST, 1969, BERKELEY, CALIFORNIA

Injection rate (g/min)			% of 50 males responding
Mycrene	*exo*-Brevicomin[a]	Frontalin[a]	
5×10^{-8}	5×10^{9}	5×10^{-9}	78
5×10^{-8}	5×10^{-10}	5×10^{-11}	52

[a]Rate is indicated for the active enantiomer; the racemate was injected at twice the indicate rate.

same or slightly higher concentrations, as estimated in the extracts at the 2×10^{-4} tree equivalents (Table 4), may indicate either that the amounts in the extracts were somewhat underestimated or that additional unidentified pheromone compounds were present in the tree extracts. However, the assays in Table 5 were conducted in different years, and thus the beetle response behavior could have been sufficiently different to account for the differences observed.

For tree A, the pheromone release rate per hour per beetle was estimated by dividing the number of beetles of the appropriate sex estimated to have attacked the tree into the rate at which the three pheromone compounds were collected from the tree. The debarking of tree A revealed an estimated 621 entrance holes, 75% of which contained mated females. The average attack density was $1.95/dm^2$ of bark surface which is comparable to the 1.25 density reported by Miller and Keen (1960) and to the 1.23–1.91 range reported by Dudley (1971). Miller and Keen (1960) report attack densities as high as 2.3 attacks/dm^2. The average attack density on the trees killed in the spring of 1970 at Bass Lake was 1.68 (DeMars, unpublished). Thus it appears that baiting to initiate the colonization process and the subsequent covering of the bole with the aluminum cylinder did not significantly alter the attack density from that previously observed.

It appears that beetles in tree A may have produced frontalin, *exo*-brevicomin, and myrcene about 8, 4, and 6 times faster (values in Table 2 times 5.24 divided by 75% of 621 entrance tunnels), respectively, than did the beetles boring in cut logs in the drums (Table 1). However, these differences may result, in part, from the different collection procedures used. A factor that possibly contributed to the lower collection rates in the cut logs was the greater head space volume in the drum than between the aluminum sheet and the tree. This resulted in less air flow through the drum and consequently less efficient recoveries of pheromones. As pheromone concentra-

tion increases in the headspace, there exists the possibility that pheromone production is modified. In addition, laboratory-emerged beetles were forced to bore into the cut bolts, which is less natural than beetles flying to, landing on, and boring into a tree.

C. Estimating Pheromone Release Rates in Nature

In calculating the amount of pheromone released in the Bass Lake Basin, an assumption is made that beetles produce pheromone at a continuous rate throughout the attack period. This assumption is probably misleading, but it is compatible with the manner in which the large headspace volume of the collection apparatus would be expected to average the release rates. Thus, by dividing the amount collected daily from tree A by the number of each sex found in galleries containing mated females (466), we estimated that a male produced frontalin at the rate of 8.6×10^{-7} g/day, and a female produced *exo*-brevicomin at 4.1×10^{-6} g/day. Myrcene, a host terpene released by the boring activity of a female, was estimated at 4.1×10^{-4} g/day/female.

It is not possible to make a probability statement about the reliability of the estimates from tree A. However, we believe the data from the drum test and tree B support the use of tree A data as reasonable estimates of release rates. In the drum study there was considerable variation between the 4-hr trapping periods for all chemicals measured. However, the averages from the drum tests, albeit less, were within an order of magnitude of tree A. Furthermore, the amounts of compounds collected from the two trees compared reasonably well with each other both by GLC analysis and laboratory assay.

DURATION OF PHEROMONE RELEASE DURING COLONIZATION

The duration of pheromone release from a tree undergoing attack by *D. brevicomis* reflects both the duration of production by individual beetles and the duration of recruitment of new beetles. If the number of attacking beetles is estimated in an area, then the rate and duration of pheromone production per beetle would be the only other parameters necessary to estimate total pheromone production. Duration of pheromone release per beetle was not estimated in the release-rate experiments. However, some conclusions about the duration of pheromone release can be made from field experiments. Bolts cut from newly attacked trees and bolts artificially infested with both sexes were wrapped with screen to exclude boring activity from the recruited beetles and then placed in sticky traps (Bedard, unpublished). From the start, these bolts were attractive and remained very attractive for an average of 3 days, after which the attractiveness declined rapidly. Thus,

it appears that individual beetles produce pheromone at a maximum rate for approximately three days.

The decline in attraction after three days could be caused by a reduction in release of one or more of the attractive compounds, or by the release of antiattractive or repellent compounds. In the latter case the attractants may continue to be released for an unknown period of time. Each of these factors would cause an underestimation of total natural pheromone production. Stephen and Dahlsten (1976) showed that, in the spring, 70% of the beetles trapped per tree under attack were caught within an average period of 8.8 days. The longer period of attraction of a whole tree than a bolt would be expected in view of the host colonization behavior of this insect. Miller and Keen (1960) report that "when a tree is attacked, usually the first attacks are made in the upper mid-bole and then progress up and down the trunk." This continuous attack process would cause the tree as a whole to remain attractive, while the portions first attacked may decline in attractiveness.

AREA-WIDE ESTIMATES OF PHEROMONE RELEASED

During the spring of 1970, in the 65-km^2 area of the Bass Lake Basin, an estimated 610,000 adult beetles (1:1 sex ratio) emerged from 283 overwintering trees and attacked 90 trees (DeMars, unpublished) during a 30-day period during May and June. The adults of this generation overlap less with preceding or subsequent generations than do other generations (Miller and Keen, 1960).

The rate at which pheromone is produced per insect per hour (tree A) multiplied by the duration of production (72 hr, the period of undiminished attractiveness) should yield the amount of pheromone that each beetle releases during the colonization phase. The amount of each compound is multiplied by 305,000 to estimate the total amount produced by the attacking adults of the overwintering generation in the Bass Lake Basin (Table 6).

TABLE 6. ESTIMATES OF TOTAL PHEROMONE RELEASED BY *D. brevicomis* IN THE BASS LAKE BASIN DURING SPRING 1970

Compound	Estimates based on tree A pheromone release rate (g)	Estimates based on pheromone release rate in drums (g)
Myrcene	370.5	61.75
exo-Brevicomin	3.7	0.975
Frontalin	0.78	0.104

DISCUSSION

We did not field assay the condensates from the laboratory drums but did assay the condensates from tree A and B as well as from several similar studies with *D. brevicomis*. In all these assays where the condensate was released from traps at rates calculated to be equivalent to beetles boring in bolts in adjacent traps, the condensates attracted very few beetles compared to the bolts. Thus, confinement in a container may have caused a marked reduction in attraction either through a reduction in the amounts of pheromones released, or by causing the release of antiattractants or repellents. In subsequent experiments with another bark beetle species, *Ips paraconfusus* Lanier, we have evidence to indicate that low headspace air exchange rates above the boring beetles lowered the attractiveness of the exhaust air stream (Browne, unpublished). The drums may have excluded normal environmental stimuli from the beetles thus effecting diel or other periodicities in pheromone release rates. We believe that the pheromone release rates reported here represent the minimum amounts that *D. brevicomis* produces in nature. Certainly myrcene would occur in greater amounts than indicated in this study, as it is released from trees and other plants not colonized by *D. brevicomis*.

The density of the Bass Lake population compared to average or typical densities of *D. brevicomis* is difficult to assess. During the 1970 pheromone test, several factors, including the suppression effort, reduced the 1969 overwintering generation, which emerged from an estimated 283 trees to kill only 90 trees throughout the 65-km^2 basin. These 90 trees represent an average mortality rate of 1.4 trees/km^2. However, a population this size could occur on an area as small as one hectare. Thus it is possible for the quantities of pheromone released over 65 km^2 shown in Table 6 to be exceeded in a single hectare.

We chose as a population model the Bass Lake 1969 overwintering generation because these data represent the only area-wide *D. brevicomis* population estimates available. Previous estimates of bark beetle incidence have been made on the basis of crop loss either in number or volume of trees killed. The 90 killed trees would certainly be considered an "endemic" population, when dispersed over 65 km^2.

CONCLUSIONS

The estimates of natural pheromone production have been presented with sufficient evidence to demonstrate that only gram quantities of these compounds were released over a considerable period of time throughout

the forest of Bass Lake Basin from natural sources. These kinds of data should be useful in predicting potential pest problems that could be caused by improper pheromone release. Thus, we may now compare the 0.78, 3.7, and 370.5 g each of frontalin, *exo*-brevicomin, and myrcene (Table 6), respectively, released by an estimated 610,000 *D. brevicomis* adults in competition with 150 g of each of these compounds released from 341 traps (Browne, 1978) throughout the basin. These traps caught an estimated 594,000 other *D. brevicomis* during this same 30-day period (Bedard and Wood, 1974). By comparison, a different pest control strategy, such as permeating the forest atmosphere with pheromones to cause interruption of aggregation behavior, would require more, perhaps orders of magnitude more, pheromone than the mass trapping strategy.

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SYNTHESIS OF THE MALE STABLE FLY POLYENE (*Z,Z*)-1,7,13-Pentacosatriene and Its Geometrical Isomers

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Abstract—The four geometrical isomers of 1,7,13-pentacosatriene were synthesized from 1,7-octadiyne. Two of the required isomers were synthesized using known methodology. Hydrogenation of acetylenic linkages were employed to produce predominantly cis alkenes, and alkali metal/amine reduction was used to produce a trans alkene. It was thus possible to produce the (*Z,Z*) and the (*Z,E*) isomers. Methods for olefin inversion were then employed to obtain the other two isomers.

Key Words—Stable fly, *Stomoxys calcitrans* (L.), pheromone, sex pheromone, polyene, mating stimulant, (*Z,Z*)-1,7,13-pentacosatriene.

INTRODUCTION

The major component (70% of the unsaturated cuticular lipids of the male stable fly, *Stomoxys calcitrans* (L.), is (*Z,Z*)-1,7,13-pentacosatriene (Sonnet et al., 1977), a material absent in virgin female stable flies. This triene may function as a sex pheromone by affecting the mating behavior of this species (for information regarding the transfer of this material when the sexes are not isolated see Harris et al., 1976). However, this assignment of the (*Z,Z*), or cis,cis structure rested on the absence of the 975 cm^{-1} IR band diagnostic for the presence of a trans double bond. Our original synthesis provided only the (*Z,Z*) isomer and required a final preparative GLC collection for purification. The procedure made possible the assignment of structure, but a route providing other isomers for a more detailed biological investigation seemed of value. Also, the syntheses described demonstrate

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the utility of recently developed olefin inversion techniques in pheromone synthesis.

METHODS AND MATERIALS

Gas–liquid chromatography (GLC) was performed with a Hewlett-Packard 5730A chromatograph employing flame ionization detection, a nitrogen carrier, a model 3380 integrating recorder, and columns and conditions as described below. Infrared data were obtained with a Beckman Acculab-3 instrument (CCl_4) and a Nicolet 7199 Fourier-transform IR instrument; nuclear magnetic resonance data were obtained with a Varian HA-100-A instrument; and mass spectra (MS) were obtained on a Varian MAT CH5 Spectrometer. Thin-layer plates (silica gel G) were purchased from Brinkmann Company. The reaction schemes are shown in Figure 1.

1,7-Nonadecadiyne (2). A solution of 1,7-octadiyne (Farchan Chem. Co.), (21.2 g, 0.20 mol) in 100 ml of THF was cooled to 0°, and 45.5 ml of 2.2 M *n*-butyllithium in hexane was slowly added while the temperature was kept below 10°. To the resulting slurry of lithium salt was added a solution of 23.5 g (0.10 mol) of 1-bromoundecane (Chem. Samples Co.) in 25 ml of HMPA. The mixture was stirred overnight at ambient temperature

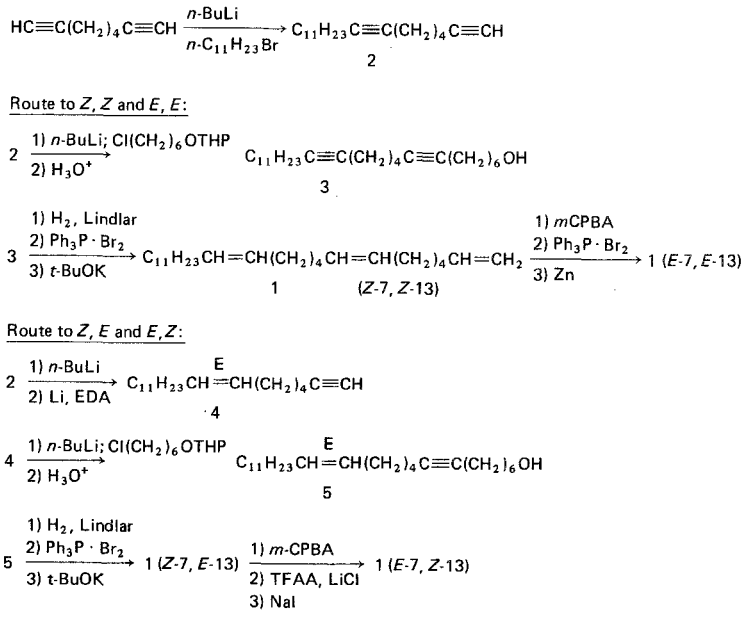

FIG. 1. Synthesis of the four geometrical isomers of 1,7,13-pentacosatriene.

in an inert atmosphere. The product was obtained by dilution of the mixture with water, extraction into hexane, and concentration of the extract on a steam bath. Fractional distillation yielded 11.5 g, bp 60–65°/15 mm (54%), of recovered 1,7-octadiyne containing a little hexane and 13.6 g, bp 160–175°/1 mm (52%), of 1,7-nonadecadiyne: n_D^{20} 1.4616 (corr); IR, 3310 (≡CH), 2090 (C≡C) cm^{-1}; NMR, 0.87 (t, CH_3), 1.75 (t, ≡CH), 2.10 (m, $CH_2C\equiv$) ppm; MS, M^+ 260.

7,13-Pentacosadiyn-1-ol (*3*). 1,7-Nonadecadiyne (*2*) (5.2 g, 20 mmol) was converted to a lithium derivative in 40 ml of THF (0–10°) by using 9 ml of 2.2 M *n*-butyllithium. The tetrahydropyranyl ether of 1,6-chlorohexanol (Aldrich Chem. Co.) (4.4 g, 20 mmol) was added as a solution in 20 ml of HMPA. Sodium iodide (3 g, 20 mmol) was added, and the mixture was stirred overnight at ambient temperature in an inert atmosphere. The mixture was worked up as described for 2 and chromatographed on 150 g of silica gel. The product was obtained by eluting with 450 ml each of hexane, 5% ethyl acetate, and 10% ethyl acetate. The condensation product, which was obtained in the last eluate, was dissolved in 40 ml of THF to which were then added several drops of 70% $HClO_4$ and enough water to saturate the mixture. The resulting mixture was warmed under reflux for 4 hr, cooled, diluted with water, and extracted with hexane. The diynol was recrystallized from hexane (low temperature precipitation and filtration) to yield 2.7 g (38%): mp, 35°; IR, 3300 (OH) cm^{-1}; NMR, 0.87 (t, CH_3), 2.08 (bs, $CH_2C\equiv$), 3.48 (t, CH_2O) ppm.

(Z,Z)-1,7,13-Pentacosatriene (*1*) (*Z-7,Z-13*). The diynol was quantitatively reduced over Lindlar's catalyst (Mozingo, 1955) by using 20 ml of hexane/g of diynol. The progress of the reaction was followed by GLC with a UCW-982 column, 10% on 80–100 WAW-DMCS, 3 mm × 0.51 m operating at 230°. The (*Z,Z*)-dien-1-ol was obtained as an oil: IR, 3300, OH; no band at 975 cm^{-1}; NMR, 0.87 (t, CH_3), 3.45 (t, CH_2O), 5.24 (t, CH=CH) ppm.

The dien-1-ol (1.0 g, 2.75 mmol) was added to a solution of triphenylphosphine dibromide (3 mmol) in 25 ml of methylene chloride. After 2 hr at room temperature, a few drops of methanol were added and the mixture was stripped of its solvent. The residue was extracted with hexane to obtain the resulting diene bromide. The bromide was added to slurry of potassium *t*-butoxide (0.7 g, 6 mmol) in 6 ml of DMSO. A few milliliters of benzene were employed as a rinse. The mixture was stirred overnight and then diluted with water. The product was extracted with hexane and chromatographed on 40 g of neutral alumina (activated at 160° for 24 hr). Elution with 120 ml of hexane gave 1 (*Z*-7,*Z*-13) (0.64 g, 67%), a sample of which was purified by bulb-to-bulb distillation (bp 170°, 0.01 nM). GLC characterization is described subsequently: n_D^{20} 1.4642 (corr.); IR, 920s, 995 ($CH=CH_2$), very weak 975 cm^{-1} band; NMR, 0.88 (t, CH_3); olefinic

protons included 5.26 (t, CH=CH) and a complex absorption 4.8–5.8 ($CH=CH_2$) ppm; MS, M^+ 346.

(E,E)-1,7,13-Pentacosatriene (1) (E-7,E-13). Compound 1 (*Z*-7,*Z*-13) (320 mg, 0.92 mmol) was epoxidized in 25 ml of methylene chloride with 0.81 g (4 mmol) of *m*-chloroperbenzoic acid. The crude trisepoxide was isolated by washing the reaction mixture with 5% NaOH and removing the solvent. Treatment with a slurry of triphenylphosphine dibromide (2.1 g, 5 mmol) in 25 ml of benzene at ambient temperature overnight converted the trisepoxide to a hexabromide. The halide was isolated by concentrating the mixture, extracting the residue with hexane, and then removing the hexane. Propionic acid, 20 ml, and powdered zinc, 1 g, were added to the bromide. After the mixture had been stirred for 0.5 hr, the product was isolated by dilution with water and extraction with hexane. From this procedure was obtained 151 mg (44%) of 1 (*E*-7,*E*-13) which was purified by bulb-to-bulb distillation. GlC characterization is described subsequently: n_D^{20} 1.4626 (corr.); IR, 920s, 995($CH=CH_2$), very strong 975 cm^{-1} band (*trans*-CH=CH), NMR, indistinguishable from its isomers; MS, M^+ 346.

(E)-7-Nonadecen-1-yne (4). 1,7-Nonadecadiyne 2 (5.24 g, 20 mmol) was dissolved in 65 ml of ethylenediamine (distilled from CaH_2) and 25 ml of THF in an inert atmosphere. The solution was cooled to 10° with mechanical stirring and *n*-butyllithium (9.1 ml of 2.2 M) was injected slowly. Lithium (2.5 equivalents) was added at one time. Stirring was continued for 4 hr at 10° after which time the mixture had lost its blue color. Methanol was carefully added (total 5 ml), and the mixture was then worked up by dilution with water and extraction with hexane. The extract was washed with 2.5 N HCl to remove EDA, and then with water. The crude product which had been obtained essentially quantitatively was analyzed by GLC by using an OV-275 column, 15% on 100-120 Chromosorb P AW-DMCS, 3 mm × 6.1 m, operating at 220° and was found to contain 7% recovered diyne, 13% of a single overreduction product (identified after purification by chromatography as the diene: IR, 920s, 995, $CH=CH_2$; 975, *E* – CH=CH), and 80% of the desired enyne, 4. Purification of 4 was accomplished by column chromatography with silica gel (5% loading) and hexane eluant. Several preparations were combined; fractions were monitored by GlC. In this fashion, material that was 92% enyne (contaminated only by the diene) was obtained. A sample was purified by bulb-to-bulb distillation. Addition of sodium benzoate to the reduction reaction to destroy excess lithium did not avoid the formation of the diene. The yield of 4 was approximately 40%: n_D^{20} 1.4566; IR, 3310 (C≡CH), 2090 (C≡CH), 975 (*trans*-CH=CH) cm^{-1}; NMR, 0.88 (t, CH_3), 1.72 (t, ≡CH), 1.94 (m, $CH_2C\equiv$), 5.30 (m, CH=) ppm; MS, M^+ 262.

(E)-13-Pentacosen-7-yn-1-ol (5). Compound 4 was carried through the sequence: (a) alkylation with the THP ether of 6-chloro-1-hexanol (84%

yield); and (b) hydrolysis (95% yield), as described for the preparation of 3. Impure 4 containing the diene overreduction product could be carried through this sequence, and the resulting alcohol 3 was easily separated from the diene by filtration through silica gel. The yield was not materially improved, although the procedure saved time. Overall yield of 5 from 1 was 30–35% either way: mp, 42–43° (hexane); IR, 3300 (OH), 975 (*trans* CH=CH) cm^{-1}; NMR, 0.88 (t, CH_3), 2.0 (m, $CH_2C\equiv$), 3.49 (t, CH_2O), 5.30 (m, CH=) ppm; MS, M^+ 362.

(Z,E)-1,7,13-Pentacosatriene (1) (Z-7,E-13). Compound 5 was converted to the triene 1 (*Z*-7,*E*-13) by the sequence: (a) hydrogenation over Lindlar's catalyst (quantitative yield); and (b) treatment with triphenylphosphine dibromide followed by dehydrobromination with potassium *t*-butoxide in DMSO (55% yield after chromatographic purification) by methods already described. A sample was purified by bulb-to-bulb distillation: n_D^{20} 1.4652 (corr.); IR, 920s, 995 ($CH=CH_2$), 975 (*trans* CH=CH intensity equal to the 920 band) cm^{-1}; NMR, indistinguishable from its isomers; MS, M^+ 346.

(E,Z)-1,7,13-Pentacosatriene (1) (E-7,Z-13). Compound 1 (*Z*-7,*E*-13), 0.35 g (1 mmol) was dissolved in 20 ml of methylene chloride containing 1.2 g (2 equivalents) of metachloroperbenzoic acid. The solution was allowed to stand overnight at ambient temperature. The crude trisepoxide was obtained by washing the organic phase sequentially with 2 N NaOH and brine. After the methylene chloride solution had been dried ($MgSO_4$), the solvent was removed. The crude epoxidized product was employed directly for the next step.

Trifluoracetyl chloride was generated in situ by adding 200 mg of lithium chloride (oven-dried at 110° for 24 hr) to a solution of 0.30 ml of trifluoroacetic anhydride in 10 ml of dry dimethylformamide (DMF). The trisepoxide was added at one time employing 1 ml of DMF as a rinse. The resulting mixture was stirred magnetically overnight at ambient temperature. Sodium iodide (1.8 g oven-dried at 110° for 24 hr) was added, and the mixture was heated at 130° for 20 hr. The resulting inverted triene was obtained by diluting the reaction mixture with water and extraction into hexane. The hexane extract was washed sequentially with 5% $NaHSO_3$ and water. After drying ($MgSO_4$), the solvent was removed, and the product was chromatographed on 15 g of silica gel (activated at 150° for 24 hr). The triene was obtained by elution with 60 ml of hexane, 0.28 g (80%). Bulb-to-bulb distillation was again performed: n_D^{20} 1.4651 (corr.); IR, indistinguishable from the *Z*-7,*E*-13 isomer; NMR, indistinguishable from its isomers; MS, M^+ 346.

Reproducible and distinct GlC retentions were obtained for each of the isomer preparations as described below. Although GlC separation was incomplete, the knowledge of the stereochemistry of the reactions employed

coupled with the visible distortions of peak symmetry allowed an assessment of geometrical purity. Retention times on the order of 30 min were observed using the 15% OV-275 column described operated at 230° with a nitrogen carrier flow of 10 ml/min. The relative retentions were: (*E*-7,*E*-13) 1.000, (*E*-7,*Z*-13) 1.009, (*Z*-7,*Z*-13) 1.015, and (*Z*-7,*E*-13) 1.024. Retention times were on the order of 18–20 min. The *Z,Z* isomer prepared by catalytic hydrogenation of a diyne would be expected to contain 2–4% each of *Z,E* and *E,Z* isomers. Slight distortions of the *Z,Z* GLC peak gave evidence for their presence, but no estimate of purity could be made from the chromatograms. Infrared analysis of the small 965 cm^{-1} band intensity was hampered by the 990 cm^{-1} vinyl band. The *E,E* isomer was contaminated by $< 2\%$ of the *Z,E* isomer (GLC). Its method of preparation therefore requires a like amount, 1–2%, of the *E,Z* isomer. GLC analysis of the *Z,E* isomer demonstrated 2.7% of the *E,E* isomer; the *E,Z* isomer should contain therefore approx. 3% of the *Z,Z* compound and a small shoulder on the late side of the GLC peak was observed.

RESULTS AND DISCUSSION

The lithium salt of 1,7-octadiyne was allowed to react with 1-bromoundecane to produce 1,7-nonadecadiyne, 2 (Figure 1). The formation of the dialkylation product was readily suppressed by the usual technique of employing an excess of the 1,7-octadiyne. Unreacted diyne was easily recovered from the reaction product by fractional distillation. A second alkylation with the tetrahydropyranyl (THP) ether of 6-chloro-1-hexanol established the chain length with the desired internal unsaturation. Hydrogenation of the diyne–THP ether was unsuccessful despite repeated attempts to purify this compound by gravity liquid–solid adsorption chromatography, but the corresponding alcohol was hydrogenated over Lindlar's catalyst after it had been recrystallized to produce 3. It has been reported that the hydrogenation of an acetylene can be made to proceed to almost pure *cis*-olefin (99.5%) by lowering the temperature of the reaction mixture to −10 to −30° (Henrick, 1977). Unfortunately, the diynol precipitated from the hexane solution at temperatures lower than 10°; the resulting heterogeneity stopped the hydrogenation. Thus the (*Z,Z*) isomer obtained is expected to contain small amounts of (2–4%) both of the (*Z,E*) and (*E,Z*) isomers (see Methods and Materials). The dienol was converted to a bromide with triphenylphosphine dibromide, and elimination of HBr with potassium *t*-butoxide in DMSO yielded 1 (*Z*-7, *Z*-13) accompanied by 5–10% of the t-butyl ether which was readily removed by column chromatography.

A method of inverting olefin geometry especially useful for the inversion of cis to trans involves epoxidation of the olefin, such as with *m*-chloro-

perbenzoic acid (mCBA), followed by reaction of the *cis*-epoxide with triphenylphosphine dibromide (Sonnet and Oliver, 1976a). The *vic*-dibromide obtained is formally the result of cis addition of bromine to the olefin: trans elimination effected by zinc in propionic acid produces the *trans*-olefin. In this instance a trisepoxide was converted to the corresponding 1,2,7,8,13,14-hexabromide with triphenylphosphine dibromide, and reductive dehalogenation with zinc generated triene *1* (*E*-7,*E*-13).

Compound 2, 1,7-nonadecadiyne was converted to a lithium derivative and treated with metallic lithium in ethylenediamine (EDA). Analogous procedures have been previously employed to protect a terminal acetylene from reduction while permitting an internal triple bond to reduce exclusively to a *trans*-olefin (Dobson and Raphel, 1955). In previously reported cases, liquid NH_3 was the solvent, and the reaction temperature, −78°, served to prevent side reactions since at temperatures above 0°, lithium in amine solvents can reduce olefins to saturated hydrocarbons (Benkeser et al., 1955; and Reggel et al., 1957). The lithium salt of the diyne, 2, was too insoluble in NH_3 and the THF–NH_3 solvents, and reduction was therefore carried out in EDA at 10° (just above the temperature at which the EDA began to solidify). The enyne, 4, was contaminated by a small amount of the diene (reduction of terminal acetylene probably occurred when methanol was added to the reaction mixture to destroy any remaining lithium) and some residual diyne. Alternative destruction of excess lithium with sodium benzoate was not successful. The enyne was purified by column chromatography, and alkylation with the THP ether of 6-chloro-1-hexanol produced the (*E*) enynol 5 after hydrolysis. Hydrogenation of 5 gave the (Z-7,*E*-13)-dienol. This alcohol was transformed to the bromide and then was dehydrobrominated giving 1 (*Z*-7,*E*-13).

Reaction of 1,2-disubstituted epoxides with trifluoroacetyl chloride (or bromide) produces β-chloro- (bromo-) trifluoroacetates which, upon further treatment with sodium iodide in hot dimethylformamide, undergo a displacement–elimination sequence to produce an olefin of configuration opposite to that of the initial epoxide (Sonnet, 1978). Accordingly, the (*Z*-7,*E*-13) isomer was allowed to react with excess metachloroperbenzoic acid thus forming a trisepoxide. Reaction with excess trifluoroacetyl chloride (trifluoroacetic anhydride–lithium chloride) followed by addition of sodium iodide and heating to 130° produced the (*E*-7,*Z*-13) isomer.

Each isomer was obtained in $>94\%$ purity. Candidate sex pheromones must in general be more highly purified than state-of-the-art synthetic procedures produce in any case (usually $\geqslant 99.5\%$ in order to neutralize considerations of effects due to the "impurities"). In that respect, the synthetic procedures described here provide quantities of reasonably pure materials which are adequate for most purposes, and for pheromone evaluation would require ultimate purification by, for example, high-performance liquid

chromatography. In addition, the inversion of olefin geometry by techniques as those described offers the opportunity to significantly decrease the synthetic effort involved in preparing a set of isomers for structure confirmation and biological evaluation.

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IDENTIFICATION OF A FOUR-COMPONENT SEX PHEROMONE OF THE FEMALE ORIENTAL FRUIT MOTH, *Grapholitha molesta* (LEPIDOPTERA: TORTRICIDAE)[1]

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Abstract—The female-emitted pheromone of *Grapholitha molesta* (Busck), the Oriental fruit moth, was collected by holding females in glass flasks during calling. Flask washes were found to contain four pheromone components: (*Z*)-8-dodecenyl acetate and (*E*)-8-dodecenyl acetate in a 100:7 ratio, and (*Z*)-8-dodecen-1-ol and dodecanol in a 100:20 ratio. The ratio of (*Z*)-8-acetate to (*Z*)-8-dodecen-1-ol was approx. 100:30. Approximately 0.1–0.2 ng of pheromone was recovered per female per hour of calling.

Key Words—*Grapholitha molesta*, Oriental fruit moth, attractant, pheromone, (*Z*)-8-dodecenyl acetate, (*E*)-8-dodecenyl acetate, dodecyl acetate, (*Z*)-8-dodecen-1-ol, dodecanol.

INTRODUCTION

One component of the female sex pheromone of the Oriental fruit moth, *Grapholitha molesta* (Busck), was characterized as (*Z*)-8-dodecenyl acetate (Z8–12:Ac) by Roelofs et al. (1969). A number of congeners of Z8–12:Ac have been reported as contributing to male trap catch when these analogs were emitted simultaneously with Z8–12:Ac. Trap catch has been reported to be elevated by the addition of (*E*)-8-dodecenyl acetate (*E*8–12:Ac) by Beroza et al. (1973), dodecanol (12:OH) by Roelofs et al. (1973), and (*Z*)-8-dodecen-1-ol (Z8–12:OH) by Cardé et al. (1975). However, other than

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*Z*8–12:Ac, the female's natural blend has remained undefined. We report here the identification of three new components of the female's pheromone.

METHODS AND MATERIALS

The laboratory culture of *G. molesta* was derived from material collected in apples from abandoned orchards in East Lansing in the fall of 1975. The insects were maintained on thinning apples in a regime of 16:8 hr L:D, 25°C, and 75% relative humidity. Adults for pheromone collection were obtained by segregation of pupae by sex; females were held as adults in 30 × 30 × 40 cm screen cages with access to water.

Female-emitted pheromone was obtained by placing 50 2-to-6-day-old females in stoppered 250-ml round-bottom 24/40 flasks just prior to the initiation of calling, similar to the technique of Weatherston et al. (1971). After approx. 3 hr of calling, the flasks were cooled to −5° for 5 min and the females were dumped out of the flasks. Flasks were given 3 consecutive rinses with 30 ml of redistilled hexane. The hexane was filtered through glass wool, concentrated to approx. 1 ml volume, and transferred to an ampoule where it was evaporated to dryness with N_2. Immediately 100 μl of redistilled methylene chloride was added, and the extracts were stored at −5°C until analyzed.

Electroantennograms (EAGs) of GLC fractions of airborne pheromone were conducted according to the procedures of Roelofs (1977). Analytical GC columns were glass, 2 mm × 1.8 m, and were packed with either 3% OV-1 on Gas-Chrom-Q (100/120 mesh) or 10% XF-1150 on Chromosorb DMCS AWW (100/120 mesh). A 4-mm × 1.8-m OV-1 column was utilized for initial purification of the airborne material. Mass spectra were obtained on a LKB 9000.

RESULTS

Approximately 0.1–0.2 ng of pheromone was recovered per female per hour of calling. The crude airborne pheromone (10,000 female hours) was collected from the OV-1 column at 132°. The 5.4–6.8 min area, which would contain saturated and unsaturated 12-carbon alcohols (standard *Z*8–12:OH = 5.9 min), elicited a 6.6-mV EAG response, somewhat above the response of adjacent fractions (5.6 mV), whereas the 10–14 min area, representing saturated and unsaturated 12-carbon acetates (standard *Z*8–12:Ac = 11.2 min, 12:Ac = 11.7 min), gave good EAG activity, eliciting a 7.0 mV (adjacent fractions 3.2 mV) with a very slow 10-sec return to baseline.

Analyses of the Acetate Region. The acetate region was reinjected on an OV-1 column at 135° (standards: *Z*- and *E*8–12:Ac = 10.0, 12:AC = 10.5

min) and gave a single broad peak at 9.8 min. Injection of this material on an XF-1150 column at 120° (standards: 12:Ac = 10.0, *E*8–12:Ac = 11.1, *Z*8–12:Ac = 12.1 min) showed components with retention times of 10.0, 11.0, and 12.1 min in relative quantities of 5:8:100. These components were isolated by collection from XF-1150 under the same conditions.

Dodecyl Acetate Region. An aliquot of the area collected at 9.4–10.4 min, which would contain dodecyl acetate and which showed low EAG activity, was reinjected on XF-1150 at 130° (standards: 12:Ac = 8.2, *E*8–12:Ac = 9.0, *Z*8–12:Ac = 9.9 min) and showed a single peak at 8.25 min. Injection of an aliquot of this material on OV-1 at 125° showed one peak at 17.7 min (standards: *Z*8–12:Ac = 16.8, 12:Ac = 17.7 min). Microozonolysis of this material (Beroza and Bierl, 1967) and reinjection on OV-1 at 125° (standards: 12:Ac = 18.1, 8-acetoxyoctanol = 6.1) gave the original peak at 18.2 min, indicating the material is the saturated 12-carbon acetate.

(E)-8-Dodecenyl Acetate Region. An aliquot of material collected from 10.5–11.4 min, which would contain the *E*8–12:Ac and which showed EAG activity was reinjected on XF-1150/130° (standards: 12:Ac = 8.2, *E*8–12:Ac = 9.0, *Z*8–12:Ac = 9.9) and showed 1 peak at 9.0 min. Injection of the same material on OV-1 at 125° (standards *Z*8–12:Ac = 16.8, 12:Ac = 17.7) gave one peak at 16.6 min. Microozonolysis of the component and reinjection on OV-1 at 125° [standards: *Z*8–12:Ac = 17.0, 8-acetoxyoctanal (obtained from ozonolysis of standard *Z*8–12:Ac) = 6.1] gave a peak at 6.1 min and no discernable peak at 17.0, indicating that this 12-carbon acetate is unsaturated in the 8-position.

(Z)-8-Dodecenyl Acetate Region. The *Z*8–12:Ac area (11.4–13 min) which showed EAG activity was reinjected on XF-1150 at 120° and gave a major peak at 11.9 with a 1.5% impurity at 11.0 min (standards: 12:Ac = 9.9, *E*8–12:Ac = 11.0, *Z*8–12:Ac = 12.1 min). Injection of the same material on OV-1 at 125° gave 1 peak at 16.7 (standards: *Z*8–12:Ac = 16.8, 12:Ac = 17.7 min). A mass spectrum of approx. 500 ng of this component was identical with a mass spectrum of synthetic *Z*8–12:Ac. Microozonolysis and subsequent injection on OV-1/125° [standards: *Z*8–12:Ac = 16.8, 8-acetoxyoctanal (from ozonolysis of standard *Z*8–12:Ac) = 6.05 min] did not give any peak at 16.8 and showed a new peak at 6.05 min, indicating unsaturation in the 8-position and confirming the presence of *Z*8–12:Ac.

Analyses of the Alcohol Region. Reinjection of an aliquot of the alcohol region on a preparative OV-1 column at 135° gave a major peak at 5.4 min and a small one at 6.4 min (standards: *Z*8–12:OH, *E*8–12:OH and 12:OH = 5.4 min). The same material on XF-1150 at 135° (standards: 12:OH = 6.7, *E*8–12:OH = 7.3, *Z*8–12:OH = 8.1) showed two major peaks at 6.7 and 8.2 min, respectively, in a ratio of 33:67. Acetylation of the material and subsequent injection on OV-1/135° (standards: *Z*8–12:Ac and *E*8–12:Ac = 10.0, 12:Ac = 10.6 min) gave a new peak at 10.1 min with a

shoulder at 10.6, in addition to the two small peaks with retention times of 5.4 (possibly unreacted 12-carbon alcohols) and 6.4 min. Injection of the acetylated material on XF-1150 at 135° showed 2 major peaks at 6.0 and 7.3 min in a relative ratio of 40:60 (standards: 12:OH = 6.7, *E*8–12:OH = 7.3, *Z*8–12:OH = 8.1, 12:Ac = 6.0, *E*8–12:Ac = 6.7, *Z*8–12:Ac = 7.3 min). To remove any unreacted alcohols from this product, it was again collected from OV-1 at 135° (standards: *Z*8–12:OH = 5.5, *Z*8–12:Ac = 10.3). The acetate area from OV-1 (9.5–12 min) showed EAG activity and two discrete areas were collected from XF-1150 at 130° (standards: 12:Ac = 6.3, *E*8–12:Ac = 6.9, *Z*8–12:Ac = 7.55) in an effort to isolate the presumed two different acetates.

Dodecyl Acetate Region (of the Acetylated Alcohol). An aliquot of the material collected from 5.9–6.6 min on XF-1150 gave no appreciable EAG activity, and upon reinjection of XF-1150/120° only a single peak of 6.3 min was noted. The same material on OV-1 at 125° gave a single peak at 17.7 min (standards: *Z*8–12:Ac = 16.5, 12:Ac = 17.7, 8-acetoxyoctanal = 6.05 min). After microozonolysis the product showed no additional peaks on OV-1 and gave the same original peak at 17.7, indicating that there is no unsaturation in this acetate. Therefore the original 12-carbon alcohol component was saturated.

(Z)-8-Dodecenyl Acetate Region (of the Acetylated Alcohol). The region collected from 7.3 to 8.5 min on XF-1150 gave appreciable EAG activity, and an aliquot of this material reinjected on XF-1150/130° gave a single peak at 7.5 min. On OV-1 at 125° [standards: *Z*8–12:Ac = 16.5, 8-acetoxyoctanal (obtained from ozonolysis of standard *Z*8–12:Ac) = 6.0 min] the material showed a peak of 16.4 min. After microozonolysis this product gave no peak at 16.4 min and a fragment showed up at 6.0 min (8-acetoxyoctanal) indicating a double bond in the 8-position in the 12-carbon acetate. Thus, the original 12-carbon alcohol was unsaturated in the 8-position.

DISCUSSION

The characterization of *Z*8–12:Ac, *E*8–12:Ac, *Z*8–12:OH and 12:OH as pheromone components represents the second, 4-component, female-emitted pheromone system in the Lepidoptera. Dodecyl acetate was found to be emitted in relatively low amounts by the female but, because it is not known to elicit a pheromone-related response, this compound cannot be termed a pheromone. Previously, *Archips argyrospilus* (Walker) was shown to be maximally attracted to a mixture of (*Z*)-11-, (*E*)-11- and (*Z*)-9-tetradecenyl acetates and dodecyl acetate (Cardé et al., 1977).

Other *Grapholitha* species may use attractant pheromones that share components in common with *G. molesta*. Males of *G. prunivora* (Walsh) have

been reported to be attracted to mixtures of *Z*8–12:Ac and *E*8–12:Ac in a 100:2 ratio (Roelofs and Cardé, 1974). Males of *G. funebrana* (Treitschke) appear to be maximally attracted to a 100:4 ratio of *Z*8–12:Ac and *E*8–12:Ac, whereas the maximal trap catch of male *G. janthiana* (Duponchel) is elicited by a 100:27 ratio of these compounds (Biwer, 1977). These species are broadly sympatric with *G. molesta* and reproductive isolation among these species may be achieved by differences in secondary pheromone components, in close-range courtship behaviors (Baker and Cardé, 1979), and in time of sexual activity (Biwer, 1976).

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ALLELOPATHIC POTENTIAL OF *Salsola kali* L. AND ITS POSSIBLE ROLE IN RAPID DISAPPEARANCE OF WEEDY STAGE DURING REVEGETATION[1,2]

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Abstract—*Salsola kali* L. is one of the species that invades during early successional stages on mine-spoil material, but tends to disappear rapidly from the weedy stage. What controls its rapid disappearance from the weedy stage becomes a logical question. *Salsola* was found to be an allelopathically active species, which also decreased the growth of selected associated species during its decaying process. However, leaf leachate did not play an important role in depressing the growth of associated species. *Salsola* was autotoxic, but its germinatoin was not inhibited by any of the isolated phytotoxins applied. The phytotoxins identified from *Salsola* leaves were caffeic, ferulic, chlorogenic, isochlorogenic, and neochlorogenic acids, and quercetin. Allelopathy may play an important role in the rapid disappearance of the weedy stage, similar to what is established for old farm lands. However, further studies are desirable.

Key Words—Allelopathy, weedy stage, succession, mine-spoil, nutrient, phytotoxins, physioecology, *Salsola kali*, germination.

INTRODUCTION

Revegetation in old agriculture fields has been reviewed by many researchers (Booth, 1941; Drew, 1942; Rice et al., 1960). It follows a certain pattern and the first stage of succession is always weed dominated. However, none of these report the weed stage lasting for more than 3–7 years. It seems that

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[2]The work was partially completed when the author was a visiting research associate professor at the University of North Dakota.

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during the weedy stage, the annuals can survive in soils which are low in minerals, and during their residence prepare the habitat for perennials. The appearance of different species during successional stages in old fields in Oklahoma has been attributed to their relative requirements for nitrogen and phosphorus (Rice et al., 1960).

The question of the rapid disappearance of the weedy stage remained unanswered for a long time. The competitive mechanism was thought to play an important role in rapid disappearance of the weedy stage. Even when competitive mechanisms were controlled, the weedy stage disappeared at a rapid rate. However, in the last decade enough evidence has been gathered to show that plant–soil–plant interaction in terms of phytotoxity plays an important role (Muller, 1966; Rice, 1974). By producing toxins, weeds inhibited their own germination and seedling growth as well as those of other species (Rice, 1974).

Areas disturbed by mining also followed a certain successional pattern (Leftwich, 1974; Wali and Freeman, 1973). Both reports clearly presented field data showing that the pioneering vegetation on the strip-mine was of the weedy type. This stage in its first year consisted mainly of annuals and later mixed with nonannual weeds. However, the weedy stage seemed to disappear rapidly within a few years.

Subsequently, Wali and Iverson (1978) reported that *Kochia scoparia* was the dominant bushy plant in its first year growth after mining, and in the following year, its growth was retarded to only a few centimeters. Under controlled environmental conditions, it was extremely autotoxic. Lodhi and Wali (1979) reported further that *Kochia,* in its second year, produced a large number of plants due to its rapid seed germination rate. Germination of *Kochia* seeds was not autotoxically affected, but the radicle and seedling growth were significantly decreased. *Salsola kali* L. is one of the species that invades during early successional stages on strip-mine spoil material, but tends to disappear rapidly from the weedy stage (Wali and Freeman, 1973). A similar pattern is also exhibited by *Kochia scoparia.* However, the question remains: What controls the rapid disappearance of the weedy stage in the surface mine spoils? Appropriate experiments were designed to determine the inter-/ and interspecific effect of allelopathy due to *Salsola kali* L.

METHODS AND MATERIALS

Study Area. All plant material and soil collections were obtained from Beulah in western North Dakota. Based on the Braun-Blanquet scale analysis of vegetation, *Salsola kali* L. was one of the important secondary

species in the freshly mined areas (1–3 years old), *Kochia scoparia* being the dominant in this stage (Wali and Freeman, 1973).

Effects of Decaying Leaves of Salsola. Seeds of *Salsola*, *Kochia*, and *Melilotus offcinalis* (sweet clover), were planted in each of five 10-cm glazed pots containing 1 g of air-dried fresh *Salsola* leaf powder per 454 g of a 3:2 soil and sand mixture. One gram of peat moss per 454 g of the soil–sand mixture was used in the control pots in order to keep the organic matter content the same as in the test pots. The leaves and peat moss were ground in a large Wiley mill with a 2-mm sieve. All experiments were run in growth chambers with a 16-hr photoperiod at 29°C and an 8-hr dark period at 21°C. The seedlings were allowed to grow for 3 weeks, after which the plants were thinned to the five largest seedlings per pot. These were allowed to grow for 3 additional weeks and harvested, separated into shoots and roots, and oven-dried for 48 hr at 36°C.

Effects of Leaf Leachate of Salsola. A mist of distilled water was sprayed over freshly collected leafy *Salsola* branches, and the drip was collected in a bucket. This leachate was used to water 5 pots of each test species. Seeds of each of the test species were planted as explained above. Five control pots of each species were treated in the same manner but were irrigated with distilled water. Seedlings were allowed to grow for 3 weeks, after which the plants were thinned to the five largest seedlings per pot. The seedlings were allowed to grow for 3 additional weeks, harvested, separated into shoots and roots, and oven-dried for 48 hr and weighed.

Isolation and Identification of Phytoxins from Salsola Leaves. The procedures used to isolate the phytotoxins from *Salsola* leaves were the same as used by Lodhi (1975a) and the alkaline hydrolysis method of Guenzi and McCalla (1966).

Biological Activity of Identified Phytotoxins. The phytotoxic activities of identified compounds were further verified by the following bioassay: a 10^{-3} M solution of each of the identified compounds was prepared in a 5.65 pH phosphate buffer solution. All solutions were added to petri plates containing 25 seeds each of *Salsola* or radish (a commonly used bioassay species) on filter paper. Controls were treated in the same manner, except the seeds were watered with toxin-free buffer solutions. Germination was determined after 24 hr, and the seedlings were thinned to ten with the longest radicles per dish. Radicles were allowed to grow further and measured at 40 and 60 hr.

RESULTS

Decaying leaves of *Salsola* significantly reduced the oven-dry weight of roots, shoots, and the whole plant of *Kochia* and *Salsola* (Table 1). How-

TABLE 1. EFFECT OF *Salsola* DECAYING LEAVES ON GROWTH OF SELECTED TEST SPECIES

	Oven-dry weight (mg, mean ± SD)[a]					
	Sweet clover		*Kochia*		*Salsola*	
Plant parts	Control	Test	Control	Test	Control	Test
Roots	3785 ± 813	3324 ± 356	3613 ± 480	829 ± 231[b]	1368 ± 478	426 ± 115[b]
Shoots	4853 ± 299	4857 ± 815	9056 ± 1118	3792 ± 1053[b]	11068 ± 3173	5607 ± 1934[b]
Whole plants	8646 ± 977	8171 ± 795	12669 ± 1091	4622 ± 1233[b]	12436 ± 3545	6035 ± 2026[b]

[a]Mean of five pots with five seedlings per pot.
[b]Significantly different at .05 level from control.

TABLE 2. EFFECTS OF *Salsola* LEACHATE ON GROWTH OF SELECTED TEST SPECIES

	Oven-dry weight (mg, mean ± SD)[a]					
	Sweet clover		*Kochia*		*Salsola*	
Plant parts	Control	Test	Control	Test	Control	Test
Roots	2403 ± 848	2471 ± 246	1492 ± 743	907 ± 236	862 ± 889	310 ± 105
Shoots	3729 ± 354	2698 ± 419[b]	4805 ± 1021	3335 ± 450[b]	5672 ± 2729	4775 ± 2284
Whole plants	5669 ± 1497	5170 ± 587	6297 ± 1502	4242 ± 521[b]	6534 ± 3591	5084 ± 2386

[a]Mean of five pots with five seedlings per pot.
[b]Significantly different at .05 from control.

TABLE 3. CHROMATOGRAPHY OF PHYTOTOXINS FROM *Salsola*

	R_fs on Whatman No. 1[a]				Fluorescence[b]			Reagent colors[b,c]		
	6%		BAW	IBW	IAW	Long UV	Short UV	P. Nit.	Sulfan. acid	$FeCl_3$-$K_3Fe(CN)_6$
Caffeic acid	0.34	0.67	0.82	0.74	0.08	bl	bl	bn black	none	bl
Suspected caffeic acid	0.32	0.66	0.81	0.74	0.07	bl	bl	bn black	none	bl
Chlorogenic acid	0.58	0.74	0.66	0.35	0.01	bl	bl	bn	tan	bl
Suspected chlorogenic acid	0.57	0.74	0.66	0.36	0.01	bl	bl	bn	tan	bl
Ferulic acid	0.42	0.70	0.88	0.79	0.14	bl	bl	f, bn black	f, tan	bl
Suspected ferulic acid	0.41	0.70	0.89	0.79	0.14	bl	bl	f, bn black	f, tan	bl
Isochlorogenic acid	0.18	0.32	0.79	0.76	0.03	bl	bl	bn	tan	bl
Suspected isochlorogenic acid	0.18	0.32	0.79	0.76	0.03	bl	bl	bn	tan	bl
Neochlorogenic acid	0.73	0.83	0.55	0.50	0.05	bl	bl	bn	tan	bl
Suspected neochlorogenic acid	0.72	0.83	0.55	0.52	0.05	bl	bl	bn	tan	bl
Quercetin		0.12	0.72	0.80	0.10	yell	yell	bn	yell	bl
Suspected quercetin		0.12	0.70	0.79	0.10	yell	yell	bn	yell	bl

[a]6% = 6% acetic acid; BAW = 63:10:27, butanol–acetic acid–water; IBW = 140:20:60, is opropanol–butanol–water; IAW = 200:10:20, isopropanol–ammonia–water.
[b]bl = blue, bn = brown, f = faint, yell = yellow.
[c]Diazotized sulfanilic acid; ferric chloride–potassium ferricyanide; and diazotized *p*-nitraniline.

TABLE 4. EFFECTS OF VARIOUS PHYTOTOXINS AT 10^{-3} M CONCENTRATION ON SEED GERMINATION AND RADICLE GROWTH AT 40 HR TIME[a]

Phytotoxins	Radish radicle length			*Salsola* radicle length		
	Control	Test	Germination	Control	Test	Germination
Caffeic acid	78.6 ± 15.2	53.0 ± 11.1[b]	62.0	28.5 ± 8.2	26.4 ± 9.2	92.0
Chlorogenic acid	78.6 ± 15.2	48.5 ± 13.2[b]	58.0	28.5 ± 8.2	32.5 ± 7.8	100.0
Ferulic acid	78.6 ± 15.2	28.3 ± 9.5[b]	48.0	28.5 ± 8.2	19.6 ± 10.1[b]	81.0
Isochlorogenic acid	78.6 ± 15.2	43.8 ± 10.3[b]	67.0	28.5 ± 8.2	23.2 ± 13.2	96.0
Neochlorogenic acid	78.6 ± 15.2	51.7 ± 7.6[b]	55.0	28.5 ± 8.2	31.2 ± 10.0	104.0
Quercetin	78.6 ± 15.2	54.2 ± 8.1[b]	73.0	28.5 ± 8.2	30.8 ± 8.8	94.0

[a]Radicle length as mm ± SD germination as % of control.
[b]Significantly different at 0.05 level from control.

TABLE 5. EFFECTS OF VARIOUS PHYTOTOXINS AT 10^{-3} M CONCENTRATION ON RADICLE GROWTH AT 60 HR TIME[a]

Phytotoxins	Radish radicle length		*Salsola* radicle length	
	Control	Test[b]	Control	Test[b]
Caffeic acid	95.8 ± 19.6	54.1 ± 13.2	43.5 ± 11.0	29.3 ± 9.8
Chlorogenic acid	95.8 ± 19.6	60.6 ± 18.2	43.5 ± 11.0	36.8 ± 10.6
Ferulic acid	95.8 ± 19.6	59.0 ± 21.0	43.5 ± 11.0	24.3 ± 6.7
Isochlorogenic acid	95.8 ± 19.6	67.7 ± 23.2	43.5 ± 11.0	29.5 ± 13.1
Neochlorogenic acid	95.8 ± 19.6	52.3 ± 18.6	43.5 ± 11.0	35.1 ± 7.0
Quercetin	95.8 ± 19.6	76.5 ± 13.3	43.5 ± 11.0	34.5 ± 8.2

[a]Radicle length as mm ± SD.
[b]Significantly different at 0.05 level from control.

ever, the root, shoot, or the total growth of sweet clover was not significantly affected (Table 1). On the other hand, the *Salsola* leachate in most cases did not decrease growth with the exception of the shoot and whole seedling of *Kochia* and the shoot of sweet clover. (Table 2).

Colors in UV light and in various reagents, absorption spectra, and R_fs in different solvent systems indicated that the chief phytotoxins in water extracts of fresh *Salsola* leaves were quercetin, ferulic acid, chlorogenic acid, and small amounts of neochlorogenic and isochlorogenic acid (Table 3). Caffeic and ferulic acids were identified in alkaline hydrolysate (Table 3). The same toxins at 10^{-3} M concentration were found to be very inhibitory to seed germination and radicle growth of radish (Tables 4 and 5). *Salsola* seed germination at 24 hr and the growth of radicles at 40 hr were not significantly inhibited by any of the phytotoxins applied except ferulic acid (Table 4). However, *Salsola* radicle length was significantly reduced at 60 hr at the same concentration of all the phytotoxins (Table 5).

DISCUSSION

Decaying leaves of *Salsola* in soil significantly reduced the root, shoot, and seedling growth of *Kochia* and *Salsola;* the root, shoot, and seedling growth of sweet clover was not reduced. The lack of appreciable allelopathic effects of *Salsola* on sweet clover is possibly of significance as the latter grows well when planted in spoil soil even in the presence of voluntary species. Even though the actual mineral requirements for sweet clover were not determined, it is known to survive well in infertile soil supplementing deficient nitrogen through the nitrogen fixation process. Leaf leachate from

Salsola significantly decreased the shoot growth of sweet clover and *Kochia* and also the seedling growth of *Kochia*. *Salsola* growth was not statistically reduced by its leachate. Inhibitory effects due to leachate may not have major allelopathic significance in this region due to the low rainfall and short growing season. On the contrary, low rainfall aids in the accumulation of phytotoxins in the topsoil and in the rooting zone of plants. Phytotoxin accumulation in soils due to the annual decaying litter cycle, even in the regions where rainfall is relatively higher, has also been documented (Lodhi, 1975b, 1978).

The phytotoxins identified were quercetin, ferulic acid, chlorogenic acid, neochlorogenic acid, isochlorogenic acid, and caffeic acid. Caffeic acid and some ferulic acid were isolated only after hydrolysis. There is no doubt that most bound compounds would be released readily by decomposers in the soil. Thus, they represent realistic inhibitors (Guenzi and McCalla, 1966). Lodhi (1978) reported that the bound phenolics in plant litter were low in the associated soil in August, but high in samples collected in April. This supports the idea that bound compounds are probably more detrimental because they are released in soils at a slower rate and are in the rooting zone in large amounts during the active growth period. Among all the phytotoxins identified, neochlorogenic and isochorogenic acid were present in relatively small quantities. However, the possible significance of such trace amounts should not be ignored.

A 10^{-3} M concentration of all the identified toxins did not inhibit seed germination or radicle growth of *Salsola* in most cases in 40 hr. However, the radicle growth of *Salsola* was significantly reduced in 60 hr at the same concentration. *Salsola* seeds germinated at a rapid rate and reached almost complete germination within 24 hr. Lodhi and Wali (1979) reported a similar germination and growth pattern for *Kochia*.

Whereas the phytotoxins mentioned above have been isolated from many weedy plants which are common in the pioneer stage of the old field succession (Rice 1974), no reports were found where of allelopathic interactions in the weedy stage during succession other than in old fields. There is no doubt that many other species in freshly disturbed soils play an important physioecological role in the rapid disappearance of the weedy stage.

It seems that most weedy pioneer species, establishing themselves on poor-soil materials, may have low nutrient requirements. However, an increasing density of plants can cause them to eliminate themselves through the combined effects on inter- and intraspecific competition for the poor nutrient pool and allelopathy. Allelopathic effects are known to be more pronounced under stress conditions, thus eliminating species that are more sensitive to allelopathic influences.

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SEX PHEROMONE OF THE BLACK CUTWORM MOTH, *Agrotis ipsilon*[1,2]

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Abstract—(*Z*)-7-Dodecen-1-yl acetate (I) and (*Z*)-9-tetradecen-1-yl acetate (II) have been identified as sex pheromone components of the black cutworm moth, *Agrotis ipsilon* (Hufnagel). They are emitted by the female in approximately a 5:1 ratio. Differential saturation studies with male antennae suggest that there are two different acceptor sites for the two pheromone components. The most effective lures found in field trapping tests were 30 μg of I plus 10 μg of II on a rubber septum, and a 3:1 mixture of I and II dispensed from a 0.2-mm-ID glass capillary tube sealed at one end.

Key Words—Insect sex pheromone, insect attractant, *Agrotis ipsilon*, Lepidoptera, Noctuidae, black cutworm moth, *Cucullia intermedia*, (*Z*)-7-dodecen-1-yl acetate, (*Z*)-9-tetradecen-1-yl acetate.

INTRODUCTION

The black cutworm, *Agrotis ipsilon* (Hufnagel), is an agricultural pest of significant economic importance on numerous crops in many areas of the

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world. The availability of a synthetic sex lure could facilitate decisions regarding whether or not to apply pesticidal sprays for the larvae of this insect and aid in the timing of these sprays. Although blacklight traps can be used for this purpose with some insect species, *A. ipsilon* is usually found in blacklight traps in low numbers that are not considered to be representative of its population levels. Furthermore, since no electrical source is required to operate a sex pheromone trap it can be used for monitoring moths in remote areas where use of blacklight traps would be impractical.

It has been known for some time that *A. ipsilon* females emit a sex pheromone that attracts the males (Flaschentrager and Amin, 1950), but the chemicals involved in this communication system were not known. Research on the identification of the sex pheromone system of this insect was initiated as part of a multistate, multidisciplinary program on the bionomics and management of soil arthropods attacking corn.

A preliminary statement of our results has been published (Hill et al., 1977b), wherein we reported (*Z*)-7-dodecen-1-yl acetate (*Z*7-12:Ac) and (*Z*)-9-tetradecen-1-yl acetate (*Z*9-14:Ac) to be the two components required for sustained upwind anemotactic flight by the males and for trapping the males in the field. Herein we present the details of the pheromone identification, which required observations of male moths in flight, and the results of field trapping tests, together with other pertinent data and observations.

METHODS AND MATERIALS

*Z*7-12:Ac and *Z*9-14:Ac were purchased from Farchan Chemical Co. and were purified by high-performance liquid chromatography (HPLC) 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 unpurified compounds contained approx. 1% of the corresponding *E* isomers and the purified compounds contained no detectable amounts of the corresponding *E* isomers (<0.1%), as determined by gas–liquid chromatography (GLC) on XF-1150.

The GLC columns (glass) used were 3% OV-1 or OV-101 (methyl silicone on 100–120 mesh Gas-Chrom Q, 2 m × 4 mm ID), 3% CHDMS (cyclohexanedimethanol succinate on 100–120 mesh Gas-Chrom Q, 2 m × 4 mm ID), and 10% XF-1150 (50% cyanoethyl, methyl silicone on 100–120 mesh Chromosorb W-AW-DMCS, 2 m × 2 mm ID). N_2 was the carrier gas, and H_2 flame ionization detection was used; operating temperatures are given in degrees centigrade. The HPLC steric exclusion column used was 10–50 μm Styragel, 2.5 cm OD × 1.3 m stainless steel and was eluted at approx. 7.3 ml/min with benzene. A 2 mm ID × 500 mm stainless steel LiChrosorb® (10 μm) HPLC column was eluted at the rate of approx. 1 ml/min using 1% ethyl acetate in heptane, and approx. 1 ml fractions were

collected. Mass spectra (MS) were determined with a Finnigan 3300 dual quadrupole mass spectrometer interfaced with an OV-101 column (Cornell University Mass Spectrometry Center).

The chemical tests used have been described in detail elsewhere (Hill et al., 1977a; Beroza and Bierl, 1967). Acetylations and saponifications were carried out in 1-dr vials with Teflon®-lined screw caps. For acetylations, several drops of acetyl chloride were added to the sample, left at room temperature for 10–15 min, and excess acetyl chloride was removed in a stream of nitrogen. For saponifications, several drops of approx. 5% sodium hydroxide were added to an ethanol solution (0.5–1.0 ml) of the sample, which was then heated on a steam bath for approx. 1 hr; the products were obtained by dilution with 2–3 ml water and extraction with redistilled Skelly B.

Most of the insects were reared in Ohio on an artificial diet (Reese et al., 1972), and were from a north central United States biotype, referred to in this paper as the North American biotype. The insects studied in Australia were collected in the field as adults and reared through only 2 generations on an artificial diet (Shorey and Hale, 1965). Moths were kept on a 16:8 hour light–dark cycle throughout. Sex pheromone glands and ovipositors were manually extruded and snipped from the abdomens of female moths when they were 3–6 days posteclosion and 3–5 hr into the scotophase. These excised abdominal tips were extracted with redistilled methylene chloride.

Airborne effluvia from female moths 2–7 days posteclosion were trapped on Porapak Q. The system used for collection of these airborne materials was similar to that described by Byrne et al. (1975). Compressed house line air was forced through a purification column (1.8 cm ID × 6 cm) of Porapak Q into a reservoir containing distilled water, with the air passing over the surface of the water. Two outlets from this reservoir led (a) to the atmosphere and (b) to the chamber containing the female moths. Air was pulled through the female chamber and then through a Porapak Q column (1.8 cm ID × 13 cm) with a model 3 Dyna pump at a rate of approx. 1–2 liter/min. The flow rate through the prepurification Porapak Q column was adjusted to exceed that through the collection column of Porapak Q so that there was always a flow of air emerging from the reservoir outlet leading to the atmosphere. This simple arrangement allowed use of prepurified, humidified air without pressure variations that could disturb the insects. The female insect chamber was cooled to approx. 17°C during the period of scotophase by draping a wet cloth over the chamber; ambient temperature was 20–23°C.

Male moths were used for bioassays in a wind tunnel (Miller and Roelofs, 1978) at 3–7 hr into their scotophase and 2–7 days posteclosion; each male was used only once. Two tungsten bulb lamps were covered with red cellophane and the light from these lamps, reflected from a white wall, served to backlight a white paper backdrop (3.5 m × 2 m), thus providing

moderately uniform lighting (<0.3 lux) against which moth flights and other behavior were observed.

Electroantennogram responses (EAGs) were determined and normalized against a reference standard as described by Roelofs (1977). The normalizing procedure results in a unitless value for the EAG response (Figure 1).

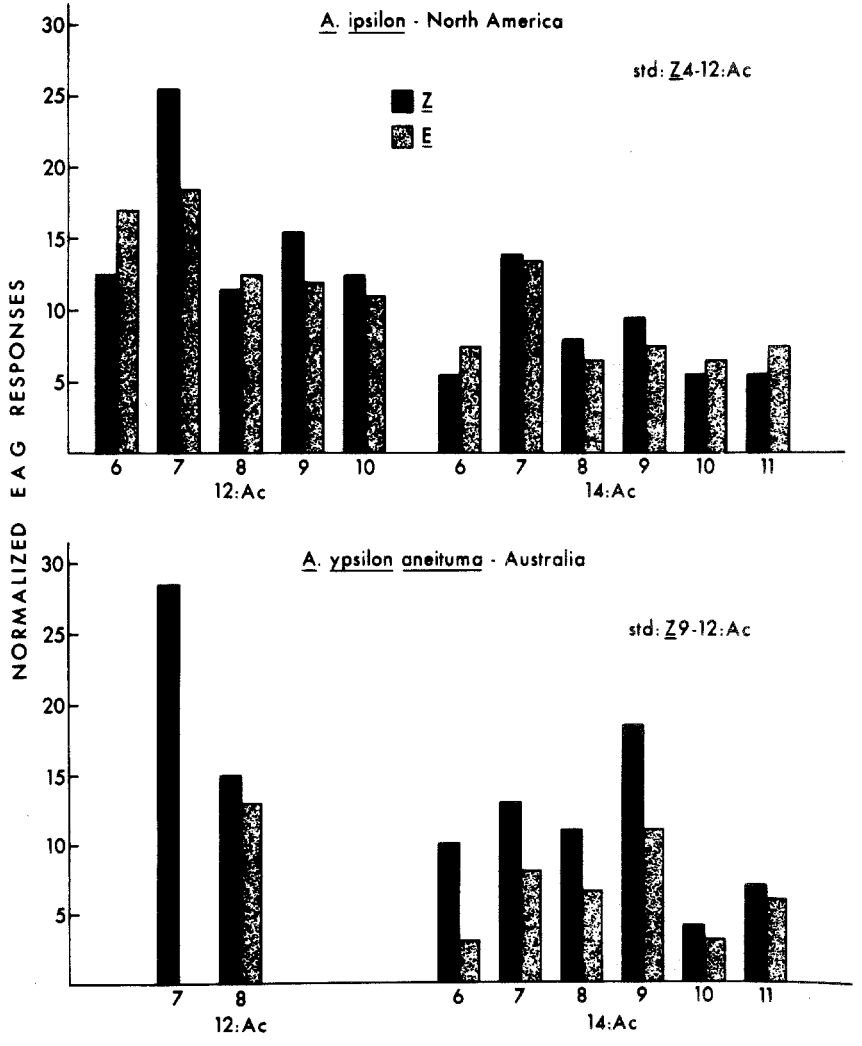

FIG. 1. Normalized EAG responses of male black cutworm antennae to synthetic chemicals.

EAG differential saturation studies were conducted as follows: filter paper with a specified amount of a test compound (30, 100, or 1100 μg) was placed in the air stream that passed continuously over the antenna and into an exhaust tube (10 cm diameter) that was placed at the downwind side of the EAG system. EAG responses to a short puff of a test compound (10 μg on filter paper in a disposable pipet: e.g., Z7–12:Ac in Table 2) were then recorded before, during, and after exposure of the antenna to the saturating compound, and the responses were compared.

Field tests were carried out in 4 fields in Wooster, Ohio, using modified Wong traps (Wong et al., 1972) hung at a height of 1.5 m and separated by 30 m. Two replicates were set out along the northern and western borders of alfalfa fields; a third in a fence row along a corn field and the fourth in a series of alternating corn–alfalfa experimental plots. For field tests carried out in Geneva, New York, Pherocon® 1C traps (Zoecon Corp.) were used; they were set out in one row along the edge of a grassy field with a minimum separation of 10 m between traps hung at a height of 0.5–1.5 m. Mean trap catches of male moths (X) were transformed to $\sqrt{X + 0.5}$, submitted to an analysis of variance, and ranked using Waller and Duncan's 1967 BSD rule. In the tables, means followed by the same letter are not significantly different at the 5% level.

Chemicals used in the tests were dissolved in methylene chloride and the appropriate amount of solution was soaked into rubber septa (5- × 9-mm rubber stoppers, sleeve-type, Arthur H. Thomas Co.). The glass capillaries used were 1-μl Microcaps® disposable micropipettes (3.2 cm × 0.2 mm ID; Drummond Scientific Co.) that had been sealed at one end and then filled using vacuum. The capillary fibers obtained from the Conrel Co. were 8 mil ID (0.2 mm ID) made of biodegradable Celcon® (polyoxymethylene).

RESULTS

Identification of Z7–12:Ac

A crude female tip extract was injected onto OV-1, 150°, collected in 1- or 2-min fractions up to 15 min, and these fractions assayed for antennal response by EAG. One area of activity at 9–11 min (2.2 mV; with responses to other fractions of 0.9–1.3 mV) was evident, and this corresponded to the retention time of normal 12-carbon acetates (9.7 min for Z7–12:Ac). At a higher temperature (190°), the active material was collected at 2–3 min, and there were no other obvious areas of activity out to 30 min. Numerous similar collections (10 FE to approx. 100 FE) were made during the course of this investigation, but they always revealed only this one definite area of activity. A similar collection and assay of a crude extract from CHDMS,

150°, up to 19 min also revealed only one EAG active area at 7–9 min (2.6 mV; other fractions ranged from 0.9–1.3 mV), which coincided with the GLC retention of *Z*7–12:Ac (7.95 min).

When the 9–11 min fraction from OV-1, 150°, was recovered, injected onto XF-1150, 150°, collected in 1 min fractions up to 25 min and assayed by EAG, activity was found at 14–15 min (1.5 mV; other inactive fractions 0.5–0.9 mV); the retention times of (*E*)-7-dodecen-1-yl acetate (*E*7–12:Ac) and *Z*7–12:Ac were 13.6 min and 14.7 min, respectively. Repeated examination of OV-1-collected, EAG-active materials on XF-1150 consistently revealed a peak with a retention time coincident with that of *Z*7–12:Ac, but no evidence was found for the presence of *E*7–12:Ac or any other positional isomers at levels of 1% or more of the *Z*7–12:Ac.

Confirmation of the presence of the acetate functionality in the EAG-active material was obtained as follows: a GLC-collected sample of the active material, after it was saponified using aqueous ethanolic NaOH, was collected from OV-1, 140°, assayed by EAG (no active fraction was found), the materials collected at 7–8.5 min (GLC retention of (*Z*)–6-dodecen-1-ol was 7.3 min) were recovered, acetylated with acetyl chloride and, after collection from OV-1, 140°, EAG activity at 13.5–15.5 min was found (5.4 mV) which coincided with the GLC retention time of *Z*7–12:Ac (14.4 min).

Ozonolysis of a GLC-collected (OV-1 and XF-1150) sample of the EAG active material produced 7-oxohept-1-yl acetate, determined by comparison of the GLC retention times of this ozonolysis product on OV-101, 145° (2.5 min) and on XF-1150, 155° (8.0 min) with those of this acetoxy aldehyde produced by ozonolysis of synthetic *Z*7–12:Ac (2.45 min and 8.0 min, respectively).

The GLC-CI-MS (methane) of the EAG-active material from a female tip extract (purified by GLC) and of synthetic *Z*7–12:Ac were the same, which confirmed the straight-chain monounsaturated structure of the active compound.

In addition, the largest EAG response from a series of normal 12-carbon acetates (Figure 1), alcohols, and aldehydes, using male *A. ipsilon* antennae, was from *Z*7–12:Ac, which, by analogy to previous examples (Roelofs, 1977), indicates that this isomer is the 12-carbon acetate component of the *A. ipsilon* sex pheromone system.

Field Trapping Tests with Z7–12:Ac

Field tests carried out at Wooster using Wong traps and filter paper impregnated with a range of dosages (10, 100, and 1000 ng) of *Z*7–12:Ac were unsuccessful in trapping male *A. ipsilon* moths, even though a female tip extract (20 FE) from this moth impregnated on filter paper attracted 5 male *A. ipsilon* moths. A test in a large screened field cage resulted in the

capture of 40 of 100 released male moths with 20 FE of a crude extract in a trap, whereas Z7–12:Ac in a trap caught none. Although no additional components had been detected by EAG assay or by GLC analysis of the 12-carbon acetate material from female tips, it was clear that there is at least one additional sex pheromone component required for attraction of male moths of this species into traps. To discover this component, it was necessary to utilize a more discriminating and/or sensitive bioassay than the EAG. Because of its availability throughout the year (in contrast to field testing), we decided to use observations of male behavior in a wind tunnel (Miller and Roelofs, 1978) for this purpose.

Identification of Z9–14:Ac; Observations of Male Moth Flights in a Wind Tunnel

Female tip extract (10–25 FE) on filter paper in the wind tunnel elicited male moth upwind anemotactic flight terminating at the chemical source by approximately one fourth of the males tested (completed flights, Table 1). Under the same conditions 10-ng samples of synthetic Z7–12:Ac on

TABLE 1. WIND TUNNEL RESPONSES OF MALE *A. ipsilon* MOTHS TO VARIOUS NATURAL AND SYNTHETIC SAMPLES ON FILTER PAPER

Sample (quantities)	Partial flights (%)	Completed flights (%)	Total no. of moths
1. Female tip extract (10–25 FE)	4	23	542
2. Female effluvia, trapped on Porapak-Q (10–25 FE)	8	38	24
3. Sample 1 + AcCl	6	19	16
4. Sample 1 + NaOH, H_2O, EtOH, Δ	0	0	48
5. Sample 4 + AcCl	0	21	48
6. Sample 1, 12-carbon acetate area collected from OV-1 (10–25 FE)	0	0	15
7. Sample 1, 14-carbon acetate area collected from OV-1 (10–25 FE)	0	0	8
8. Sample 6 + sample 7	6	6	16
9. Sample 6 + Z9–14:Ac (10 ng)	0	62	16
10. Sample 7 + Z7–12:Ac (10 ng)	0	25	8
11. Z7–12:Ac (10 ng)	6	2	115
12. Z7–12:Ac (20 ng)	6	0	83
13. Z7–12:Ac (50 ng)	10	0	42
14. Z9–14:Ac (10 ng)	0	0	4
15. Z7–12:Ac (10 ng) + Z9–14:Ac (10 ng)	4	15	240
16. Z7–12:Ac (100 ng) + Z9–14:Ac (10 ng)	0	32	32
17. Z7–12:Ac (10 ng) + Z7–14:Ac (10 ng)	<1	10	207

filter paper elicited almost no completed flights, and no completed flights were observed with 20- and 50-ng samples. However, about 5–10% of the male moths hovered in the chemical plume for short periods (seldom exceeding 5 sec), making little or no forward progress, after which they suddenly flew out rapidly from the chemical plume in a sideways and upwards direction. During these periods of hovering flight, the male moth faced the upwind direction, held his body at about a 45° angle from the vertical with the terminal portion of his abdomen arched slightly backward, his claspers well extended, and his antennae held upward in a V-shape, with the ends slightly bent toward the horizontal (Figure 2a). With the female tip extract as a lure, the male was seen frequently in this same posture, but during rapid upwind progress the abdomen was often closer to the horizontal, the claspers obviously still extended and the antennae held upright (Figure 2b).

Having established that a female tip extract elicits a sustained upwind anemotactic response, whereas *Z*7–12:Ac alone does not, the extract was treated in several ways and the male moth response to these treated materials was determined in the wind tunnel (Table 1).

Treatment of the crude female tip extract with acetyl chloride did not appreciably alter its capacity to elicit completed male moth flights to the source. No flights of male moths were observed when saponified female tip extract (10–25 FE) were used as a lure. Combination of saponified extract (approx. 10 FE) with *Z*7–12:Ac (10 ng) elicited the response expected from *Z*7–12:Ac alone, and no completed flights were observed using this combination as a lure in the wind tunnel. However, treatment of these saponified materials with acetyl chloride restored in full the attractivity of the extract to male moths in the wind tunnel. These tests established that the second sex pheromone component was not an alcohol and was probably an acetate. Further support for the ester structure was obtained when a crude extract was chromatographed on a Florisil (Floridin Company) column (36 cm × 2 cm, ID) using successive 150 ml portions of 0, 5, 10, 20, 50, and 100% diethyl ether in hexane (Skellysolve B). Completed flight activity was elicited by the 20% diethyl ether fraction, the fraction containing esters, but not by earlier or later fractions.

Combinations of synthetic *Z*7–12:Ac (10 ng) with some selected acetates [10 ng each of (*Z*)-5-decen-1-yl acetate, (*Z*)-7-decen-1-yl acetate, (*Z*)-5-dodecen-1-yl acetate, (*Z*)-9-dodecen-1-yl acetate, 11-dodecen-1-yl acetate, (*Z*)-8-tetradecen-1-yl acetate, and *Z*9–14:Ac] were tested in the wind tunnel and completed upwind flight was seen only for the combination with *Z*9–14:Ac. Subsequent repeated testing showed that this combination at the 10 ng + 10 ng and the 100 ng + 10 ng level elicited about the same response as a crude female tip extract or Porapak trapped materials from female effluvia (Table 1).

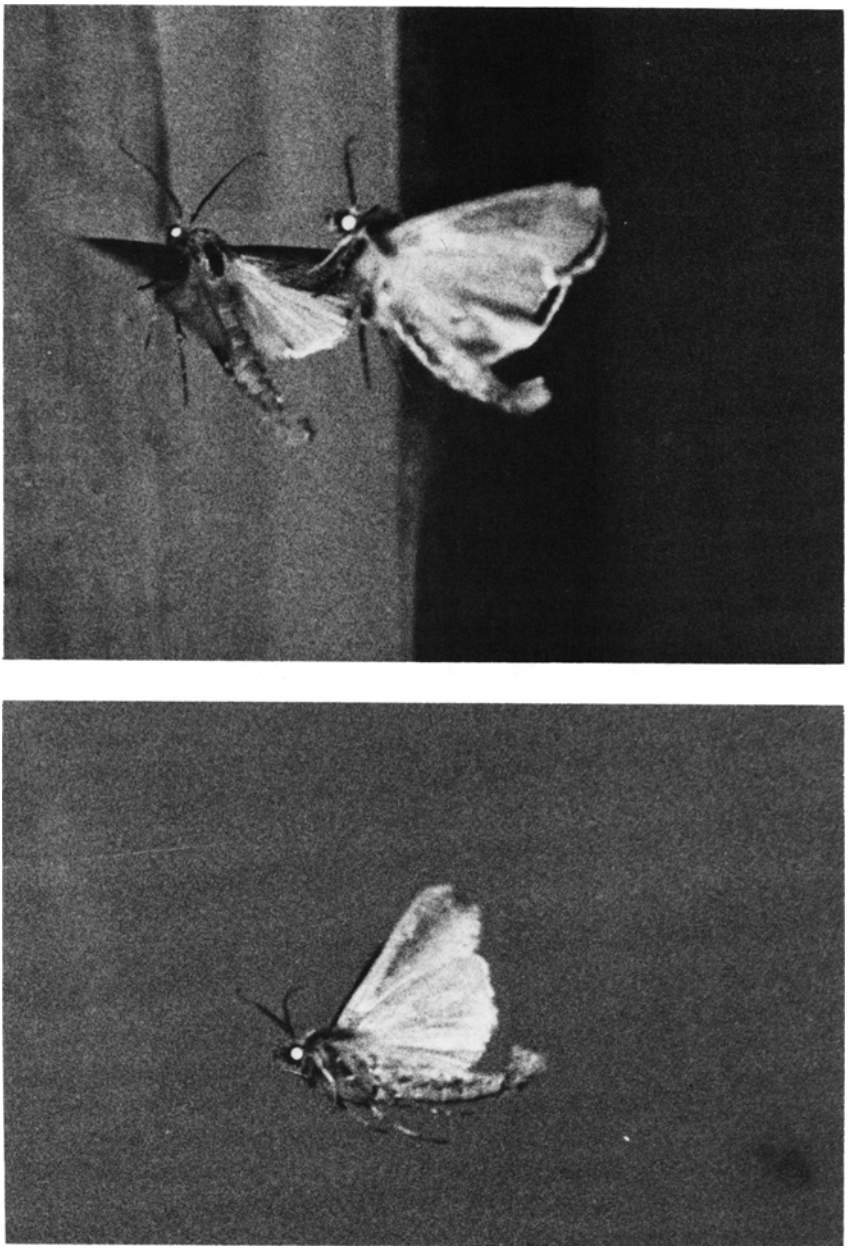

FIG. 2. (a, top; b, bottom) Male *A. ipsilon* flying towards a chemical sex lure (*Z*7–12:Ac + *Z*9–14:Ac) in a wind tunnel.

About 100 FE of a crude female tip extract, known to elicit completed flight responses in the wind tunnel, was collected from OV-1, 170°, at 4.5–6 min (12-carbon acetates) and at 11–12 min (14-carbon acetates). Separately, each of these (approx. 10-FE aliquots) was inactive in the flight tunnel, but activity was restored, albeit at a somewhat reduced level, when the two were recombined (Table 1). Additionally, another aliquot of the active crude female tip extract was collected from OV-101, 170°, at the retentions of 12-carbon and 14-carbon acetates. The 12-carbon acetate fraction (approx. 10 FE) was combined with 10 ng of *Z*9–14:Ac, whereas the 14-carbon acetate fraction (approx. 10 FE) was combined with 10 ng of *Z*7–12:Ac. Both combinations were tested in the wind tunnel and found to elicit a completed flight response from male *A. ipsilon* moths (Table 1).

Confirmation of *Z*9–14:Ac in *A. ipsilon* female tip extract and effluvia was obtained by GLC retention times, saponification–reacetylation reactions, mass spectrometry, and ozonolysis, as follows.

GLC tracings of crude female tip extracts on OV-101 or OV-1 usually showed the *Z*7–12:Ac peak, but not that corresponding to a 14-carbon acetate. To remove interfering materials of high molecular weight, the extract from approx. 3000 female tips was chromatographed (HPLC) on Styragel using benzene. The fractions eluting approximately where materials of molecular weight 150–300 were expected (fractions at 27–28, 28–29, and 29–30 min, with an elution rate of approx. 7.3 ml/min) were analyzed by GLC-EAG for the presence of *Z*7–12:Ac and were found to contain this acetate. In addition, these fractions elicited completed flights by male moths in the wind tunnel. The fractions were combined, the 14-carbon acetate area was collected from OV-1, and this material was used for the subsequent identification of *Z*9–14:Ac. A GLC tracing on XF-1150, 170° showed a peak at 10.1 min, corresponding to *Z*9–14:Ac (10.0 min); no peak at the retention of *E*9–14:Ac (9.3 min) was seen. A GLC-CI-MS (methane) of this material was essentially the same as that of synthetic *Z*9–14:Ac, confirming that the natural material is an unbranched monounsaturated 14-carbon acetate. Ozonolysis of the natural material produced 9-oxonon-1-yl acetate, as determined by its GLC retention time on OV-1, 150° (4.85 min), which matched that of an authentic sample (4.75 min) prepared by ozonolysis of *Z*9–14:Ac. Confirmation of the presence of the acetate group in the natural material was obtained by saponification to produce (*Z*)-9-tetradecen-1-ol (5.8 min on OV-101, 170°), which was collected from OV-101 and acetylated with acetyl chloride to regenerate *Z*9–14:Ac (9.75 min on OV-101, 170°). This last test was carried out on a sample of the natural material obtained from female effluvia trapped on Porapak, and purified as described in the next section.

Although a mixture of *Z*7–12:Ac and (*Z*)-7-tetradecen-1-yl acetate (*Z*7–14:Ac) elicited a completed flight response in the wind tunnel, this

combination did not attract males into traps in the field nor was any evidence found for the presence of Z7–14:Ac in female tip extracts or in female effluvia. The unreplicated series tested in the field consisted of 2, 6, 20, 60, and 200 μg in rubber septa of a 1:1 mixture of Z7–12:Ac + Z7–14:Ac; at the same time 10 μg Z7–12:Ac + 10 μg Z9–14:Ac trapped 30 male *A. ipsilon*.

Airborne Female Effluvia Trapped on Porapak Q

Various preparations of female effluvia (100–150 "female nights," or FN, each) were used. Each preparation elicited completed flights by male *A. ipsilon* moths in the wind tunnel at the 10–25 FN level (Table 1). The GLC retentions, GLC-EAG determinations of active components, saponification–reacetylation reactions, and ozonolyses of both sex pheromone components were essentially the same as those already described, and fully confirmed the presence of Z7–12:Ac and Z9–14:Ac in the female effluvia.

The crude preparations, however, were not suitable for direct GLC analysis of the two components due to the large number of interfering peaks, so one preparation (approx. 200 FN) was purified by HPLC on LiChrosorb®. The 6–7 min fraction, known to contain acetates by comparison with a previous chromatogram using saturated standards, was examined on OV-1, 180°, and found to contain about a 5:1 ratio of Z7–12:Ac to Z9–14:Ac.

EAG Responses of Male A. ipsilon Antennae

Series of monounsaturated 10-, 12-, 13-, 14-, and 16-carbon chain acetates, alcohols, and aldehydes were tested for antennal interaction with male *A. ipsilon* antennae. Of the compounds tested, Z7–12:Ac produced the largest depolarization. Figure 1 presents the depolarization response profile for the 12- and 14-carbon acetates. Of the 14-carbon acetates, Z7–14:Ac elicits the largest response, although Z9–14:Ac is the natural pheromone component. It is of interest to note that the same series of compounds, when tested on antennae of male Australian black cutworm. *A. ypsilon aneituma* (Walker) (Common, 1958), produced a slightly different profile (Figure 1), in which Z9–14:Ac caused the largest depolarization. No further investigation of the sex pheromone system of the Australian insect has been carried out.

Differential antennal saturation studies were conducted to determine what effect continuous exposure of male antennae to various acetates would have on the antennal response to Z7–12:Ac (Table 2). At the three concentrations used (30, 100, and 1100 μg), (Z)-7-decen-1-yl acetate (Z7–10:Ac) effectively saturated antennal acceptors and thus suppressed further depolarization by a standard sample (10 μg) of Z7–12:Ac.

TABLE 2. EAG RESPONSES OF MALE *A. ipsilon* ANTENNAE TO *Z*7-12:Ac WHILE EXPOSED CONTINUOUSLY TO VARYING AMOUNTS OF OTHER TEST COMPOUNDS

	% of full response to 10 μg of *Z*7-12:Ac[a]		
Test compound at	30 μg[b]	100 μg[b]	1100 μg[b]
*Z*7-10:Ac	13	5	5
*Z*7-12:Ac	18		9
*Z*7-13:Ac	53	19	19
*Z*7-14:Ac	83	85	66
*Z*9-12:Ac	70	39	28
*Z*9-13:Ac		74	62
*Z*9-14:Ac	112	82	81
*Z*8-12:Ac		68	28
*Z*8-13:Ac		69	
*Z*6-12:Ac		39	22
*E*7-12:Ac		36	16
*Z*10-12:Ac		61	45

[a]This is the relative EAG response to 10 μg of *Z*7-12:Ac on filter paper, determined as the ratio of the mean response during exposure to the test compound (2 determinations/sample) to the mean response before and after exposure to the test compound (2 determinations before and 2 after exposure, per sample). The number of samples varied from 1 to 3/test compound.

[b]Amount of the test compound on filter paper.

*Z*7-12:Ac also suppressed further antennal response to itself, whereas (*Z*)-7-tridecen-1-yl acetate (*Z*7-13:Ac), although less effective than *Z*7-10:Ac at 30 μg, approached the suppressing effect of *Z*7-10:Ac at 100 μg and 1100 μg. *Z*7-14:Ac was relatively ineffective at 30 μg and 100 μg, but a partial suppression of further depolarization was seen at 1100 μg. With the *Z*9 isomers, the 12-carbon acetate (*Z*9-12:Ac) suppressed antennal responses to *Z*7-12:Ac at 100 μg and 1100 μg, but not at 30 μg. The 13-carbon acetate (*Z*9-13:Ac) was somewhat less effective than the 12-carbon acetate at the two higher concentrations; the 14-carbon acetate (*Z*9-14:Ac) showed very little or no suppressing effect even at 1100 μg. The specificity of the *Z*7-12:Ac receptors for the *Z*7 position is apparent from comparison of the greater suppressing effect that *Z*7-10:Ac, *Z*7-12:Ac, and *Z*7-13:Ac have compared to other positional isomers or to *E*7-12:Ac.

Although continuous exposure of male *A. ipsilon* antennae to *Z*9-14:Ac, even at the 1100 μg level, did not suppress the EAG response to *Z*7-12:Ac, it greatly reduced the response to itself, indicating saturation of the acceptor sites for *Z*9-14:Ac but not of those for *Z*7-12:Ac un-

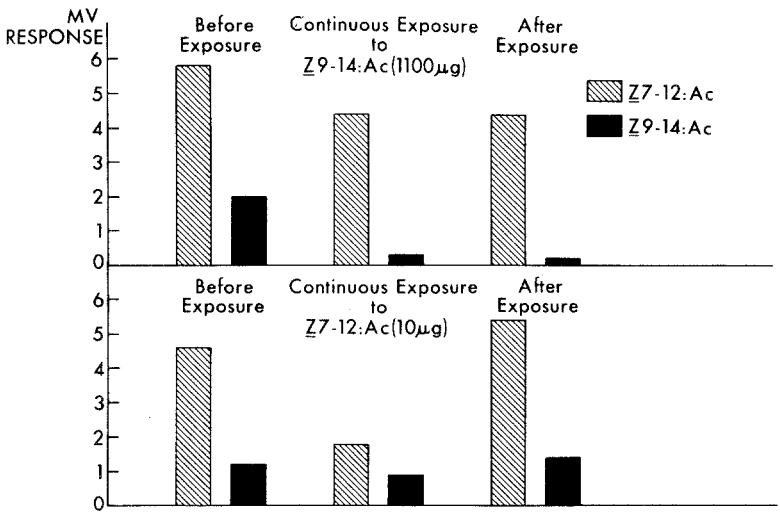

FIG. 3. EAG responses of male *A. ipsilon* antennae to 10 μg of test chemicals on filter paper.

der these conditions (Figure 3). Alternatively, continuous exposure of antennae to Z7–12:Ac at the 10-μg level effectively suppressed further response to itself but not to Z9–14:Ac, indicating that the acceptor sites for Z7–12:Ac can be saturated without significantly affecting those for Z9–14:Ac. These data suggest the presence of two different acceptor sites, one for each of the two sex pheromone components.

Field Tests

A preliminary unreplicated field test was carried out at Geneva using rubber septa impregnated with Z7–12:Ac and Z9–14:Ac in a 1:1 ratio. Traps with 6 μg and 20 μg of chemicals caught the most male *A. ipsilon* moths (23 and 30 moths each, respectively); those at 60 and 200 μg caught lower numbers (18 and 16 moths each, respectively), and those with 2 μg and 600 μg caught almost no moths (1 moth in each).

In another preliminary test carried out in Geneva, males of another noctuid, *Cucullia intermedia* (Speyer), known to be trapped with Z9–14:Ac alone (Roelofs and Comeau, 1971) were found in traps baited with the higher quantities of Z9–14:Ac (1 mg or when dispensed from capillary fibers). Totals of 154 *C. intermedia* males and 950 *A. ipsilon* males were trapped during the summer of 1977 in Geneva and in only one instance did a trap contain individuals of both species (one each in a trap baited with 4 glass fibers containing a 1:1 mix of Z7–12:Ac and Z9–14:Ac). *A. ipsilon*

males were not trapped with the high quantities, and *C. intermedia* were not trapped with the lower quantities of *Z*9–14:Ac, with the one exception already noted. Thus, the appearance in traps of *C. intermedia* provided a convenient indication of release rates of *Z*9–14:Ac that were too high to trap *A. ipsilon* males.

Results of tests conducted in Wooster, Ohio, are presented in Tables 3 and 4. In these tests, the best combination of *Z*7–12:Ac and *Z*9–14:Ac in rubber septa was 30 μg and 10 μg, respectively. This was the only combination with which male *A. ipsilon* moths were trapped in numbers that are statistically the same as those trapped with four live virgin female *A. ipsilon* moths. It is apparent from the data in Table 3 that trap catches diminish significantly as the quantities of the two components are either increased or decreased, suggesting that this insect requires a narrow concentration range of sex pheromone for maximum sexual responsiveness. It is clear that maximum trap catches are dependent on having an optimum release rate of chemicals from the dispenser. Because release rates of chemicals from dispensers such as the rubber septa used in these tests are known

TABLE 3. TRAP CATCHES OF MALE *A. ipsilon* WITH VARIOUS COMBINATIONS OF *Z*7–12:Ac AND *Z*9–14:Ac DISPENSED FROM RUBBER SEPTA IN WONG TRAPS

Treatment (*Z*7–12:Ac, μg + *Z*9–14:Ac, μg)		Mean no. males/trap
Test 1[a]		
1	1	4.8 de
3	3	13.0 cd
10	10	17.2 cd
30	30	28.2 bc
10	3	16.0 d
30	10	36.2 ab
4 virgin females		58.2 a
Unbaited		1.2 e
Test 2[b]		
30	30	7.0 gh
30	10	11.2 fg
30	3	6.8 gh
100	30	3.2 hj
100	10	1.8 j
300	30	0.8 j
4 virgin females		17.5 f
Unbaited		0.2 j

[a]Conducted May 18, to May 26, 1977, at Wooster, Ohio.
[b]Conducted May 27, to June 3, 1977, at Wooster, Ohio.

TABLE 4. TRAP CATCHES OF MALE *A. ipsilon* WITH VARIOUS COMBINATIONS OF *Z*7–12:Ac AND *Z*9–14:Ac, EMITTED FROM VARIOUS DISPENSERS AND TWO TYPES OF TRAPS

Treatment *Z*7–12:Ac, μg + *Z*9–14:Ac, μg (or ratio)		Dispenser	Trap	Mean no. males/trap
Test 1[a]				
30	5	RS[d]	Wong	36.2 bc
30	10	RS	Wong	47.8 ab
30	15	RS	Wong	28.2 c
30	20	RS	Wong	33.5 c
30	10	RS	Pher. 1C[d]	27.0 c
4 virgin females			Pher. 1C	36.2 bc
4 virgin females			Wong	50.5 a
Unbaited			Wong	1.8 d
Test 2[b]				
30	10	RS	Pher. 1C	25.8 efg
30	10	RS	Wong	15.0 gh
30[c]	10[c]	RS	Wong	10.8 hj
(3:1)[c]		1 Gl.Cap.[d]	Wong	34.2 ef
(10:1)[c]		1 Gl.Cap.	Wong	17.0 fgh
(30:1)[c]		1 Gl.Cap.	Wong	14.2 hj
4 virgin females			Wong	45.5 e
Unbaited			Wong	2.8 j

[a]Conducted June 24, to July 4, 1977, at Wooster, Ohio.
[b]Conducted July 12, to July 22, 1977, at Wooster, Ohio.
[c]Untreated chemicals from Farchan Chemical Co.; each contained approximately 1% of the corresponding *E* isomer.
[d]RS = rubber septa; Gl. Cap. = glass capillary; Pher. 1C - Pherocon 1C trap, Zoecon Corp.

to be greatly affected by environmental conditions, such as temperature and wind velocities, capillary dispensers were tried. Capillary-dispensed chemicals are known to be released at rates that are relatively unaffected by environmental conditions (Brooks et al., 1977). Initial tests using capillary dispensers were carried out in Geneva using 8 mil ID (0.2 mm ID) capillaries (Conrel Co.) filled with either *Z*7–12:Ac or *Z*9–14:Ac and placed in traps in various combinations. Even the lowest release rate obtainable with these (1 fiber *Z*7–12:Ac + 1 fiber *Z*9–14:Ac) was too high to attract *A. ipsilon*, but did attract numerous *C. intermedia*. Consequently, different capillaries were tested, in expectation of obtaining a lower release rate. The capillaries used were 1 μl Microcap® disposable pipettes with one end sealed; the inside diameter of these was calculated to be approx. 0.2 mm from their length (3.25 mm) and volume (1 μl = 0.001 cm^3). In field tests carried out in New York, the two acetates lured fewer *C. inter-*

media when dispensed from the glass capillaries than did comparable mixtures dispensed from the Conrel fibers. In one of the Ohio tests, a 5:1 mixture (*Z*7–12:Ac to *Z*9–14:Ac) lured significantly more *A. ipsilon* males into Wong traps when it was dispensed from the glass capillaries (39 moths) than when it was dispensed from the Conrel fibers (7 moths). A mixture of the two chemicals in a 3:1 ratio (*Z*7–12:Ac to *Z*9–14:Ac), was the most attractive mixture in a capillary dispenser found. Test 2, Table 4, demonstrated that the 3:1 mixture dispensed from the glass capillary in a Wong trap is as effective for luring males into traps as the best rubber septum dispenser in a Pherocon 1C trap or four virgin females in a Wong trap. Further testing of capillary dispenser systems is being carried out.

The two tests in Table 4 are at variance with regard to which of the two types of traps is the most effective for trapping *A. ipsilon* moths. The commercially available acetates, each containing approx. 1% of the corresponding *E* isomer, were as effective in these tests (Table 4) as the isomerically pure materials used for all the early tests. In an additional test carried out in Geneva, New York, isomerically pure *Z*7–12:Ac and isomerically pure *Z*9–14:Ac in rubber septa at the 30 μg + 30 μg and 10 μg + 10 μg levels caught a total of 87 and 59 males, whereas the chemicals from Farchan at these same levels caught 62 and 52 males, respectively; treatments were replicated three times and rerandomized eight times over 19 trapping nights. Statistical analysis of these data failed to show any significant difference between trap catches.

DISCUSSION

Two components of the primary sex pheromone system of *A. ipsilon* have been identified as *Z*7–12:Ac and *Z*9–14:Ac. These are emitted by the female moths in the approximate ratios of 5 to 1, respectively. A number of other noctuids are known to utilize *Z*7–12:Ac as part of their sex pheromone systems. These include *Trichoplusia ni* (Hübner) (Berger, 1966), and *Pseudoplusia includens* (Walker) (Tumlinson et al., 1972). There are also noctuids known to use *Z*9–14:Ac as a primary sex pheromone component. These include *Spodoptera eridania* (Cramer) (Jacobson et al., 1970), *Spodoptera frugiperda* (J.E. Smith) (Sekul and Sparks, 1967, 1975), and *Mamestra configurata* (Walker) (Underhill et al., 1977). *Z*9–14:Ac is also known to be a sex pheromone component for moths other than noctuids, notably a number of Tortricidae (Tamaki, 1977). *A. ipsilon* is the first insect for which the two chemicals in combination are known to comprise a primary sex pheromone system.

The EAG technique is frequently relied on to indicate which members of a series of compounds are involved as primary components in sex phero-

mone systems of lepidopteran insects (Roelofs, 1977). For *A. ipsilon* it was possible to select Z7–12:Ac as the monounsaturated 12-carbon acetate involved in this sex pheromone system using the EAG assay, but this was not the case for the less abundant Z9–14:Ac component. In the monounsaturated 14-carbon acetate series, the compound that stands out is Z7–14:Ac (Figure 1). Although this compound, in combination with Z7–12:Ac has been observed to elicit a completed upwind flight response in a wind tunnel similar to that seen with Z7–12:Ac and Z9–14:Ac, the two Z7 isomers in combination failed to attract male *A. ipsilon* into traps in the field, and there was no evidence in female tip extract or effluvia for the presence of Z7–14:Ac. Most likely the relatively high antennal response is due to its interaction with the Z7–12:Ac acceptor cells (Roelofs, 1977).

Differential antennal saturation studies indicated the probability that two types of acceptor cells are present on *A. ipsilon* antennae for perception of its sex pheromone: one type for Z7–12:Ac and the other for Z9–14:Ac. Additional information obtained from these differential saturation studies also showed that the Z7–12:Ac receptor site is capable of accommodating not only a shorter chain compound, such as Z7–10:Ac, but also the longer chain of Z7–13:Ac, and that it is rather specifically attuned to the Z7 positional isomer.

At present, the reason for the low EAG response of male *A. ipsilon* antennae to Z9–14:Ac is not clear. However, the small amounts of this 14-carbon acetate present in female-produced sex pheromone preparations and its low EAG response account for the difficulties in locating this component by the GLC-EAG technique. In the case of *A. ipsilon*, flight responses of the males in a wind tunnel proved to be a very sensitive and discriminating bioassay for the minor component. Of interest is the 14-carbon acetate EAG profile for the Australian black cutworm moth, *A. ypsilon aneituma* (Walker). Male antennae of this insect biotype show their highest response to Z9–14:Ac in this series rather than to Z7–14:Ac, as found for the North American biotype. The most intense EAG response for both insects was to Z7–12:Ac. No field tests with the Australian biotype or analysis of its female-produced sex pheromone have been carried out, but it seems reasonable to expect that it will utilize the same compounds as the North American biotype.

The best lures found for *A. ipsilon* males in the field were either a rubber septum with 30 μg of Z7–12:Ac plus 10 μg of Z9–14:Ac or a 0.2-mm (ID) glass capillary tube containing a 3:1 mixture of Z7–12:Ac and Z9–14:Ac. *A. ipsilon* males were trapped by a relatively narrow range of dispenser loadings, indicating a requirement by this insect of a rather specific pheromone concentration in the atmosphere for maximum sexual responsiveness. Other noctuids are known to have similar pheromone concentration requirements (Kaae et al., 1973). The emission rates of chemicals from

capillaries are less sensitive to changes in environmental conditions, such as temperature and wind speed, and thus should be more reliable for attraction of *A. ipsilon* males into traps than either rubber or polyethylene dispensers.

The noctuid *Cucullia intermedia* is known to be attracted to Z9-14:Ac. (Roelofs and Comeau, 1971). In the present study, male *C. intermedia* were trapped with dispenser loadings of Z9-14:Ac that were higher than those used to trap *A. ipsilon*, but not with the lower loadings that attracted *A. ipsilon*.

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REVIEW ARTICLE

CHEMICAL DEFENSE BY TERMITE SOLDIERS

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Abstract—The chemistry of the defensive secretions of termite soldiers is reviewed. The structural variety of the chemical weapons, the uses of these substances in defense, and some potential biosynthetic interrelationships are discussed. Classification of the monoterpenes, sesquiterpenes, diterpenes, and various acetate-derived compounds according to termite subfamilies places the known defense substances in a phylogenetic perspective.

Key Words—Termite soldiers, defense compounds, chemical defense, frontal gland, terpenes, Isoptera.

INTRODUCTION

Termites possess rigid totalitarian societies in which morphologically specialized individuals execute specific tasks: the king and queen reproduce, the workers forage and feed, and the soldiers defend (Wilson, 1971; Harris, 1971; Behnke, 1977; Prestwich, 1978a). Descended from cockroaches over a hundred million years ago, these mostly blind, soft-bodied insects rely heavily on chemical communication for caste determination and recognition, trail-following and food-finding, building, alarm, and defense (Stuart, 1966, 1970; Moore, 1969, 1974). Recently, Meinwald et al. (1978) described the results of chemical ecological studies of termite building pheromones and termite defense secretions, based on work performed at the International Centre of Insect Physiology and Ecology in Nairobi, Kenya. In this review, I will cover the chemistry of the substances isolated from termite

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soldier frontal glands in more detail, with reference to the morphological variety of the "delivery systems" for these chemical weapons, the deployment and efficacy of these secretions in defensive behavior, the hypothetical modes of action of the secretions, and the biosynthetic origins postulated for these substances. The format will reflect a phylogenetic perspective with respect to the development of more sophisticated chemical defense systems by soldiers of higher termite genera.

BIZARRE WEAPONRY

Maeterlinck (1939) called termite soldiers "a caste of nightmarish monsters, which recall the most fantastic revelries of Hieronymous Bosch, Breughel the Elder, and Callot." Recent studies have used scanning electron microscopy (SEM) to examine the gross morphology of the frontal weapon; in addition, these workers have used histological techniques and transmission electron microscopy (TEM) to unravel the fine structure of the secretory cells themselves (Deligne, 1965, 1973, 1975; Deligne and Quennedey, 1977; Noirot, 1969; Noirot and Quennedey, 1974; Quennedey, 1973, 1975a–c; Quennedey and Deligne, 1975; Sannasi, 1969). Drawings of representative heads of termite soldiers discussed in this review are shown in Figure 1.

Quennedey (1973, 1975a) recognizes three fundamental mechanisms for soldiers employing chemical defense. First, a soldier may be capable of biting with its mandibles with the simultaneous addition of a toxic, irritant, or oily chemical secretion from the frontal gland reservoir (e.g., *Amitermes*, *Cubitermes*, *Macrotermes*). Most soldiers employing this mechanism have thin, sharp mandibles which cross over each other with a slashing motion ("*soldats faucheurs*"—"reaping" or "mowing" soldiers) (Deligne, 1965, 1971). Second, if the mandibles cannot cause a wound, copious quantities of a topically active poison may be applied using an extended labrum, or "upper lip," terminating in broad spatulate hairs—a "daubing brush" (e.g., *Schedorhinotermes*) (Quennedey, 1975a–c). Finally, physical contact with the enemy can be avoided totally by the nasute soldiers (e.g., *Nasutitermes*). They eject a viscous, sticky solution which irritates and mechanically immobilizes small assailants. I will allude to these basic techniques of chemical defense during the discussion of the chemical structures and physical properties of the substances employed.

Nonchemical defenses (Deligne, 1971; Wilson, 1971) include the crushing mandibles of many lower termites (e.g., *Kalotermes*), the asymmetric and symmetric snapping mandibles of several higher termites (e.g., *Pericapritermes* and *Termes*, respectively), and the hole-plugging behavior of the phragmotic soldiers (e.g., *Cryptotermes*). These will not be discussed further in this review.

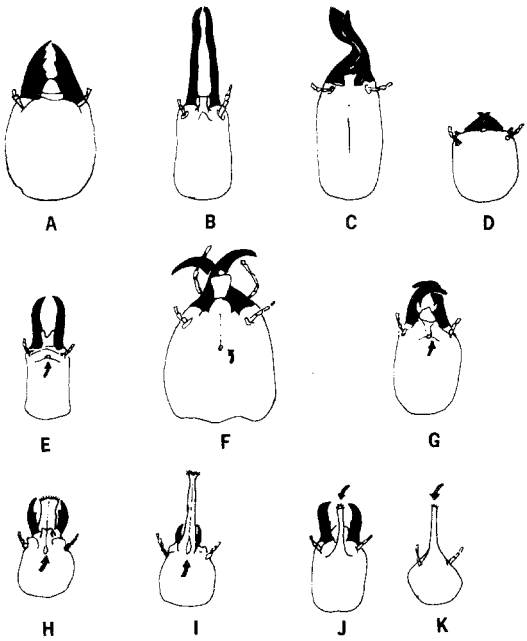


FIG. 1. Representative cephalic weapons of soldier termites (redrawn by the author). Nonchemical: A, biting (*Neotermes*); B, symmetric snapping (*Termes*); C, asymmetric snapping (*Pericapritermes*); D, phragmotic (*Cryptotermes*). Chemical: E, reaping (*Cubitermes*); F, reaping (*Macrotermes*); G, biting-reaping (*Amitermes*); H, daubing (*Schedorhinotermes*); I, daubing "nasutoid" (*Acorhinotermes*); J, biting-squirting (*Armitermes*); K, squirting (*Nasutitermes*). Some antennal segments and mouthparts are not shown. Arrows indicate the location of the frontal pore (fontanelle) of soldiers possessing defense secretions.

LOWER TERMITES

The insect order Isoptera was first divided into two superfamilies (Krishna, 1970; Wilson, 1971)—lower (primitive) and higher termites (Table 1). The former consists of six families: Mastotermitidae, a largely fossil family with a single surviving species; Kalotermitidae, the dry-wood termites; Hodotermitidae, the harvester termites; Termopsidae, or rotten-wood termites; and the diversified Rhinotermitidae. The new family Serritermitidae is monotypic and is believed to have its origins from a primitive rhinotermitid ancestor (Emerson and Krishna, 1975).

Mastotermes darwinensis (Mastotermitidae) possesses, in addition to its powerful mandibles, a rudimentary chemical defense system. Glands presumed to be in the buccal cavity supply a mixture of neat benzoquinone

TABLE 1. TERMITE TAXONOMY

Phylum:	Arthropoda		
Class:	Insecta		
Order:	Isoptera		
Family:	Mastotermitidae		
	Kalotermitidae		
	Hodotermitidae		
	Termopsidae		
	Rhinotermitidae		
Subfamily:	Coptotermitinae		
	Heterotermitinae		
	Psammotermitinae		
	Termitogetoninae		
	Stylotermitinae		
	Rhinotermitinae		
	Prorhinotermitinae		
	Serritermitidae		
	Termitidae[1]		
	Amitermitinae		Apicotermitinae
	Termitinae	(or)	Termitinae
	Macrotermitinae		Macrotermitinae
	Nasutitermitinae		Nasutitermitinae

(major component, 1) and toluquinone (2) for admixture with an aqueous protein mixture secreted by the salivary glands (Moore, 1968). This odorous secretion turns dark and rubbery shortly following its application to the attacker. The use of quinones as nonspecific toxicants and repellents is widespread among arthropods (Eisner, 1970) and also occurs in several genera of higher termites. Soldier frontal glands are absent in Mastotermitidae, Kalotermitidae, Hodotermitidae, and Termopsidae, so it is not surprising that no further chemical defenses have been reported for these lower termite families.

The panglobal family Rhinotermitidae is divided into seven subfamilies. Quennedey and Deligne (1975) have described the frontal weapon, consisting of the frontal gland and associated cephalic structures used in defense, in this family. The highly developed soldier frontal glands which contain defensive secretions are described below.

Coptotermitinae. This primitive subfamily has only a single genus, *Coptotermes*, which includes several widely distributed pest species in tropical and subtropical areas. Large (250 μm) frontal pores were observed in four species examined by Quennedey and Deligne (1975). Soldiers of the Australian *Coptotermes lacteus* secrete a milklike fluid from massive abdominal reservoirs (Moore, 1968, 1969). Although it dries quickly to form a colorless, resilient film on the attacker, it can be reconstituted by addition

of water, thus ruling out a chemical hardening process as seen above in *Mastotermes*. Chemical analysis of the secretion shows it to be a heterogeneous suspension of saturated *n*-alkanes (C_{22}–C_{27}) in an aqueous solution of mucopolysaccharides based on glucosamine and glucose units.

Heterotermitinae. The head morphology of *Heterotermes convexinotatus convexinotatus* (Quennedey and Deligne, 1975) is indicative of the presence of a defense secretion, but its chemistry is unknown. *Reticulitermes* spp. also exhibit a differentiated frontal gland which opens through a 10-μm-diameter frontal pore into a depression with small channels leading to the labrum (Quennedey, 1975b; Quennedey and Deligne, 1975).

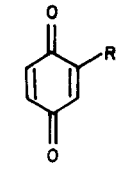
Psammotermitinae and Termitogetoninae. Major and minor soldiers of *Psammotermes* both possess frontal glands and sizable fontanelles; however, no secretions have been chemically identified (Quennedey and Deligne, 1975). Only two species of the unusual flat-headed *Termitogeton* are known (Deligne and Quennedey, 1977). A secretion is exuded from a minute (3-μm) hole in a depression in the center of the head, and it interferes with SEM examinations of surrounding hairs unless removed with solvent. No chemical data are available.

Rhinotermitinae and Prorhinotermitinae. Quennedey and Deligne (1975) recently separated these two subfamilies which contain the most highly modified soldiers of the rhinotermitids. Minor soldiers of several genera are characterized by a reduction of the mandibles and an elongation of the labrum to enable dispersal of volatile repellent and toxic fluids from the frontal gland (Scheme I).

Soldiers of the primitive species *Prorhinotermes simplex*, found exclusively in the New World tropics, possess a large (50-μm) frontal pore but lack the brushlike labrum of their more advanced cousins (see below). Nonetheless, a unique nitroolefin, *trans*-1-nitro-1-pentadecene (3) was discovered to be the major component of the monophasic secretion (Vrkoč and Ubik, 1974). The toxicity of this secretion was demonstrated in topical applications of acetone solutions to houseflies (LD_{50} = 13 μg/fly), and was found to be equivalent in potency to several monoterpene hydrocarbons (Hrdý et al., 1977).

Parrhinotermes aequalis soldiers exhibit a small labral brush and a 20-μm frontal pore. No chemical secretion has been isolated yet. The soldiers of *Dolichorhinotermes*, *Rhinotermes* (see also Sannasi, 1969), and *Schedorhinotermes* are dimorphic, with the minor soldiers exhibiting reduced mandibles, enlarged labrums, and well-developed frontal gland–fontanelle systems. *Acorhinotermes* (Figure 1) possesses a monomorphic "nasutoid" soldier believed to be the result of regressive evolution by loss of the major soldier of a dimorphic pair.

Chemical, morphological, and histochemical analyses are reported for two species of *Schedorhinotermes* (see also Figure 1). Both major and minor



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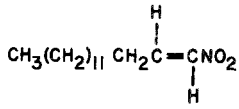

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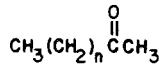
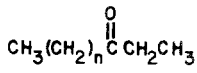
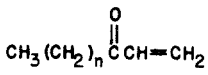


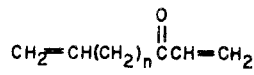
4a, n = 8
b, n = 10
c, n = 12
d, n = 14



5a, n = 8
b, n = 10
c, n = 12



6a, n = 8
b, n = 10
c, n = 12



7a, n = 7
b, n = 9
c, n = 11

SCHEME I. Defense substances of lower termites.

soldiers of *S. lamanianus* were found to possess massive frontal gland reservoirs—invaginations of the cephalic epidermis—containing 1000 μg and 200 μg, respectively, of a mixture of twelve-, fourteen-, and sixteen-carbon 3-alkanones (5), 1-alken-3-ones (6), and α, ω-alkadien-3-ones (7) (Prestwich, 1975; Prestwich et al., 1975). The major component in both secretions (>70%) was the C_{14} enone 6b; traces of 2-tridecanone (4b) were found in minor soldiers only. In earlier work, Quennedey et al. (1973) showed that *S. putorius* soldiers produced three major compounds: enone 6b (75%), enone 6c (15%), and 2-tridecanone (4b) (8%). The behavior associated with this chemical weapon was studied in detail by Quennedey (1975c). Release of the secretion is controlled by the use of mechano- and chemoreceptor hairs ("flow monitors") in the groove and on the daubing-brush tip of the labrum of the combative termite soldier.

Quennedey et al. (1973) found that topical application of the *Schedorhinotermes* soldier frontal gland secretions or synthetic 6b to ants (*Lasius*, *Myrmica*, *Leptothorax*), or exposure of these ants to the vapors of these vinyl ketones, led to toxic effects in ants thus treated (loss of coordination, immobilization, and death). The fact that no wound was necessary led us to speculate that the long aliphatic side chain of the enone facilitated pene-

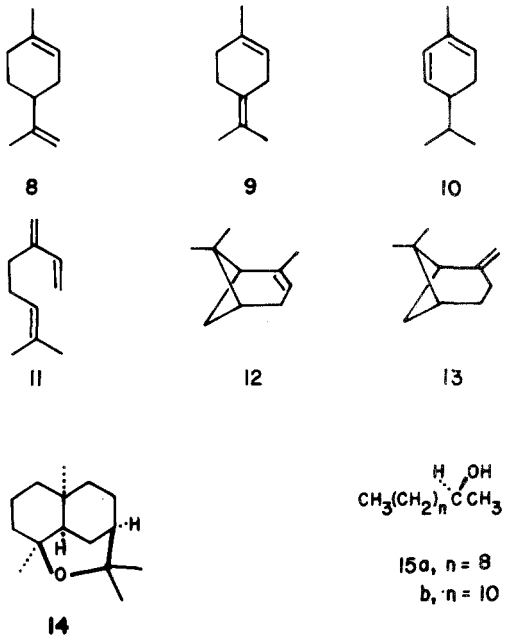
tration through the lipophilic ant cuticle. Subsequently, a Michael-type conjugate addition of a biological sulfhydryl group to the vinyl ketone moiety could result in the observed toxicity (Prestwich et al., 1975). Metabolic poisons of this type are common in cytotoxic natural products used in cancer chemotherapy (Fujita and Nagao, 1977). We are attempting to demonstrate this mode of action in toxicity studies with ants. It is interesting to note that in preliminary tests with mosquito larvae, dienone 7b was tenfold more effective (LD_{100} 25 $\mu g/ml$) than the enone 6b (Prestwich, unpublished). Soldier and worker termites of this genus are relatively unaffected by these toxic vapors, suggesting a rapid detoxification mechanism. In addition, it has been reported that the soldier secretion may inhibit soldier formation and thus help maintain the correct worker/soldier caste ratio (Renoux, 1976).

HIGHER TERMITES

The family Termitidae, the higher termites, is the largest family and contains four evolutionary advanced subfamilies: Amitermitinae, Termitinae, Macrotermitinae, and Nasutitermitinae.[1] The chemical weapons are as distinctive as the "nightmarish creatures" which produce and discharge them.

Amitermitinae. The secretions used in defense and alarm by these termites are varied in structure and function (Scheme II). Moore (1968, 1969) found limonene (8) with smaller amounts of terpinolene (9) and α-phellandrene (10) in the alarm secretion of *Drepanotermes rubriceps*. Although the monoterpene hydrocarbons are themselves toxicants (Howse, 1975; Hrdý et al., 1977) and irritants (Eisner et al., 1976), their major role in this termite is the production of a short-lived snapping frenzy which constitutes alarm behavior. The secretion of *Amitermes herbertensis* is >98% terpinolene (9) with traces of 10, and also appears only in small quantities, thereby suggesting its role only as an alarm signal. Similarly, the Australian termite *A. laurensis* has very few soldiers, and those few that can be found produce a mixture of 8, 10, and 12 in small quantities, suggesting little behavioral significance. However, another Australian termite, *A. vitiosus*, produces a sticky mixture of unknown resinous components in C_{10} solvents (10, 8, 12, 9, 11, and 13 in order of abundance) which serves both defensive and alarm roles (Moore, 1968).

[1]The question of a new classification of the Termitidae by Sands (1972) was raised by one reviewer. In this proposed change, the old subfamily Amitermitinae becomes a junior synonym of Termitinae, and a new subfamily, Apicotermitinae, is created to encompass soldierless termites (*Anoplotermes* branch) and soldiers with regressed frontal glands (*Apicotermes* branch). This reclassification then places Termitidae soldiers with developed frontal glands and chemical defenses in three subfamilies—the Termitinae, Macrotermitinae, and Nasutitermitinae—and those without largely in the Apicotermitinae.



SCHEME II. Defense substances of Amitermitinae.

Soldiers of the West African termite *A. evuncifer* have a formicidal secretion containing ⩾90% of a unique tricyclic ether, 4,11-epoxy-*cis*-eudesmane (14) (Wadhams et al., 1973, 1974; Howse, 1975). Minor components include three other unidentified sesquiterpenoids and two monoterpene hydrocarbons. The structure of the major component was determined by microgram-scale reactions, reaction GC, proton NMR, and GC-MS. The absolute configuration shown in 14 was unequivocally established by an enantiospecific total synthesis starting from (−)-carvone (Baker et al., 1977). The formicidal activity appears to be selective for the major predatory ant, a large ponerine of the genus *Odontomachus* (Evans et al., 1977).

Despite close morphological and ecological similarities, three sympatric East African *Amitermes* species and one American species show a remarkable intrageneric chemical diversity (Prestwich, 1975; Meinwald et al., 1978). *A. messinae* produces a pleasant floral-scented secretion composed of 90% of the sesquiterpenoid ether 14 and 10% limonene (8). The soldiers of *A. unidentatus* produce a complex and pungent-smelling mixture dominated by methyl ketones 4a–d, with 2-tridecanone (4b) as the major component (60 μg/soldier). Also in this secretion in significant amounts are a pentadecen-2-one, 2-undecanol (15a), 2-tridecanol (15b), a tridecen-2-ol, and the corresponding C_{15} alcohols (Prestwich and Engstrom, unpublished). In con-

trast with the other species, *A. lonnbergianus* possesses no hexane-extractable secretion. Finally, the Arizonan termite *A. wheeleri* produces a single sesquiterpene hydrocarbon in $\geqslant$ 99% purity (Prestwich and Nutting, unpublished).

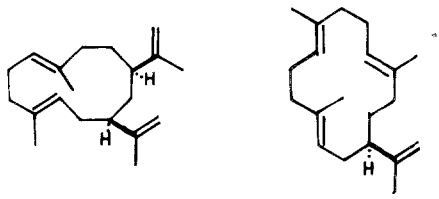
Other members of this subfamily are reputed to be examples of regressive evolution (Noirot, 1969). Thus, soldiers of *Microcerotermes turneri*, *M. exiguus* (Lubin et al., 1977), *Gnathamitermes perplexus* (Prestwich and Nutting, unpublished), and *Eburnitermes* all lack highly developed frontal glands and thus presumably have no defense secretions. Colonies of the West African termite *Anoplotermes* lack soldiers altogether.

At the opposite extreme, *Globitermes sulphureus* soldiers possess voluminous salivary reservoirs which occupy the entire front half of the abdomen and are filled with a vivid yellow liquid. During combat, the convulsive abdominal contractions which expel this secretion through the mouth often cause the entire termite to explode, thus entangling enemies and nestmates alike in a rapidly congealing and darkening liquid of undetermined chemical composition (Noirot, 1969; Wilson, 1971).

Termitinae. Termitine soldiers are noted for highly developed mandibles designed for biting and reaping or for snapping. We have found a variety of novel chemicals originating in the frontal glands of several genera of the reaping soldiers ("*soldats faucheurs*") which may play important roles in the overall effectiveness of this mode of defense.

Cubitermes umbratus is a humivorous termite which builds mounds in the coastal forests of East Africa. Soldiers of this termite produce a frontal gland secretion which is composed of four major diterpene hydrocarbons (Meinwald et al., 1978). Cubitene (16), which constituted 18% of the crude secretion, was isolated by preparative GLC and was characterized by proton and carbon NMR, IR, MS, and single-crystal X-ray diffraction experiments (Prestwich et al., 1978). This novel diterpene possesses an irregular isoprenoid skeleton and a hitherto unknown 12-membered carbocyclic ring.

A pair of isomers with a cembrenoid 14-membered ring constituted 8% and 57% of the secretion (Wiemer and Prestwich, unpublished). Comparison with an authentic sample confirmed the identity of the major isomer as neocembrene-A (17), although its absolute configuration is still undetermined. Patil et al. (1973) previously identified R-(−)-neocembrene-A as a constituent of the shrub *Commiphora mukul*, and Birch et al. (1972) found 17 to be a trail-following pheromone secreted in submicrogram quantities by the sternal glands of workers of the Australian termite *Nasutitermes exitiosus*. A total synthesis by Kodama et al. (1975) has since confirmed the E configurations of the three double bonds. Structure determinations for the minor cembrenoid compound (8%) and the fourth *C. umbratus* component (17%), a bicyclic diterpene, are now in progress.



16 17

SCHEME III. Two defense substances of Cubitermes soldiers (Termitinae). The absolute configurations of 16 and 17 are as yet unknown.

These four diterpenes and several others are widespread among other *Cubitermes* species from East and West Africa (Quennedey, 1973; Prestwich, unpublished). Four other East African species—*C. muneris*, *C. ugandensis*, *C. tenuiceps*, and an undescribed *Cubitermes* species from Kajiado—possess diterpene hydrocarbons in varying distributions. One of these, a bicyclic material with a conjugated diene chromophore [λ_{max}^{hexane} 247 nm (18,200)] constitutes over 50% of the *C. ugandensis* secretion. Its structure is currently under investigation. Finally, a different termitine, *Crenetermes mixtus*, was found living in the "basement" of a *C. umbratus* mound. Soldiers of this species also possess a diterpene hydrocarbon secretion dominated by 16, 17 (Scheme III), and the unidentified UV-absorbing bicyclic compound (Prestwich, unpublished).

The smaller humivore *Noditermes wasambaricus* produces a neat mixture of two sesquiterpene hydrocarbons in a 1:3 ratio (Meinwald et al., 1978). These bicyclic dienes appear to have the same carbon skeleton, and further characterization is in progress (Prestwich and Spanton, unpublished). Finally, some termitine soldiers lack developed frontal glands, e.g., the "snappers" *Termes* and *Pericapritermes* (see Figure 1) (Moore, 1968; Prestwich, unpublished). Others have frontal gland secretions which are as yet chemically uncharacterized, e.g., *Proboscitermes* (Deligne and Quennedey, 1977) and *Thoracotermes* (Howse, 1975).

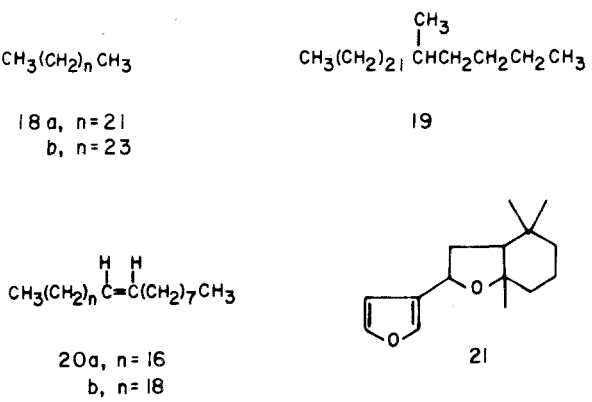
Macrotermitinae

> The chitin armored head has been deliriously, portentiously developed. . . . The whole insect is practically one buckler of horn, with a pair of lobster-like pincer-clippers worked by powerful muscles . . . [Maeterlinck, 1939].

In the fungus-growing termites, secretions from the soldier salivary glands are used most frequently in defense (Noirot, 1969; Quennedey, 1975b); development of the frontal gland is the exception rather than the rule. Soldiers of the Oriental termite *Macrotermes carbonarius* stab sickle-shaped mandibles into the skin of the enemy; rhythmic contractions of the

abdomen pump an aqueous solution of benzoquinone (1) and toluquinone (2) from massive labial glands into the wound (Maschwitz et al., 1972). Five other species of the Oriental macrotermitine genera *Microtermes*, *Macrotermes*, *Odontotermes*, and *Hypotermes* also produce nonproteinaceous quinone-containing secretions. In two species, *Macrotermes gilvus* and *Odontotermes horni*, however, quinones were conspicuously absent (Maschwitz and Tho, 1974). Wood et al. (1975) reported an aqueous mixture of benzoquinone and protein as the defensive secretion of the East African pests *Odontotermes badius* and *O. stericorivorus*. The aggressive soldiers of the West African mound-building termite *Macrotermes bellicosus* are reported to produce a solution of toluquinone in an unidentified volatile "solvent" (Howse, 1975). Cmelik (1971) found unusually large frontal glands in the major soldiers of *Macrotermes goliath*, which contained copious amounts of normal and isoalkanes (C_{22}–C_{34}) in addition to free fatty acids, sterols, and phospholipids.

Recently, Prestwich et al. (1977) reported that the soldiers of *Macrotermes subhyalinus* (closed mound type[2]) release a frontal gland secretion composed of long-chain saturated and monounsaturated hydrocarbons into wounds inflicted by their powerful mandibles (Scheme IV). Chemical analysis of the secretion showed the paraffin fraction to consist primarily of *n*-tricosane (18a), *n*-pentacosane (18b), 3- and 5-methylpentacosanes, and 5-methylheptacosane (19). The major olefins were identified as (*Z*)-9-heptacosene (20a) and (*Z*)-9-nonacosene (20b). Major soldiers possess a hypertrophied frontal gland reservoir containing over 2.8 mg (>8% of dry



SCHEME IV. Defense substances of Macrotermitinae.

[2]Sands has proposed that the closed-mound type should be called *Macrotermes* sp. near *subhyalinus* and may be synonymous with *M. michaelseni*. The open-chimney type is believed to be *Macrotermes subhyalinus* (sensu stricto).

weight) of secretion, a factor of 500 more than that of the minor soldiers. Termite-eating ponerine ants (*Megaponera foetens*) which had been wounded and treated with this secretion suffered greater mortality and morbidity over a 24- to 36-hr period than did ants that were only chemically treated or only wounded. This was tentatively attributed to an uncontrolled loss of hemolymph ("bleeding") from the unhealed wounds. We have since discovered that a partially sympatric *Macrotermes* species which builds multiple open-chimney mounds[2] possesses a copious hydrocarbon secretion consisting of C_{27} to C_{35} alkanes and alkenes (Prestwich and Bruinsma, unpublished). In fact, the difference in the gas chromatograms of the defensive secretions of these two species enable us to distinguish them without examination of the soldier or mound morphology.

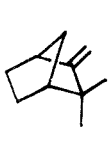
Finally, an unusual furanosesquiterpenoid structure 21 has been proposed for the most abundant component (3 μg/soldier) of the labial gland secretion of major soldiers of the West African termite *Ancistrotermes cavithorax* (Evans et al., 1977). This compound, along with several other unidentified mono- and sesquiterpenes, appears to effectively deter predation by the obligate termitophagic ant, *Megaponera foetens*.

Nasutitermitinae

> They have no mandibles, and where their head should be is a huge, weird apparatus, as heavy as the rest of their body and exactly similar to the injection bulbs sold by chemists . . . [They are] a kind of perambulating artillery . . . [Maeterlinck, 1939].

Soldiers of the highly-evolved nasute termites eject a viscous gluey secretion from a nozzle-like frontal gland contained in an elongated rostrum (the "nasus"). Until recently, only the pungent-smelling monoterpene hydrocarbons were chemically identified (Moore, 1964, 1968, 1969) and their roles as alarm and defense compounds were poorly understood (Ernst, 1959; Maschwitz, 1966; Blum, 1969). Since 1970, considerable progress has been made in the elucidation of the structures of the individual diterpenoid "resins," the overall chemical composition of the secretions, the occurrence of chemically discrete geographic races, and the use of the secretions in alarm and in defense (Scheme V).

Our initial work was carried out using two species of grass-feeding nasutes from East Africa—*Trinervitermes bettonianus* and *T. gratiosus*. We first identified several well-known monoterpenes (8, 11, 12, 13, 22) as the solvents for the defensive glue produced by both major and minor soldiers of these species (Prestwich, 1975; Meinwald et al., 1978). In addition, we found two monoterpene alcohols (as yet unidentified) in different ratios in major and minor soldiers of *T. gratiosus* (Prestwich, 1977). These were replaced in *T. bettonianus* by a nonisoprenoid compound identified as 3-ethyl-2-octanol (26) by GC-MS comparison with synthetic material (Gebreysesus, Chong, and Meinwald, unpublished). Apparently, these al-



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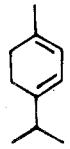

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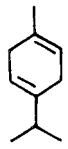

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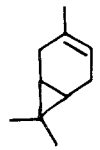

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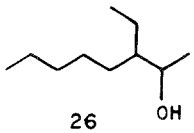

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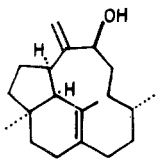

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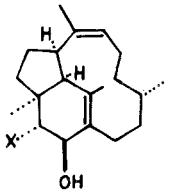
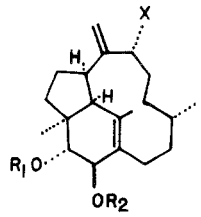
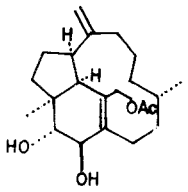


28a, X = H
b, X = OH



29a, X = R1 = R2 = H
b, X=OAc, R1 =R2 = H
c, X=OAc, R1 =R2 = Ac
d, X=OH, R1 =R2=Ac
e, X = H, R1 or R2=Ac



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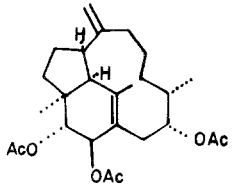

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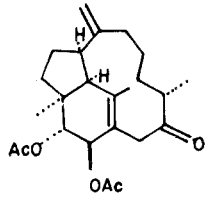

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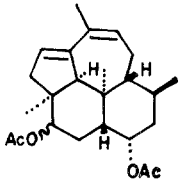

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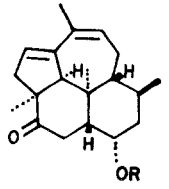


34a, R = H
b, R = Ac

SCHEME V. Defense substances of Nasutitermitinae.

cohols act as cosolvents for the more polar diterpenes; no biological role has yet been determined.

Dissolved in these C_{10} compounds was a series of previously unknown crystalline diterpenes. Single-crystal X-ray diffraction experiments with compound 29b established the new cembrene-related trinervitene skeleton (Prestwich et al., 1976a). The absolute stereochemistry was determined by observing a negative chirality in the circular dichroism spectrum of the $Pr(dpm)_3$–diol complex. A combination of microspectrometric methods then allowed the elucidation of structures 27–30 as further members of this new class of unusual dome-shaped molecules (Prestwich et al., 1976b).

An attempt was made to determine the variation of the *Trinervitermes* soldier frontal gland secretions (1) within a given mound, (2) between mounds in the same population (sympatric), (3) between mounds in geographically isolated populations (allopatric), and (4) between different species of the same genus. In the first two instances, major and minor soldier secretions were substantially different from each other but were essentially invariant within one mound or one sympatric population. Allopatric populations of *T. gratiosus* and of *T. bettonianus*, however, could be readily distinguished on the basis of the chromatographic profiles of their soldier secretions (Prestwich, 1978b, and unpublished). For example, one population of *T. gratiosus* lacked the "characteristic" monoterpene alcohols in the other two populations, lacked the major constituent 29d in the minor soldier secretion, and had a new major component, isotrinervi-2β-ol (28a), in the major soldier secretion. Interspecific variation was examined for *Trinervitermes* species from East Africa [*T. bettonianus*, *T. gratiosus*, *T. rapulum* (*dispar?*)] and from West Africa (*T. geminatus*, *T. occidentalis*, *T. oeconomus*, *T. togoensis*, *T. trinervius*) (Prestwich and Kaib, unpublished). All possessed C_{10} hydrocarbons and trinervitenes in varying ratios, often with a greater difference between major and minor soldiers of the same species than among major soldiers of different species.

An arboreal East African nasute, *Nasutitermes kempae*, had trinervi-2β,3α,9α-triol-9-*O*-acetate (29b) as a primary component of its frontal gland secretion, but in addition afforded two members of still another new diterpene type (Prestwich et al., 1977b). Kempene-1 (33) and kempene-2 (34b) have novel tetracyclic structures suggestive of a kinship with the trinervitenes via an additional cyclization of a cembrene-derived intermediate (see below). The absolute configuration, as evidenced by the positive helicity of the diene chromophore, is the same as that of the trinervitenes (except for an inversion at C-12). The relatively low molar absorptivity ($\epsilon = 6500$) of the diene is attributed to the twisted geometry (about 20° out of planarity) conferred by the rigid tetracyclic array of 5-, 6-, and 7-membered rings (Figure 2). Reduction of the ketone followed by acetylation gives a pair of epimeric acetates 33, which both exhibit a pronounced hypochromicity ($\epsilon = 85$) re-

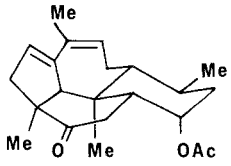


FIG. 2. Stereostructure of kempene-2 (34b) based on X-ray diffraction data.

sulting from a loss of planarity caused by the rehybridization of C-3 from sp^2 to sp^3.

Interspecific variation among *Nasutitermes* soldiers is quite remarkable. Although the distribution of the arboreal nasute *N. infuscatus* overlaps with that of *N. kempae*, no kempenes are detectable in this species. Instead, a trinervitene distribution reminiscent of *T. gratiosus* major soldiers is observed (Prestwich, unpublished). Similarly, *N. ephratae* from Panama has only trinervitenes (27, 29, and an unidentified isomer 29), while *N. luzonicus* from the Phillippines has almost exclusively kempenes (33, 34a,b) (Prestwich, unpublished). The Cuban species *N. ripperti* and *N. costalis* were found to have traces of kempenes amidst two new C-13-oxygenated trinervitenes (31, 32) and several known trinervitenes (27, 29a–c,e) and a complex array of C_{10} hydrocarbons (8–13, 22–25) (Vrkoč et al., 1973, 1977, 1978a).

Current interest has also focused on behavioral aspects of the nasute secretions. Alarm has been conclusively demonstrated in *Hospitalitermes monoceros* by Maschwitz and Muehlenberg (1972), in *Tenuirostritermes tenuirostris* by Nutting et al. (1974), in *Nasutitermes exitiosus* by Eisner et al. (1976), and in *N. ripperti* and *N. costalis* by Hrdý et al. (1977), and Vrkoč et al. (1978b). In the latter two species, analysis of the volatiles produced in response to chemical challenges confirm species-specific responses. The major C_{10} component of *N. ripperti*, α-pinene (12), elicits the strongest response from soldiers of this species. Similarly, 3-carene (25), the major C_{10} constituent of *N. costalis* soldiers evokes a maximal emission of volatiles and a high level of alarm activity in these soldiers (Vrkoč et al., 1978b).

Alarm in *N. exitiosus* was manifested by recruitment of small soldiers to a freshly squirted adversary (Eisner et al., 1976). Firing of the secretion did not occur unless the soldier was contacted directly. In an interesting behavioral dimorphism, large soldiers of this termite do not participate actively in defense. Instead, they retreat to the central nest in response to contact or to alarm scent, perhaps serving as "alarm messengers" for the rest of the colony. The nonusage of their frontal gland secretion, however, is not well understood (McMahan, 1974; Kriston et al., 1977).

Early experimental data supporting the increased efficacy of nasute chemical secretions relative to biting with the mandibles has been reported by Emerson (1961) for the primitive mandibulate nasute soldiers of the

genera *Cornitermes*, *Rhynchotermes*, and *Armitermes*. More recently the defensive roles of the individual chemicals in the nasute secretion have now been analyzed in detail by several workers. As a whole, the spray is (1) a viscous entangling agent; (2) an irritant, thus promoting its spread by the sprayed attacker; (3) a topical poison; and (4) a physical blocking agent for sensillae and spiracles of the attacker (Eisner et al., 1976). In Eisner's study, α-pinene was found to be a potent irritant in tests with cockroaches and houseflies. Howse (1975) and Hrdý et al. (1977) found α-pinene to be a topical poison for ants (*Formica rufa*, $LD_{50} = 1\ \mu g$) and flies (*Musca domestica*, $LD_{50} = 5\ \mu g$). The diterpenes substantially retard the evaporation of monoterpenes, thus prolonging the effective stickiness and irritancy of the secretion (Eisner, Aneshansley, Brattsten, and Prestwich, unpublished). These secretions are analogous to pine resin, in which diterpenes with one or two polar groups are dissolved in monoterpene hydrocarbons. It would appear that nature's plant glues are reiterated with variations in the animal kingdom. Hrdý et al. (1977) reported that the trinervitenes also appear to be genuinely toxic ($LD_{50} = 22\ \mu g$ for topical application of an acetone solution of 31 to houseflies); we feel, however, it is unlikely that this is their primary role.

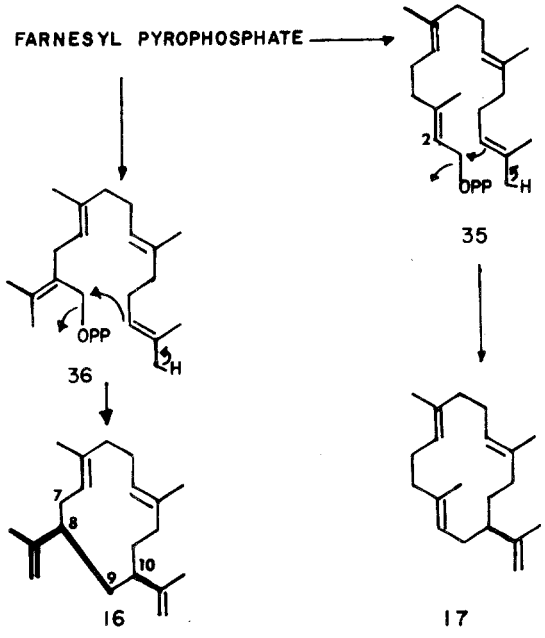
BIOSYNTHESIS

Although many of the details of terpene biosynthesis in plants are well understood, relatively little is known about these processes in insects. In some cases, insects are known to appropriate their defense substances from plants by sequestration (Eisner, 1970). For example, the resinous defense secretion of larvae of the pine sawfly *Neodiprion sertifer* consists of a complex of pine resin acids dissolved in α- and β-pinenes (Eisner et al., 1974). The majority of insect defense substances thus far studied are, however, bona fide secretions of specialized glandular structures (Noirot and Quennedey, 1974; Quennedey, 1975b), and their de novo biosynthesis by the insect must be considered.

Termites are unique among insects which produce defense substances. Not all individuals of the species possess the glandular apparatus for making the secretion; this is a common result of sociality in insects. More important, the morphological adaptation of the soldier's head as a weapon makes it impossible for the soldier to feed itself. All nourishment must be obtained by either oral (stomatodeal) or anal (proctodeal) trophallactic exchange from workers. In other words, predigested raw materials are provided in liquid form to the soldiers which then manufacture the chemical weapons. Although no incorporation data are available yet for termites, the case against sequestration is substantial: (1) the frontal gland is not directly connected to the digestive system to allow facile selective filtration of the defense

substances from the food (Noirot, 1969); (2) the gland tissues contain secretory cells, in which lipid droplets may be observed (Noirot and Quennedey, 1974; Quennedey, 1975b); (3) worker termites of all species we have examined lack extractable mono- or diterpenes; (4) at least two species of *Nasutitermes* do not attack woods rich in monoterpenoid hydrocarbons (Hrdý and Zelený, 1967), while other nasutes (*Trinervitermes* spp. and *N. triodae*) feed on harvested dry grass; and (5) monoterpene and diterpene contents are species-specific and independent of food source (Vrkoč et al., 1973; Prestwich, 1978b). Nonetheless, the demonstration that termite soldiers are capable of performing diterpene biosynthesis will be a topic for further research.

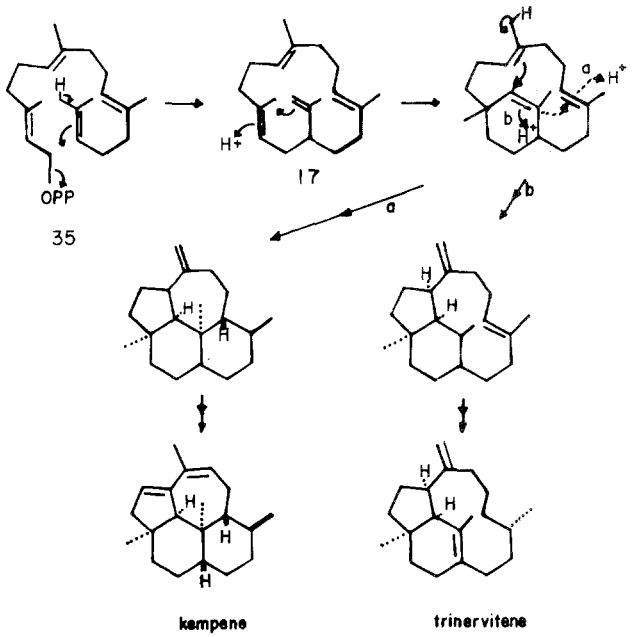
Despite the lack of hard evidence, it is instructive to consider potential biosynthetic interrelationships for the mono-, sesqui-, and diterpenes found in numerous species of higher termites. In fact, on the basis of our current knowledge of termite chemical defense substances, the higher and lower termites can be separated by the ability or inability of at least one member of the subfamily to biosynthesize terpenes. A general trend is beginning to appear in which subfamilies may be characterized by their mechanical and chemical defenses. Indeed, a chemosystematic approach to termite phylogeny should provide fascinating information about the evolution of chemical defense in termites.



SCHEME VI. Possible biosynthetic relationships for two *Cubitermes* diterpenes.

Of the two known diterpenes from *Cubitermes*, neocembrene-A (17) would be expected to arise from cyclization of geranylgeranyl pyrophosphate (35) (Scheme VI). Cubitene (16), however, contains one isoprene unit which is joined irregularly (bold) to the other three. Thus, coupling of farnesyl pyrophosphate with dimethallyl pyrophosphate could provide a precursor which could suffer proton loss to give tetraene 36. Solvolytic cyclization followed by double-bond isomerization could lead to cubitene. Alternatively, we proposed a second route to (16) which involves the scission of 17 followed by recyclization and deoxygenation (Prestwich et al., 1978).

It is also reasonable to postulate cembrene-like structures as intermediates in the biogenesis of the trinervitenes and kempenes (Scheme VII). Thus, either two or three transannular cyclizations may occur to generate these two skeletal types, respectively. Subsequent stereospecific hydroxylation of the former at C-2, C-3, C-9, C-13, or C-17 would generate the known trinervitene congeners, while oxidation at C-3 and C-14 with concomitant dehydrogenation at C-7/C-8 of the latter would afford the isolated kempene derivatives. Experiments to test these hypotheses will involve incorporation studies using live termites or crude enzyme preparations from frontal glands and isotopically labeled precursors.



SCHEME VII. Potential biogenetic routes to trinervitenes and kempenes.

SUMMARY

Its own weapons have not been borrowed, like ours, from the external world; it has done better. . . . It has created those weapons out of its own body. . . . And indeed there can be no doubt that in this case, and in certain others, the termite knows more than we do [Maeterlinck, 1939].

Termite soldiers have provided a rich harvest of new natural products with unique structural features. The morphological variety of their armaments and the chemical variety of their frontal and salivary gland secretions reflect a wide range of defensive behaviors and evolutionary trends. The specific functions of these chemicals, individually and in concert, in alarm behavior, control of caste ratios, and protection of the colony from predators, molds, diseases, parasites, and competitors (Bouillon, 1970), as well as the biosynthetic origins of these compounds are topics currently under investigation. We hope that, with a more complete knowledge of the chemical entities involved, results with regard to the biology of these secretions and their phylogenetic and chemosystematic implications will be forthcoming.

Acknowledgments—It is a pleasure to acknowledge my collaborators and mentors at ICIPE, Cornell University, Columbia University, Iowa State University, and State University of New York at Stony Brook, whose names appear in the references. In particular, I thank Professor Jerrold Meinwald and Dr. David Wiemer for their critical reading of an earlier draft of this manuscript. This work was begun with financial support by the ICIPE and an NIH postdoctoral fellowship (AI 05076). Awards by the Department of Chemistry, the Biomedical Research Support Grant Committee, and the University Awards Committee/Joint Research Council of the State University of New York at Stony Brook have assisted in its continuation.

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ANNOUNCEMENT

CURT P. RICHTER PRIZE IN PSYCHONEUROENDOCRINOLOGY

Through the generosity of the Irish Foundation for Human Development, an annual prize has been established for meritorious research in the area of psychoneuroendocrinology. The aim of this essay prize is to encourage younger scientists to contribute to this interdisciplinary field, and the sum of $1,000 will be awarded annually for the best manuscript or essay submitted by a scientist or physician under 35 years of age. The winning paper will be published in the journal, *Psychoneuroendocrinology*.

Submissions should be made in writing to Dr. Fleur L. Strand, Secretary, International Society of Psychoneuroendocrinology, New York University, Washington Square, New York, New York 10003. All submissions will be screened by a broad committee of established psychoneuroendocrinologists.

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SOCIAL BEHAVIOR AND CHEMICAL COMMUNICATION IN REINDEER (*Rangifer t. tarandus* L.)

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Abstract—The social interactions of captive reindeer were observed for one year. The seasonal changes of agonistic behavior, the social rank order, social affinity during rest, social sniffing and licking, solitary sniffing and licking, hindleg-head contact, and other motor patterns involving head and antlers are described. Social sniffing and licking correlated more with sex than with social rank. Olfactory responses during encounters and tracking were investigated experimentally. Chemical communication is particularly important in sexual and maternal behavior and during encounters.

Key words—*Cervidae*, dominance order, licking, olfactory communication, pheromones, *Rangifer tarandus tarandus*, reindeer, scent glands, sniffing, social behavior, tracking response.

INTRODUCTION

This paper describes the role of chemical communication in the behavior of reindeer (*Rangifer tarandus* L.). The rutting and maternal behaviors and social rank order of reindeer have been investigated by Espmark (1964a,b, 1971a,b, 1975). The behavior of American caribou has been studied by Pruitt (1960), Lent (1965, 1966), Kelsall (1968), and Bergerud (1961, 1975). While vocal communication in reindeer has received attention (Espmark, 1971b, 1975), we lack information on chemical communication. Urine and skin glands are the possible sources of chemical stimuli. Reindeer possess antorbital, tarsal, and interdigital glands (Schaffer, 1940; Quay, 1955). Recently a caudal gland and its role in social behavior has been described

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(Müller-Schwarze et al., 1977) for European reindeer. This gland has been found earlier in study skins of American caribou (*Rangifer tarandus caribou*) by Lewin and Stelfox (1967). The velvet of the growing antler contains sebaceous and apocrine glands (Vacek, 1955; Lojda, 1956). There are also scattered sudoriferous glands in the oral angle, foreleg pit, groin, rump patch, and on the legs and geet (Källquist and Mossing, 1977). The chemical composition of interdigital gland secretion was described by Sokolov et al. (1974) and by Andersson et al. (1975).

Responses of reindeer to interdigital secretion were described by Müller-Schwarze et al. (1978), and short aliphatic acids in interdigital secretion and their discrimination by reindeer were investigated by Brundin et al. (1978).

METHODS AND MATERIALS

Nine captive forest reindeer (*Rangifer t. tarandus* L.) (4 males, 4 females, and one calf) ranging in age from nine months (at the end of the study) to four years were kept in one of two 50- × 100-m enclosures. The males were at the start of this study 3 (δ_A and δ_G), 2 (δ_L), and 1 (δ_M) years old; the females 4 (φ_C), 3 (φ_B), 2 (φ_H), and 1 (φ_K) years. Female C had a calf on June 2, 1975.

The annual cycle of social interactions was studied from May 1975 to March 1976. Each reindeer was observed for 10 periods of 15 min each during each of the months of May, July, September, November, and March, and for 5 such periods during January/February. Since reindeer show a polyphasic circadian activity rhythm, they were observed whenever they were active and when daylight and weather permitted observation. Observation started when the focal animal was active. The 15-min period was completed if the animal reclined after more than half of that time had passed (i.e., at least 8 min), but discontinued (and completed later) if it reclined before the half-time point. Social interactions and concomitant sniffing or licking responses were recorded. Spatial relationships were recorded when the group had bedded down. Thus sex, age, rank relations, and special social bonds could be correlated with chemical communication. Other uses of skin glands, such as solitary sniffing and licking, and hindleg-head contact, were also recorded.

Social sniffing and licking during encounters between previously acquainted individuals were determined by separating one reindeer at about 15:00 hr, keeping it isolated for 66 hr and then reintroducing it to the group at about 09:00 hr on the fourth day. The interactions were observed for the first 30 min after reintroduction. Each animal was reintroduced only once.

For the tracking experiment, a new pen was built in an area where reindeer had never been before. The pen was 10.8 × 9.9 m, and contained hard soil with and without vegetation. A reindeer was let into the pen for 5 min and then lured out again with lichens. Its path through the pen was recorded by two observers on a prepared map of the pen. Within 10 min, another reindeer

was allowed to enter the pen, and its movements through the pen were recorded. Of the four tracking trials a reindeer (a male and a female) was used twice, and a female German shepherd dog twice. Each tracking animal was exposed to a male and a female track.

Reindeer in the wild and in zoos were observed on several occasions.

Sniffing and licking of conspecifics or their excrements on tracks were taken as evidence of chemical communication, i.e., transfer of social information via the chemical senses.

RESULTS

Observations of Interactions in Established Group

The systematic observations over one year resulted in quantitative data on the annual cycles of the following behaviors: (1) the kinds and frequencies of patterns of agonistic interactions between individuals of both sexes, which lead to and maintain social rank relationships; social dominance; (2) spatial associations of individuals in resting groups, indicative of social compatibility (mutual tolerance); (3) olfactory and gustatory social responses, which could be correlated with the specific relations between individuals; (4) intraindividual sniffing and licking responses to scent glands and other parts of their own bodies; (5) hindleg-head contact (HHC), a behavior in which the interdigital glands of the hind feet are brought into contact with the forehead and antlers, its function not known (the Lapps assume that this behavior aids in the growth of the antlers); and finally, (6) head rubbing, antler thrashing, and head turning, behavioral patterns which bring head and/or antlers into contact with the vegetation or ground.

Agonistic Behavior and Social Dominance. Encounters resulting in increased interindividual distance (or "spacing") are here referred to as agonistic behavior. The first 12 most common motor patterns employed by reindeer in agonistic encounters—listed in the order of increasing intensity of a specific activity on the part of the "dominator"—are as follows:

1. Keep away: An individual keeps another away from a site, especially the feeding station, by its mere presence. No particular motor pattern is employed. (In the tables of this paper this behavior is included in "yielding".)
2. Supplant: Approach by one animal, followed by withdrawal or veering off by another. When focusing on the latter individual, another term for this type of interaction is "yielding."
3. Head up: Vertical upward thrust of the head.
4. Head turn: Lateral twist of head that is visually amplified by the antlers ("antler dipping"). The antler tips may touch the ground. This movement seems identical with Bubenik's (1975) "head tilt."
5. Head lowering: Head is quickly moved vertically, often as a prelude

to antler-locking (see point 1.). In the tables, this behavior is included under "antler-locking."

6. Pushing: Forward thrust with extended head.

7. Butting: Forward thrust of the forehead, often with body contact.

8. Lip smacking: Both sexes may rapidly open and close their mouths while the head is raised. This occurs when another individual passes closely.

9. Striking: Hitting with one or both raised forelegs.

10. Lunge: Short, quick rush at a conspecific with a sudden stop. It can be viewed as an aborted "chase." (In the tables, this behavior is included under "chase.")

11. Chase: Driving an opponent at a fast trot or gallop over distances ranging from about 5 m to several rounds through the enclosure, totaling several hundred meters.

12. Antler locking: Two facing animals lower their heads, touch each other's antlers, push forward and turn their heads laterally. Although dominance may be assumed for the animal remaining on the site after an antler fight, this behavior pattern was not used in the dominance matrices.

The following three patterns typically occur during the rut:

13. Grunt: Uttering of a short call, performed by males during the rut. It may be repeated, and results in the moving away by another male. The grunt also serves in keeping the females together in the "harem."

14. Tramp urinating: A rutting bull will rhythmically tramp with his hindlegs and urinate simultaneously so that urine soaks the metatarsi and hooves. In captivity, this behavior is directed at both other bulls and people.

15. Pawing: During the rut, the harem bull was observed to paw with his forelegs and sniff the ground when facing people. Pawing may also accompany thrashing a tree with the antlers.

Some agonistic patterns, such as head lowering, lip smacking, grunting, or pawing, are accompanied by opening the antorbital gland pouch. Some motor patterns may have other functions in addition to the agonistic one. Lip smacking, grunt, and some head movements also serve in courtship.

Table 1 includes the frequencies of agonistic patterns during the months when they were more prevalent, July and September. The average number of agonistic encounters per individual during 20 hr of observation ranged from 15.9 in May to 49.6 in January/February and 52.8 in November. Not all of these agonistic patterns, especially those typical for the rut, were frequent enough to be included in these quantitative data.

The 8 animals were active for 18.3 hr of observation in May, 19.5 in July, and 19.4 in September; 9 were active for 22.5 of the 22.5 hr in November and in March; and 9 were active for 11.3 of the 11.3 hr observation in January/February. The number of agonistic encounters per animal and hour of time active are 6.9, 12.8, 17.9, 18.8, 22.3, and 16.7 for the six observation months,

TABLE 1. AGONISTIC PATTERNS IN REINDEER; FREQUENCIES IN BOTH SEXES OF A GROUP OF FOUR MALES AND FOUR FEMALES IN JULY AND SEPTEMBER

	Actor and target										
	♂ → ♂		♂ → ♀		♀ → ♀		♀ → ♂		Total		
	July	Sept.	July	Sept.	July	Sept.	July	Sept.	July	Sept.	Total
Pattern											
Yield	30	44	16	28	19	51	32	7	97	130	227
Head up	8	6	4	7	2	5	6	0	20	18	38
Head turn	7	13	3	0	2	10	7	4	19	27	46
Pushing	5	1	1	1	2	12	13	1	21	15	36
Butting	6	2	0	3	1	19	4	7	11	31	42
Striking	9	2	18	0	14	2	22	4	63	8	71
Chasing	3	10	0	0	2	4	13	5	18	19	37
Antler locking	0	10	0	7	1	0	0	7	1	24	25
Miscellaneous*	0	3	0	8	0	7	0	3	0	21	21
Grunts	0	17	0	44	0	0	0	0	0	61	61
Totals	68	108	42	98	43	110	97	38	250	354	604
Number of combinations	3	3	4	4	3	3	4	4			
Frequency per combination	22.7	36	10.5	24.5	14.3	36.7	24.3	9.5			

starting with May. The highest levels coincided with the rut (September and November) and the antler shedding in January/February, when social ranks were redetermined. The annual cycle of the frequencies of agonistic encounters is shown in Figure 1.

By comparison, the rates of agonistic encounters in a group of 2 males and 2 females with calves in Skansen Zoo, Stockholm, were 6.5 per hour and individual in August, and 0.07 in a free-ranging group of 90 adults, yearlings, and calves of both sexes in August in Lappland. Of the latter group, only subgroups were observed. During spring migration (April) the rate was 2.26 per animal per hour (Müller-Schwarze, in preparation).

Tabulation of the agonistic encounters between all pairs of animals results in their social dominance matrices. Tables 2 and 3 are the dominance matrices for May and September 1975. For each individual animal, the number of other individuals with whom encounters were lost is subtracted

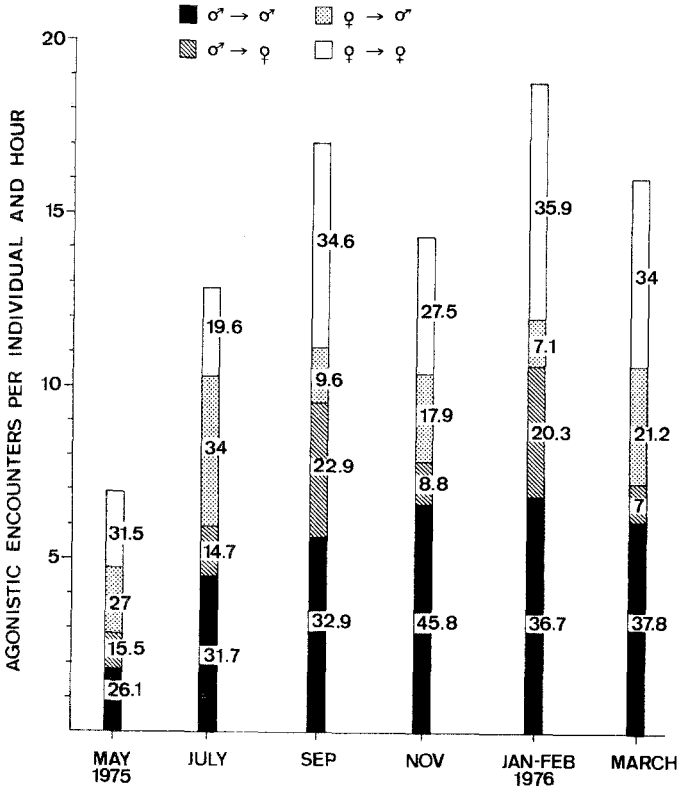

FIG. 1. Annual cycle of agonistic encounters in a group of captive reindeer (4♂♂ and 4♀♀). The figures in the bars are percentages (whole bar is 100% for the four different combinations.

TABLE 2. DOMINANCE MATRIX FOR EIGHT REINDEER IN MAY 1975

	Loser								Numbers of encounters	Rank
	A	B	G	H	C	M	K	L		
Winner										
♂A		3	11	12	1	5	4	11	50	1
♀B	0		6	17	2	1	4	7	47	2
♂G	0	1		7	5	3	5	10	52	3
♀H	0	2	0		13	12	8	13	87	4
♀C	3	1	1	0		5	4	9	44	5
♂M	0	2	0	0	0		0	3	41	6
♀K	0	1	2	1	0	8		0	37	7
♂L	0	0	1	2	0	2	0		58	8
Encounters in 20 hr of observation									127	

from the number of individuals over whom it dominated in encounters, i.e. prevailed at a certain site. Rank 1 was assigned to the highest positive value of this difference. In case of ties, a pair of individuals is ranked on the basis of the relative numbers of wins over each other. If still equal, the higher average rank of subordinate animals is used to assign higher rank. Each dominance matrix contains both sexes in a mixed order, with older animals dominant over younger ones, as was described by Espmark (1964b).

The basic dominance order, with the younger individuals at the bottom, persisted throughout the year, except for the antler-shedding season in February. However, different individuals were at the top during the different months (Figure 2). In May, the 3-year-old δ_A was at the top of the dominance

TABLE 3. DOMINANCE MATRIX FOR EIGHT REINDEER IN SEPTEMBER 1975

	Loser								Numbers of encounters	Rank
	G	C	B	M	A	L	H	K		
Winner										
♂G		18	23	21	15	22	12	14	126	1
♀C	0		31	18	0	0	6	21	113	2
♀B	0	2		8	0	0	7	11	85	3
♂M	0	0	2		0	25	10	5	93	4
♂A	1	0	0	0		20	0	0	37	5
♂L	0	0	1	0	1		1	0	60	6
♀H	0	17	1	3	0	0		6	64	7
♀K	0	0	0	1	0	0	1		70	8
Encounters in 20 hr of observation									324	

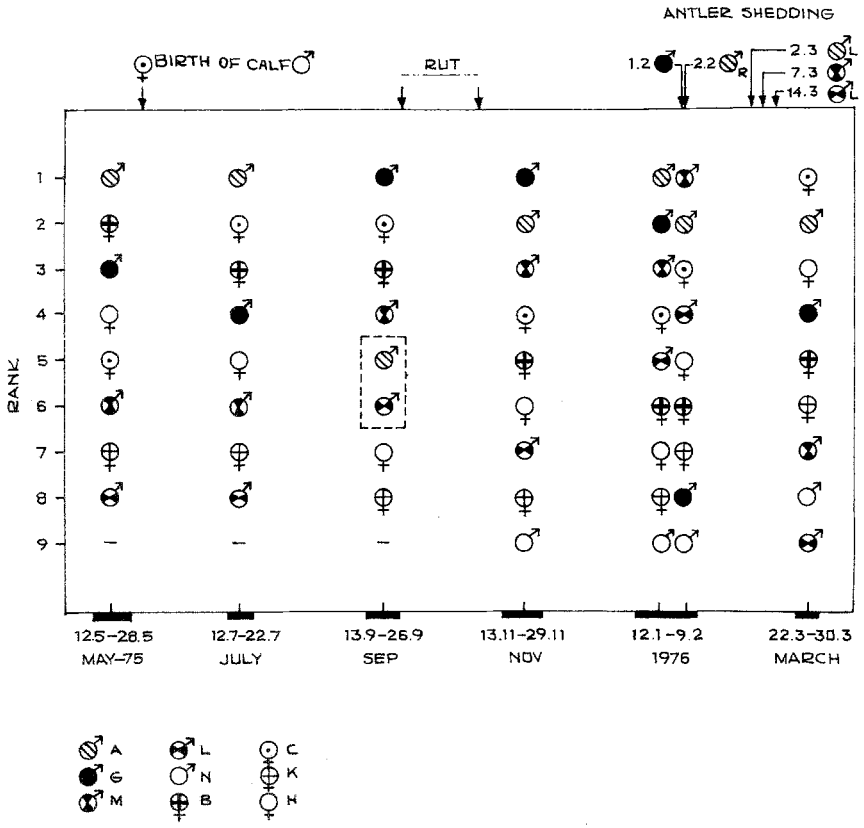

FIG. 2. Annual cycle of social rank order in a group of reindeer. Dates of antler shedding are given at upper right. (R = right, L = left antler); the pair indicated by the broken lines represent the bachelor group.

order. His antlers then were the largest in the group. From May to July, the 4-year-old ♀C moved from 5th to 2nd place in the dominance order. She had given birth to a calf on June 4th and was the only female in the group to have a calf that year. As the dominance diagram (Figure 2) shows, the remainder of the dominance order stayed the same from May to July. More changes in the rank order occurred as the reindeer approached the rut. During the prerut in mid-September the 3-year-old ♂G occupied the top rank and kept the females close to him at all times ("harem" formation). Sixty-five of his agonistic encounters were with the other three males, while 68 involved the four females. Females C and B stayed at their second and third ranks respectively. Male M moved up from seventh to fourth place.

Males A and L deserve special mention. By the percentage of wins, ♂A ranks third. However, all his wins (except one) were over ♂L. Males A and L

stayed together as a "bachelor group," separated from the main cluster formed by δ_G and the four females ("harem") in which the yearling δ_M was the only male tolerated. The group had, in fact, temporarily split into two, with δ_A and δ_L in one and all others in the other group. But the two groups still interacted, especially near and at the single feeding station.

After the rut (late November), δ_A and δ_L rejoined the main group. Male A then occupied 2nd place. Females C and B fell to 4th and 5th place. The young δ_N (calf) was now a full member of the group and ranked lowest. During the observation period in January/February, δ_G dropped his antlers on February 1, and he fell immediately to the lowest rank among adults. Only the calf δ_N ranked lower. Even this young animal could be seen attacking successfully the formerly dominant δ_G. A drop in social rank after antler dropping or experimental removal of antlers in reindeer was also demonstrated by Espmark (1964b), and loss of antlers resulted in a lower social rank in red deer, *Cervus elaphus* (Gossow, 1971). Males L and M advanced in the hierarchy. Male M arrived at top rank. At this time he had the largest antlers. This left δ_A in second place, probably because δ_A broke one of his antlers, which marred his large appearance. At the same time, the numbers of agonistic encounters per individual and hour were the highest of the year. The females also moved upward in the hierarchy, and during the observation period in March they had advanced still more. All the females were at this time pregnant, and the presence of fully grown antlers may account for their high ranks. At this time δ_A and δ_G had new antlers growing, and held 2nd and 4th place, respectively, while δ_M and δ_L, who just had shed their antlers, occupied the bottom ranks. The calf δ_N, who still had his antlers, dominated δ_L.

Social Affinity and Agonistic Behavior. The social relations between group members can, in addition to their agonistic encounters, be measured by the distances maintained between the individuals. While interindividual distances are constantly changing during activities like grazing, the static distances during resting periods can be easily recorded. After the group had reclined, the estimated distances were recorded. Of these, the minimum distances were the most meaningful for social relationships, as they indicated social tolerance. Maximum distances, on the other hand, can have many causes; the animals may not even have seen one another when reclining. Thus, as a measure of mutual tolerance, each recumbent individual's closest neighbor was recorded. When a recumbent reindeer was equally close to several individuals, they all were recorded as closest neighbors.

The frequencies at which pairs of individuals were closest neighbors during rest were subjected to cluster analysis (Sorensen, 1948; Deghett, 1975). First, a quotient of affinity (QA) was computed. The frequency J_{xy} of a pair being closest neighbors was divided by the frequency R_{xy} of their resting at the

same time: $QA = \frac{J_{xy}}{R_{xy}}$. The frequencies of individual x having individual y as closest neighbor and of y having x as closest neighbor were added to obtain the value J_{xy}. The QA values were cast into a matrix. Then a dendrogram (Figures 3-6) was constructed: the highest QA value in the matrix provided the first node of the dendrogram as starting point. In Figure 3 this value is 73.7 for the combination C, H. The next node in the dendrogram is found by a second, reduced matrix, which now contains C, H as one unit and all other individuals separately as before. Each individual of the closest pair (here C, H) is compared with all other individuals. The new values of QA are obtained by averaging: C, H: $A = [(C \times A) + (H \times A)]/2$ or, in actual numbers: C, H: $A = (27.8 + 32)/2 = 29.9$. Again, the highest value of the second matrix is selected as the next nodal point. It is 48 for the combination A × G. The third matrix is still more reduced by using A and G in a single cell. Again the largest QA is selected. It is 44.1 for C, H × B. Now these three individuals are joined at social affinity unit 44.1. The procedure is repeated until all individuals are connected. For Figure 3, it takes 7 matrices, the last one joining the group B, C, H and L with that of A, G, K and M.

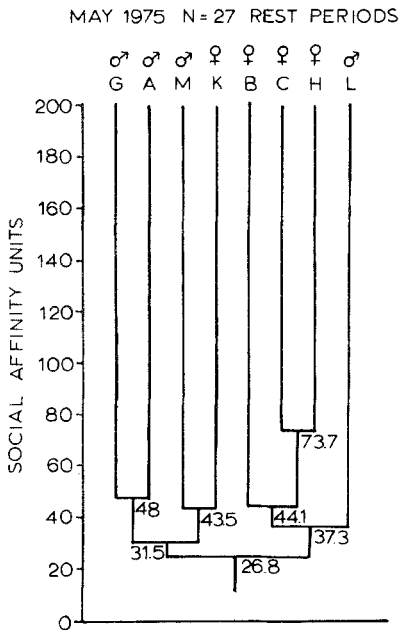

FIG. 3. Dendrogram of social affinity within a group of reindeer, based on nearest neighbors at rest, in May 1975.

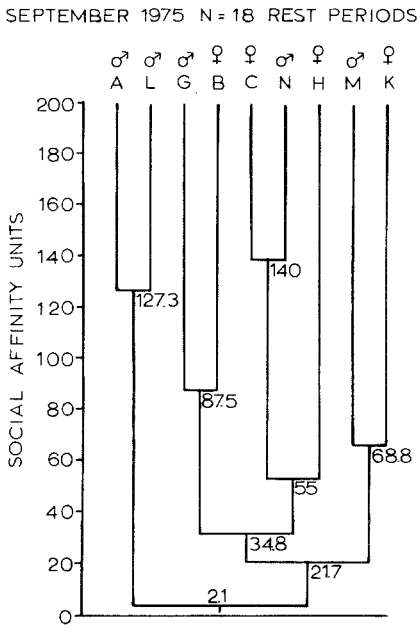

FIG. 4. Social affinities in September 1975 (prerut).

Seasonal changes in social organization are illustrated in Figures 3–6. In May (Figure 3) there is little specific social affinity (the highest number of social affinity units is 73.7) while the social affinity reaches 200 (the maximum) in January (Figure 5) and February (Figure 6). The highest number of social affinity units in July is 162.5, in September 140 (Figure 4), in November 142, and in March 146. In September, during the prerut, the group is heterogeneous: while there is a very close relationship between males A and L, they are joined to the rest of the herd only at affinity level 2.1 (Figure 4). They move and rest together as a "bachelor group," away from the harem. The dendrogram for September also shows the closeness of $♀_C$ and her calf $♂_N$ and the "harem" with $♀_G$ and all females. The yearling male M is tolerated in the harem. The range between the most distant nodes for the periods studied are 46.9 (73.7 minus 26.8) in May; 150.6 (162.5 − 11.9) in July; 137.9 (140 − 2.1) in September; 139 (142 − 3) in November; 197 (200 − 3) in January (before the dominating $♂_G$ shed his antlers); 195 (200 − 5) in February (after $♂_G$ shed his antlers); and 135 (146 − 11) in March. This is a measure of social structure: the group was least structured in May and most highly structured during the rutting and antler-shedding seasons.

There is no clear correlation between the number of wins by one animal over another (i.e., prevailing at a given site) and the frequency of their being closest neighbors. In May for instance, the highest number of wins was scored

by $♀_B$ over $♀_H$, but their *QA* of 44.1 is intermediate. On the other hand, $♀_C$ and $♀_H$ showed the highest affinity, but $♀_H$ won over $♀_C$ 13 times, while C never won over H. In September, during the prerut, agonistic behavior showed a clearer (negative) correlation with social affinity during rest.

Social Sniffing, Licking, and Affinity. The reindeer sniffed and licked one another or themselves. Social sniffing and licking occurred in the following behavior patterns:

1. Sniffing. Nasonasal approach, sniffing the tail (Figure 7), anogenital area, rump patch, tarsal gland area, fore- or hindleg, shoulder or back, side of body, and hard antlers, or urine while being voided or on the ground, often followed by lip curl (Flehmen). A close view of "Flehmen" is shown in Figure 8.

An individual may also sniff toward another. If this occurs over a distance of 2 m or less, it is unambiguous enough to be recorded as directed at a particular conspecific.

2. Licking. Unlike other cervids, there is no mutual licking between adult individuals and very little unidirectional social licking. The only observed pattern is licking of females' anogenital area and urine by males. Also, mothers do not lick the pelage of their calves once the calf has been licked dry

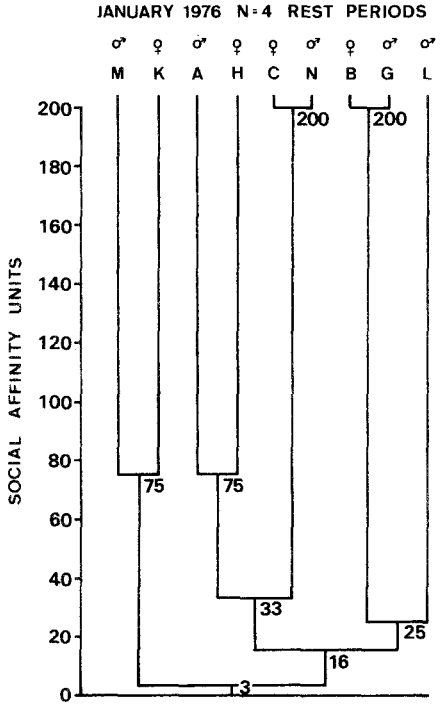

FIG. 5. Social affinities in January 1976 (before antler shedding).

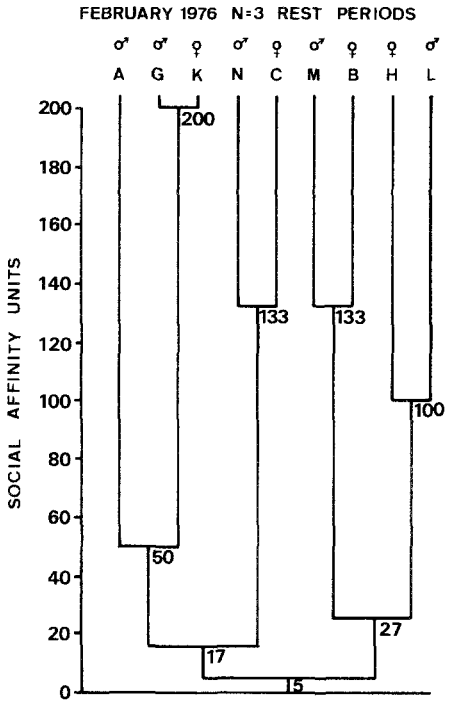

FIG. 6. Social affinities in February 1976 (after both mature bulls shed their antlers).

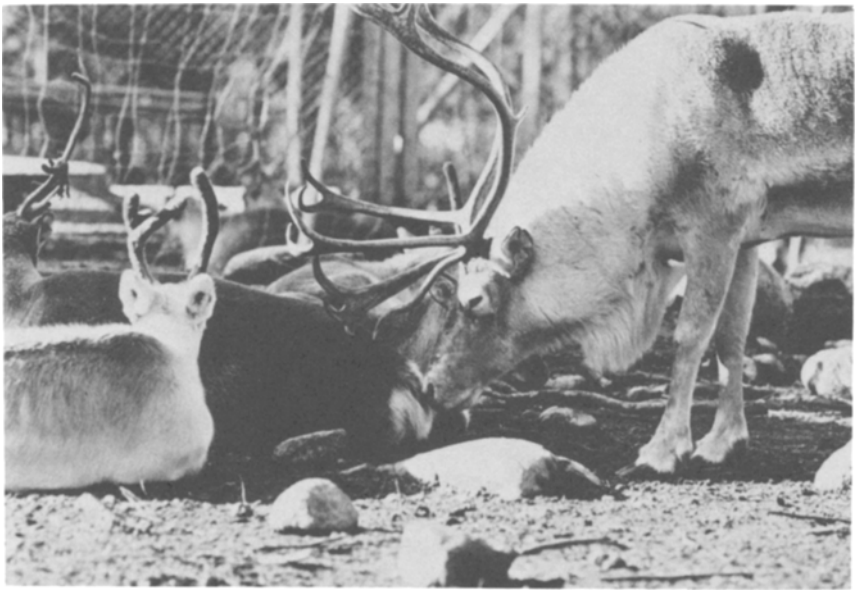

FIG. 7. A male reindeer sniffing the tail of a female.

FIG. 8. Close view of "Flehmen" posture in a male reindeer.

following birth, which was also described for reindeer by Espmark (1971) and for caribou by Lent (1966).

The relative frequencies of sniffing and licking the various areas are listed in Table 4.

The three most common body areas, anogenital, tail and nose areas combined, accounted for 62.1% of the total sniffing and licking (inclusive of urine). The interdigital gland area of conspecifics was the least often sniffed. Sniffing the mane and the long hair located posteriorly to the udder was observed only in the calf. These conspicuously white areas seem to attract the young, and a calf may occasionally suck at the mane. Sniffing the tail, anogenital area, and nose accounted for 71.1% of all sniffing and licking when responses to urine are excluded.

The overall frequencies of social sniffing and licking increased 14.6-fold from May to November. Nasonasal sniffing reached its maximum in March and its minimum in November, while sniffing and licking of the anogenital area was most frequent during the reproductive season in November. The

TABLE 4. FREQUENCIES OF SNIFFING AND LICKING VARIOUS BODY AREAS OF CONSPECIFICS IN CAPTIVE REINDEER

Body area	Adults (May 1975 to March 1976)	Reintroduction (June 1975)	Mother-calf		Calf-mother (June and July 1975)	Total	%
			June and July 1975	Sept 1975			
Anogenital	131	5	7	0	1	144	35.3
Tail	29	11	15	3	1	59	14.5
Urine	52	0	0	0	0	52	12.7
Nose	29	12	8	0	1	50	12.3
Body trunk	21	5	3	2	3	34	8.3
Tarsal gland	11	3	1	0	4	19	4.7
Rump patch	10	0	7	0	0	17	4.2
Antler	10	0	0	0	0	10	2.5
Foreleg	1	2	1	0	2	6	1.5
Hindleg	3	0	3	0	0	6	1.5
Chin and head	2	0	0	0	2	4	1.0
Mane	1	0	0	0	3	4	1.0
Udder hair	0	0	0	0	2	2	0.5
Interdigital gland	1	0	0	0	0	1	0.2
Total	301	38	45	5	19	408	100.2
Hours of observation	116.25	8	3.1	1.25	3.1	131.7	

maximum of the relative frequency of response to urine occurred in January. The annual cycle of social sniffing and licking is shown in Fig. 9.

In the encounters after reintroduction (in June), sniffing the nose and tail and "sniffing towards" accounted for 67.4% of all social sniffing and licking. They are obvious consequences of two animals approaching each other over a distance.

In Table 7 the sniffing frequencies during the encounters after separation are listed separately for the reintroduced animal and the seven other members

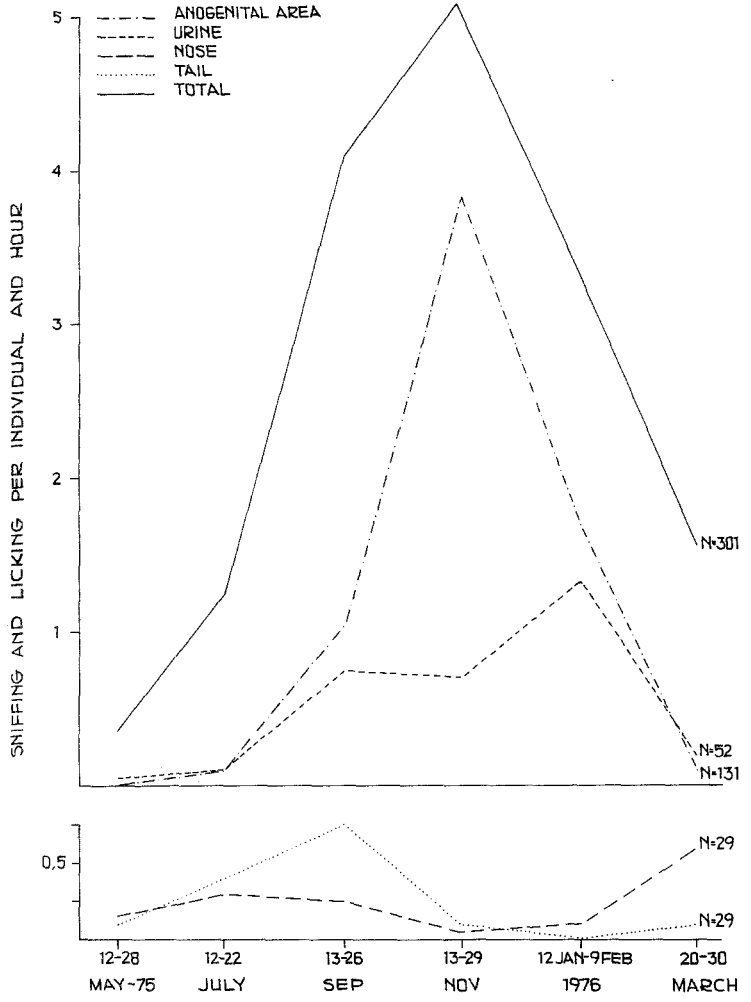

FIG. 9. Annual cycle of social sniffing and licking in reindeer.

TABLE 5. FREQUENCIES OF SOCIAL SNIFFING AND LICKING VARIOUS BODY AREAS OF CONSPECIFICS OF THE SAME AND OPPOSITE SEX

Body area	♂–♂	♂–♀	Σ♂♂	♀–♀	♀–♂	Σ♀♀	Total
Anogenital	4	125	129	0	2	2	131
Tail	6	14	20	6	3	9	29
Nose	16	3	19	4	6	10	29
Tarsal gland	5	2	7	2	2	4	11
Rump patch	3	5	8	1	1	2	10
Other parts of body	9	19	28	8	3	11	39
Urine	4	48	52	0	0	0	52
Total	47	216	263	21	17	38	301

of the group, who had remained together in their familiar enclosure. The arriving animal shows 21.5 times more sniffing (27.3 times more if the calf's responses are included) than the members of the resident group. This is in contrast to the black-tailed deer, for example, where in the same situation social sniffing is initiated by the resident animals and the newcomer explores the physical environment (Müller-Schwarze, 1971).

The sex specificity of social sniffing and licking is examined in Table 5. Overall ($N = 301$), males sniff more than females, particularly in the anogenital region. Sniffing of tail, rump patch, and nose area is also more frequent in males than in females. Males sniff females more often than males, and overall, females are more often the target of social sniffing and licking than males. In November, when social sniffing and licking was particularly frequent, the five males together sniffed or licked other individuals 92 times (4 females: 6 times), while the males were sniffed only 10 times (females: 88 times). Of 92 incidents of social sniffing and licking, 84 were directed at the anogenital area of females. Thus, the bulk of interindividual sniffing and licking incidents are part of sexual behavior. Nonsexual social sniffing ($N = 118$) is also more frequently performed by males (71.6%) than by females (28.4%). However, in nonsexual contexts, males are sniffed as often (49.4%) as females (50.6%). In November during 22.5 hr of observation, urine was licked 16 times, and by males only. Twice a young male licked urine from other males; all other cases involved female urine.

The olfactory interactions between mother and calf are listed separately in Table 4. The mother sniffed her calf most often at tail, nose, rump, and anal area. Together they account for 82.2% of the total ($N = 45$). She did not sniff or lick the area around the penis of the male calf, as other deer or bovids do. These observations in a single cow-calf pair were confirmed by observations in a herd of 500 reindeer during spring migration, when mothers and calves often separated and reunited. Social sniffing between calves and cows

occurred more often than between adults or between males and calves (Müller-Schwarze, in preparation).

The frequency of social sniffing between pairs of adult individuals and their social affinity values are not correlated. In November, for instance, males sniffed all females frequently regardless of their social distances. Male-male sniffing, on the other hand, was rare at all levels of social affinity.

Solitary Sniffing and Licking. The annual cycle of solitary sniffing and licking shows it was most frequent in May and least frequent in September and March (Figure 10). These frequencies may be related to the shedding of hair. Unlike other deer, reindeer lick or groom their own fur very little. The

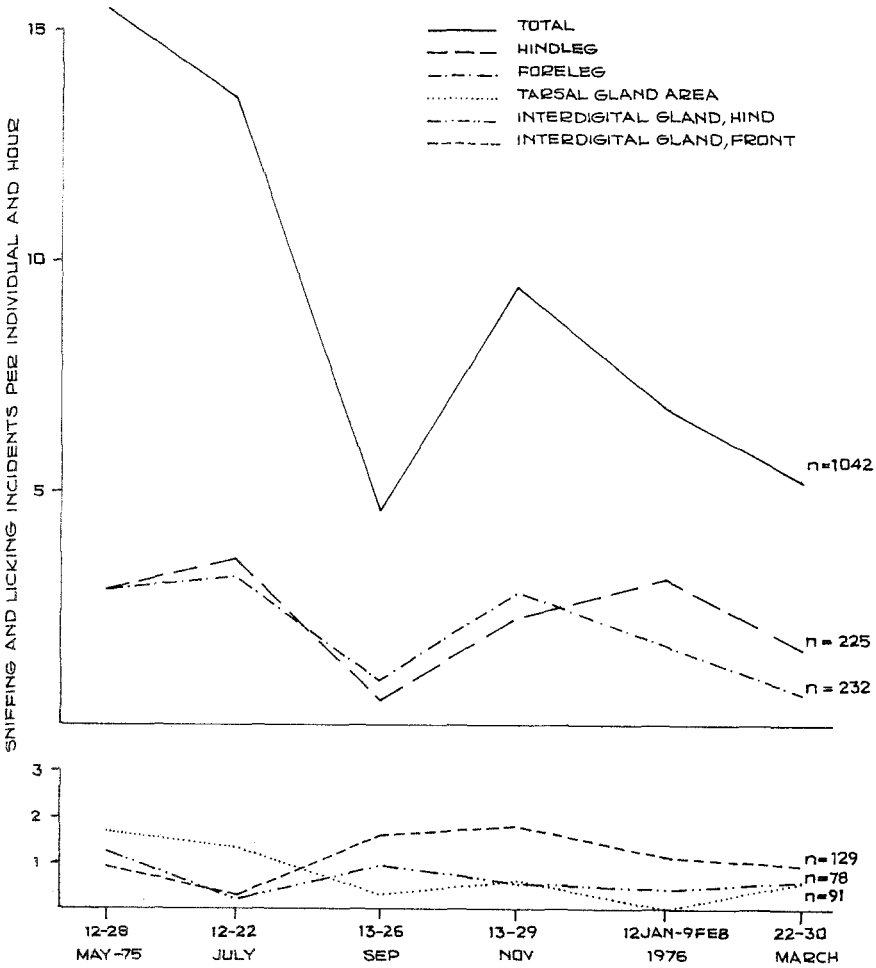

FIG. 10. Annual cycle of solitary sniffing and licking in reindeer.

FIG. 11. A reindeer licking its own interdigital gland of the forefoot.

only body areas they lick frequently are their fore- and hindlegs (24.5 and 22.3%, respectively, of all solitary licking in both sexes during entire year), their feet and their interdigital glands (12.4% front, 7.5% hindfoot). Figure 11 shows a female licking her front interdigital gland.

Sniffing its own body without subsequent licking is rare. Reindeer may occasionally show brief "nibbling" or "mouthing" of their own pelage. It occurred in May only 3 times in 10 hr and in July no animal was observed to sniff itself. In September, there were 24 cases of solitary sniffing in 20 hr, aimed at the interdigital glands (20 times), tarsal glands (2 times), and hindlegs (2 times). In November there were 54 incidents of sniffing, licking, or both

sniffing and licking of the interdigital glands (22.5 hr of observation). These included 17 incidents of sniffing, 22 licking, and 15 sniffing and licking at the same time. In January/February sniffing and/or licking occurred 18 times during 11.3 hr of observation. Eight cases were sniffing only and 10 were licking. In March (22.5 hr observation) the total was 34 incidents, of which 8 were sniffing only, 9 were licking only, and 17 both sniffing and licking at the same time.

Sniffing and licking of the interdigital glands was most frequent in May, September, and November. In September, at maximum frequency (2.6 incidents per hour and individual), the animals licked or sniffed their interdigital glands 4.5 times as often as at the minimum in July (0.6 incidents per hour and individual).

The fore- and hindlegs were most often sniffed or licked in May (5.8/indiv. and hr), July (6.7), and November (5.2), but less often in September (1.5), January/February (4.8) and March (2.3). There were no pronounced sex differences in licking skin glands and other areas of their own body. The higher rate of self-licking in May is probably related to the shedding of the winter fur and that in July to irritation by insects, such as tabanids.

Hindleg-Head Contact (*HHC*). Reindeer rub or tap their foreheads and the tips of their antlers with the lower end of their hindfeet, so that the opening of the interdigital gland comes in contact with the area touched. This had been called "tjårve-takam" (antler-making) by the Lapps, as they believe this behavior will enhance the growth of antlers by enabling the reindeer to grow new points at specified sites (Bubenik, 1956). Espmark (1971) called this behavior "antler rubbing." Bubenik (1956) suggested that the animal "scratches" where the new growth itches. Currently the function of this behavior is not understood. An analysis of 127 sequences of hindleg-head contacts showed that in 64.6% of the cases the immediately preceding behavior pattern is licking of the hindleg (knee, shank, tarsal gland area, metatarsus, interdigital gland area). These areas were licked 50 out of 127 times; the proportions of the listed parts of the hindleg were: 10:11:17:8:4. The remainder of the licking incidents were directed at: nose (2), foreleg above interdigital gland (6), belly (11), side (8), inguinal area (4), rump (1). Hindleg-head contact was preceded by scratching nose, cheek, or ear 8 times, and no licking or scratching occurred prior to hindleg-head contact in 37 cases.

For both sexes combined, the hindleg-head contact was most frequent in May (6.0/animal and hr). It dropped to 4.8 in July, 1.4 in September, and 0.3 in November. In January/February and March the frequency was higher again, 1.5 and 1.7, respectively. The frequency of HHC in males dropped steadily from a maximum in May (8.4/animal and hr) to a minimum in November (0.3). Before dropping his antlers on February 1, δ_G had a HHC frequency of 6.7 and showed no HHC at all thereafter. Males A and M showed

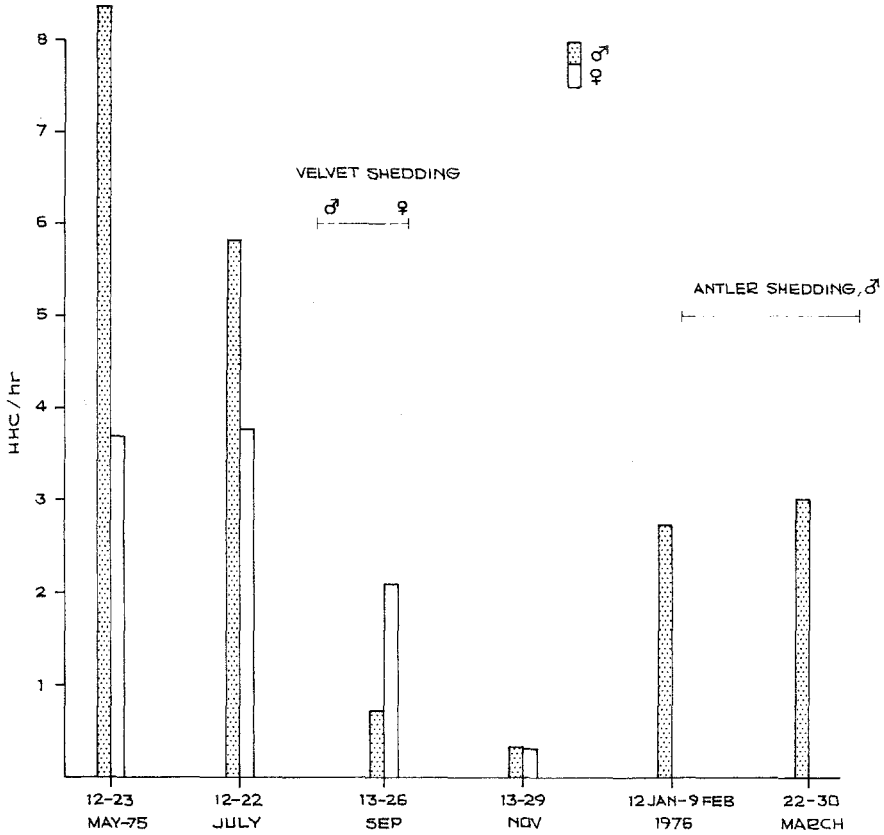

FIG. 12. Annual cycle of hindleg-head contact (HHC) in reindeer.

no HHC at all before δ_G shed his antlers, but afterwards they had a frequency of 12 and 8 HHC/hr, respectively. In March the HHC frequency for males increased to 3.0 HHC/male and hour.

The females had their HHC maximum in July (3.8 HHC/female and hr) and then it dropped steadily to no HHC at all in January/February and March. The annual cycle of HHC is shown in Figure 12. Thus, the frequency of hindleg-head contact parallels closely the growth of the antlers, with the females being about 2 months behind the males. Females C and K had the lowest frequency of hindleg-head contact in May and July, and shed their velvet last of all eight reindeer. On the other hand, the two males (δ_G and δ_L) who had most hindleg-head contact in May completed the growth of their antlers first and were the first to shed their velvet (Table 6). An analysis by Kendall partial rank correlation showed that there is a high correlation between HHC frequency (H) and antler growth (G) ($\tau_{HG} = 0.6794$) and a low

TABLE 6. FREQUENCIES OF HINDLEG–HEAD CONTACT DURING PERIOD OF ANTLER GROWTH BETWEEN MAY AND SEPTEMBER AND RANKS IN SEQUENCE OF VELVET SHEDDING (1 IS FIRST ANIMAL TO SHED; 8 IS LAST)

	Male					Female					Total	HHC/hr and individual
	A	G	L	M	Total	B	C	H	K	Total		
May	15	26	23	13	77	12	3	15	4	34	111	6.0
July	10	23	10	14	57	13	6	13	4	36	93	4.8
September	0	2	1	4	7	9	7	4	0	20	27	1.4
Totals	25	51	34	31	141	34	16	32	8	90	231	
Rank in sequence of velvet shedding	4.5	2.5	1	4.5		2.5	8	6	7			

correlation between HHC frequency and eventual antler size (S) (τ_{HS} = 0.3273). If antler size is held constant, the correlation between HHC and antler growth remains at τ_{HGS} = 0.6308. Thus, little or no influence by antler size could be demonstrated. Hindleg-head contact seems to be part of the comfort behavior syndrome, as it occurs together with licking and scratching, and mostly in situations when grooming would occur, such as midday rest. Espmark (1977) studied in detail hindleg–head contact behavior in the same reindeer that provided our data and his results confirm ours. Espmark discusses the possibility that application of interdigital secretion to the growing antlers will provide an individual odor for mutual recognition during a time when the visual appearance of the antlers changes rapidly.

Head Rubbing, Antler Thrashing, and Head Turning (Antler Dipping). Both sexes regularly rubbed their foreheads, antler bases, ears, eyes, or noses on thin trees, shoots, branches, or deadwood. This behavior is preceded and often followed by sniffing the substrate. This rubbing of parts of the head occurs particularly often directly before an animal reclines. As the summer progressed and the antlers grew, the animals performed head turning (antler dipping) on the ground prior to lying down. The head is lowered and turned, and the distal part of one antler is gently touching the ground. In September the reindeer showed intense antler thrashing of thin, resilient trees, such as mountain ash (*Sorbus aucuparia*), poplar (*Populus* sp.), birch (*Betulus* sp.) or willow (*Salix* sp.). The antler-thrashing has been termed "sham-fighting" by Espmark (1964a). Figure 13 shows the annual cycle of head rubbing, antler thrashing, and head turning.

Head turning and head rubbing was most frequent during the summer months (May to September). During May, July, and September 113 cases of head turning (95.8%) were performed and only 5 during November, January, and March. Head turning was more frequent in males, who performed 68.6% of the total. Forty-nine cases of head rubbing (86%) were performed during May–September while only 8 cases were observed during November–March. Females and males rubbed their heads about equally often (33 and 24 times, respectively). There was no thrashing in May and July, while the behavior was frequent during September–March. In September there were 147 30-sec periods of thrashing. Of these the harem bull accounted for 70 periods (53.7%). Figure 14 shows thrashing by the harem bull. Males and females thrashed equally often (157 vs. 163 periods for the entire year). The males thrashed most frequently in September (103 periods). Their thrashing frequency decreased over the months to 18 periods in March, when only the calf still had antlers and accounted for 17 periods (and male A for one period). The females thrashed most often in November (61 periods), and the frequency dropped gradually to 31 periods in March.

Head rubbing was most frequent in May when the reindeer had small or no antlers, head turning in July when the antlers were in velvet, and antler

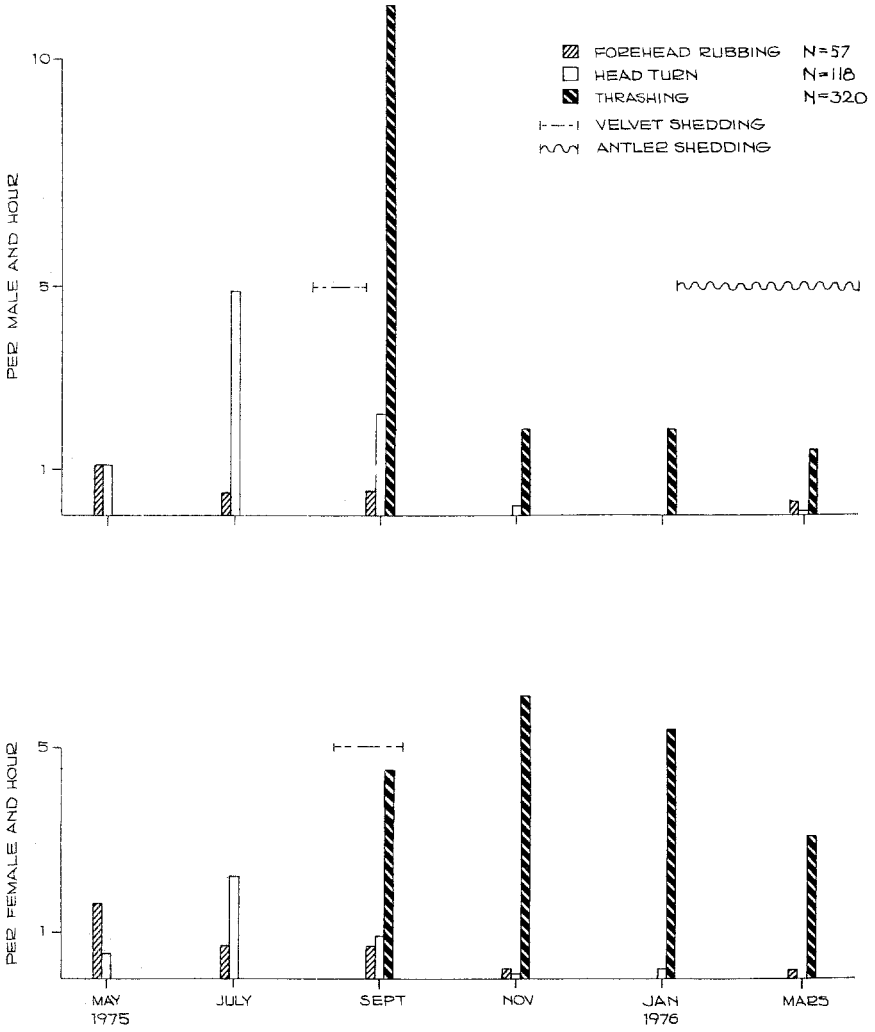

FIG. 13. Annual cycle of head rubbing, antler thrashing, and head turns in reindeer.

thrashing in September after the animals had shed their velvet from the hard antlers.

The intense thrashing not only produces alterations of the thrashed trees that can serve as visual and olfactory signals, but the behavior itself provides a distinct clatter audible over long distances. The behavior is contagious: one thrashing reindeer of either sex can induce another to thrash. As many as five of the eight adults were seen thrashing simultaneously.

As in other cervids, the head rubbing or thrashing consists of a sometimes

FIG. 14. Antler thrashing by a male reindeer.

long sequence of sniffing the tree alternating with actual thrashing movements of the head. The sniffing by the thrashing animal and by later passers-by bring this behavior into the context of scent marking, and therefore, olfactory communication. There was no behavioral evidence that a socially important odor emanated from the sebaceous glands of the antlers' velvet, as Bubenik (1966) has postulated, although to the human nose velvet has a characteristic odor.

Environmental Sniffing

Reindeer often sniff the ground when stopping briefly between bouts of locomotion, or sniff the ground continuously while walking. This sniffing can be in search of food or in response to reindeer tracks, urine, or feces. The latter can be termed "tracking." During the observations, ground sniffing was recorded. However, when the animals are moving freely in a large pen, it is very difficult to observe every case of ground sniffing. Moreover, it is often impossible to separate ground sniffing in response to footprints or urine from

ground sniffing in connection with feeding activities such as grazing or picking small pieces of lichen from the ground. Therefore, ground sniffing was recorded in the more controlled situation of a test pen of 7 m diameter for animals who had been isolated from conspecifics for 2 1/2 days, and for animals living with the group-mates. In 15 min, isolated males sniffed the ground on the average 30 times, females 29 times, while individuals taken from the group sniffed 19.8 and 17.5 times, respectively (Müller-Schwarze et al., 1978). This can be interpreted as tracking behavior with the potential function of bringing an individual back to its group (see Discussion).

Reintroduction Experiment

To determine which behavior patterns are typical for encounters between reindeer that meet after living apart, during the month of June each member of the group was separated once for 66 hr starting at 15:00 hr of the first day and ending at 09:00 hr on the fourth day. After isolation, the animal was reintroduced, and the interactions were observed for the first 30 min. The observations focused on sniffing responses between newcomer and resident animals that could serve in recognition and also agonistic encounters.

The olfactory interactions that took place between the returning animal and its former group-mates were nasonasal approach; sniffing the tail, anogenital area, general body (mane, shoulder, side); or sniffing toward an animal from a distance. Table 7 lists the frequencies of these behavior patterns for both the introduced individual and the resident group. The newcomer initiates most of the sniffing: it sniffed three times as often as the other seven

TABLE 7. SOCIAL SNIFFING AFTER RETURN OF SEPARATED GROUP MEMBER

Body area sniffed or licked	Reintroduced individual		Established group		Total		%
1. Nose	12	(16)[a]	0	(0)	12	(16)	26.1
2. Tail	7	(8)	4	(5)	11	(13)	23.9
3. "Sniffing toward"	5	(10)	3	(3)	8	(13)	17.4
4. General body	6	(9)	1	(1)	7	(10)	15.2
5. Anogenital area	3	(4)	2	(3)	5	(7)	10.9
6. Tarsal gland	2	(2)	1	(1)	3	(3)	6.5
Total	35	(49)	11	(13)	46	(62)	100
Animal hours	4	(4.5)	28	(31.5)	32	(36)	
Average per individual and hour	8.6	(10.9)	0.4	(0.4)	1.4	(1.7)	

[a] Values in parenthesis include calf.

reindeer combined. In these encounters, nasonasal and tail sniffing are the most frequent. It is, therefore, postulated that these body areas are important for mutual recognition (see Discussion).

The level of social sniffing was drastically raised in the isolated and then reintroduced reindeer: the newcomer sniffed 8.6 times/hr on the average, while the average frequency of social sniffing and licking per animal and hour in grouped reindeer was 0.4 in May, 1.3 in July, 4.1 in September, 5.1 in November, 3.3 in January/February, and 1.6 in March. The value for the main group during the reintroduction experiments in June was 0.4. After reintroduction, females sniffed more often than males. Anogenital and tarsal sniffing was directed more at females and carried out more by males, while tail sniffing is aimed at both sexes. Nasonasal sniffing was initiated by the returning animal in all cases, and tail sniffing in 7 of 10 cases.

Tracking Experiment

Reindeer produce and follow tracks during their migrations and short-range movements within the summer and winter ranges. It is not known whether secretion of the interdigital glands is deposited on the ground during walking, and if so, whether it is used by conspecifics as a chemical cue. Production of such a "trail pheromone" has been the suspected function of the interdigital glands in artiodactyls for a long time. Pruitt (1960) noted a phenol-like odor in footprints of mature caribou bulls after they had performed the "excitation jump," a rearing up on the hindlegs when startled. Lent (1966) observed caribou avoiding a spot on the trail where a conspecific had veered off with an "excitation jump." In roedeer (*C. capreolus*) it is assumed that small amounts of interdigital secretion on the track function in sexual behavior, while large amounts release escape in conspecifics (Kurt, 1966). Kurt induced roedeer to flee from experimental interdigital samples, but no control odors were used. It should be noted that reindeer paths are heavily contaminated with urine and feces, so that they can often be followed visually, and tradition plays an important part.

To determine the ability of reindeer to follow fresh tracks of conspecifics, four single trials were carried out. In all trials a reindeer laid a track (see Methods and Materials). In two trials another reindeer followed it, and in two a dog was used for tracking. In all four cases both the reindeer and dog were capable of following the track. For statistical analysis, the area was divided into 59 sections (most of them squares), and for each section and each trial it was noted whether both animals, only the donor, only the tracker, or none had been there (Table 8). Chi-square analysis showed a highly significant coincidence of the movements of the two individuals ($P < 0.01$). The dog was better in tracking a reindeer than were other reindeer.

TABLE 8. NUMBER OF SECTIONS (*N*) OF AN EXPERIMENTAL ENCLOSURE VISITED BY TRACKLAYING DONOR AND TRACKING ANIMAL

Track laying donor	*N*	Tracking animal: Visits	(%)	Does not visit	Statistical significance
	Tracker: Female dog				
Test 1: Female reindeer visits	17	13	(76.5)	4	$\chi^2 = 26.02$
Female reindeer does not visit	42	3	(7.1)	39	$P < 0.01$
Test 4: Male reindeer visits	20	17	(85.0)	3	$\chi^2 = 31.90$
Male reindeer does not visit	39	3	(7.7)	36	$P < 0.01$
	Tracker: Reindeer of opposite sex				
Test 2: Female reindeer visits	20	15	(75.0)	5	$\chi^2 = 10.44$
Female reindeer does not visit	39	20	(51.3)	19	$P < 0.01$
Test 3: Male reindeer visits	24	20	(83.3)	4	$\chi^2 = 8.06$
Male reindeer does not visit	35	15	(42.9)	20	$P < 0.01$

Occasional Observations in the Wild and in Zoos

Observations in the Wild. Between July 30, and August 2, 1975, free-ranging reindeer were observed at the northern slopes of Norra Storfjället in southern Lappland. A group of 90 individuals was within view for 26.5 hr. This group contained nine mature bulls, 25 calves, and the remainder were yearlings and 2-year-olds of both sexes, and adult females.

The 90 animals constantly formed three to four subgroups that exchanged individuals all the time. During the heat of the day they stayed on a snow slope in two groups of about 40 and 50 animals, respectively.

In a subgroup that contained on the average 20.4 individuals, solitary licking occurred at a rate of 1.08 per animal and hour, social sniffing at 0.25, and social licking not at all. This compares with 13 for solitary licking and 1.3 for social sniffing in captive reindeer in July. The four occurrences of social sniffing involved the nasal area, tarsal gland, and shoulder. Four cases of agonistic behavior were observed (0.13/animal and hr). They all involved fawns, either in encounters with other fawns or adult females. Hindleg-head contact (HHC) was observed 25 times in 45 min, or 3.2 times per adult and hour (captive reindeer in July: 4.8).

Observations in Zoos. Two mature males and two females with calves were observed at Skansen Park, Stockholm, on August 25, 1975, for 37 min. Sixteen incidents of agonistic behavior were observed, all of them by females to males. This agrees with a high level of aggression by captive females toward males in July (Table 1). Chasing and "head-up" were the most frequent motor patterns. The rate of agonistic behavior was on the average 6.5 incidents per

adult and hour. By comparison, the highest value observed in the captive herd at Umeå was 22.3 agonistic encounters per adult and hour in January/February (antler-shedding season) and the lowest value was 2.88 after reintroduction of separated group members in June. At Skansen Park head turns occurred during these encounters at a rate of 7.7 per animal and hour. Ground sniffing occurred 12 times (1.2 time per animal in 15 min) and tracking twice (0.2 per animal and 15 min). The bare ground had no food on it.

Two pairs of adult reindeer, housed separately and fence-to-fence, were observed at Amsterdam Zoo for 30 min each. No agonistic encounters or social sniffing or licking occurred between or within the pairs. During the 60 min of observation, the ground was sniffed 5 times, and tracking (ground sniffing while walking) occurred 5 times. This sniffing was in response to urine and feces on the ground, as the bare gravel surface has no food on it.

In summary, the occasional observations in the wild and in zoos corroborated the results obtained from the captive reindeer in Umeå, and showed that the frequency of agonistic behavior patterns was considerably higher in the confined animals.

DISCUSSION

This study was undertaken to determine the possible role of chemical signals in the intraspecific behavior of reindeer. As a basis for the olfactory and gustatory interactions to be observed, the social organization of a captive group was analyzed for one annual cycle. Agonistic encounters, social rank order, and social affinities were used to describe the infrastructure of the group throughout the year. Thus, seasonal changes in the frequencies of social sniffing and licking could be correlated with the changing social relationships. In captivity, social interactions, notably agonistic ones, tend to be more frequent than in the dispersed, free-ranging herds.

We gathered information on the behavioral contexts in which social sniffing and licking occurred, the possible functions of chemical signals, and their sources. It was found that reindeer use chemical signals, albeit not to the same extent as other cervids, notably the black-tailed deer, *Odocoileus hemionus columbianus* (Müller-Schwarze 1971). Particularly frequent is sniffing of the tail and anogenital area. Earlier investigators have noted the keen sense of smell in *Rangifer*, and rated it superior to vision and audition (Jacobi, 1931; Kelsall, 1968).

In reindeer, chemical communication occurs in sexual, maternal, and general social behaviors. In sexual behavior, males examine the anogenital area of females and their urine by both olfaction and taste. In maternal behavior, a mother will smell the tail of her calf, where the largest skin gland is located (Müller-Schwarze et al., 1977).

In general social behavior, conspecifics are smelled at a distance of several centimeters to several meters ("sniffing towards") rather than with close contact. When two individuals meet, especially after isolation, they sniff each other's nose, tail, and general body. A reindeer also smells footprints of another reindeer in its absence (tracking). This is particularly obvious in fresh snow.

The sniffing and licking responses by reindeer that are reported here suggest pheromone activity in several contexts. In reindeer, effects of pheromones are probably indirect and long-term, with a good possibility of the occurrence of primer pheromones in urine, interdigital, anogenital, or caudal glands. Where no immediate effects occur, the stimuli are not "releasing," but rather "informative," meaning that the receiving animal extracts information that is used to change its behavior, in an unspecific way, and usually at a later date. The behavioral effects can consist of searching behavior, staying in an area, becoming alert, changing course, accelerating or decelerating ongoing behavior, etc. The response—if it is observable at all—may depend on the context. This situation seems widespread in higher mammals with their complex behavior (Müller-Schwarze 1977).

In contrast to the simpler and more stereotyped behavior of insects, for which the pheromone definition was originally coined, in mammals there is one important additional step between the reception of the odor stimulus and a behavioral change: an evaluation of the context. An odor track may be perceived by conspecifics without any overt and specific behavioral effect if there is no pressing social, sexual, or physiological need. The animal may merely stay in the area, but show trail-following behavior later as appetitive behavior in some specific context. It may show searching behavior when it cannot find reindeer tracks. Thus, the possibility exists that the absence of an odor, not its presence, triggers a change in behavior. For instance, in experiments with interdigital secretion reindeer merely sniffed the samples, with no further responses (Müller-Schwarze et al., 1978). The behavioral contexts and possible functions of the various social odors in reindeer are summarized in Table 9.

Reindeer belong to the Telemetacarpalia branch of the Cervidae, and thus share tarsal glands with other members of that branch, such as New World deer of the genus *Odocoileus*, or moose, *Alces*. On the other hand, there is no metatarsal gland as in the genus *Odocoileus;* actually very few cervids have metatarsal glands. *Rangifer* has antorbital and interdigital glands, as most deer do. Unique among deer is the presence of antlers in both sexes. These morphological similarities and differences to other deer raise the question of whether and how scent glands are developed and specialized for sexual and social communication. Behavior traits peculiar to *Rangifer tarandus* are pronounced; it undergoes prolonged seasonal migrations and

TABLE 9. SOCIAL SNIFFING IN REINDEER: ODOR SOURCES, BEHAVIORAL CONTEXTS, AND POSSIBLE FUNCTIONS[a]

Odor source	Sex or age class of odor donor	Sex of receiver	Behavioral context	Possible function
Tail gland	Calf	Lactating ♀	Approach between mother and calf; nursing	Recognition and acceptance of calf
	Adult ♂ and ♀	Adult ♂ and ♀	Social encounter	Recognition (sex, group, or individual)
Nose (and possibly breath)	♂ and ♀	♂ and ♀	Social encounter	Recognition or appeasement
Sudoriferous glands on general body	♂ and ♀	♂ and ♀	Reclining; social encounter	Leaves scent on ground; Recognition
Interdigital gland	♂ and ♀	♂ and ♀	Migration; grazing; separation from group; HHC	Laying tracks; marking on head by HHC (Espmark, 1977); marking of vegetation by HHC and HT
Antorbital gland	♂ and ♀	♂ and ♀	Threat	Marking vegetation by HR, indicating presence in area
	Adult ♂	Adult ♂ and ♀	Rut	Threat
Anogenital area	Adult ♀	Adult ♂	Sexual behavior	Informing on sexual state
Urine	Adult ♂ and ♀	Adult ♂ and ♀	Rut	Threat (♂ to ♂) and information on sexual state (♂ to ♀, ♀ to ♀, ♀ to ♂)
Sudoriferous glands in ventral interdigital skin	♂ and ♀	♂ and ♀	Track laid	Information on presence in area
Sebaceous and apocrine glands in antler velvet	♂ and ♀	♂ and ♀	Unknown	Scenting vegetation or self-marking (Bubenik, 1966)
Tarsal gland	♂ and ♀	♂ and ♀	Unknown	Unknown

[a] HHC: Hindleg–Head contact; HT: head turn; HR: head rubbing.

extreme gregariousness, and, as the northern-most cervid, it is highly cold adapted, as evidenced by the hair cover on its muzzle and the ability to dig through 1 m of snow in search for food (Bergerud, 1967). It therefore is of great interest to compare the specific forms of chemical communication in *Rangifer* with cervids that have received more attention, such as the black-tailed deer (e.g., Müller-Schwarze 1971), and other ungulates.

One striking difference from other cervids is that a reindeer moves about more with its nose on the ground. This is related to feeding habits, but also serves in tracking. In vegetated areas, ground sniffing is difficult to separate from food searching behavior. But on bare ground that contains no food visible to humans, reindeer still sniff and track. However, during spring migration we observed reindeer sniffing footprints of conspecifics on a frozen river and then picking up a small piece of lichen that had been blown onto the snow from the woods about 0.5 km away. Thus, tracking behavior and food search are impossible to distinguish in the field.

In addition to the four different types of large scent-producing dermal glands (antorbital, tarsal, interdigital, and caudal gland), reindeer possess sudoriferous glands of the apocrine type in the hairy skin. Glands with well-developed secretory epithelium are restricted to the foreleg pit, a narrow ventral strip on the belly, the groin, and the legs. Well developed glands are also found in the oral angle and in the rump patch. The greatest numbers are found in the ventral interdigital skin of both fore- and hindfeet (Källquist and Mossing, 1977). The sebaceous and apocrine glands in the tips of the growing antler (Vacek, 1955; Lojda, 1956) have been assumed to leave scent marks by way of brushing against vegetation or rubbing the antlers against the hindlegs (Bubenik 1966).

The findings on olfactory communication have to be seen against the unique ecology of the European reindeer with its pronounced and predictable seasonal migrations. The spring migration from the low altitude forest to the mountains may last from two days to a month, depending on snow, ice, and grazing conditions (Espmark, personal communication; S. Persson, personal communication). Pregnant cows initiate the spring migration, followed by the yearlings. At that time one would expect tracking to be most important. In the forest reindeer, cows separate from the group prior to giving birth, and in the mountain reindeer calves may be dropped in "maternity groups" or in isolation. A few hours to two days after birth, the cows join the herd again (Espmark, 1971). On the summer range, both sexes and all age classes live together in herds, with subgroups maintaining their identity. With many calves present, individual recognition by smell and sound is important at that time. Mothers can recognize their calves by the calves' calls (Espmark, 1971b, 1975). The rut usually starts at the summer range, with the peak of the rut occurring in late September to early October. (Our captive bulls started to rut

in mid- and late September.) Some bulls then collect a harem and regularly check cows in the group for physiological condition. The departure for the winter range depends on the snow conditions, and may start in October, or as late as January. Glandular secretions, such as that of the interdigital gland of the hindfoot, may show seasonal variations in composition, as Brundin and Andersson (in preparation) have shown.

It should be pointed out that in captivity reindeer may not show some aspects of their chemical communication. An example is the habituation to alarming stimuli. Our captive reindeer were not alarmed by dogs outside the pen; they never escaped from them with their tails raised (exposure of tail gland) as wild reindeer do nor did they show the so-called "excitation jump" (Pruitt, 1960) which is assumed to leave interdigital secretion in the footprints of the hind feet.

Furthermore, some uses of social odors may be confined to very special circumstances that are almost impossible to duplicate in an experiment. For example, a cow may sniff the tracks of other reindeer when in search for her calf. One of us (D.M.S.) observed a caribou cow in Labrador that seemed to have lost her calf during fall migration. She first walked in the opposite direction of the migration and then trotted across the various tracks in the snow and finally followed them. While doing so, she sniffed along the ground in short bursts, each for the duration of about three strides. The ground was sniffed in this fashion 24 times in 2 min. It should be noted that only one individual of several thousand seen was in the situation of looking for her calf and that the distance over which the search took place was on the order of several kilometers. It is obvious that these conditions are impossible to create in an ordinary field laboratory.

Although the more controlled conditions of captivity allow extensive experimentation, more detailed behavior observations of free-ranging reindeer and caribou are needed before chemical communication in this species can be better understood.

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TRAPPING THE WESTERN PINE BEETLE AT AND NEAR A SOURCE OF SYNTHETIC ATTRACTIVE PHEROMONE
Effects of Trap Size and Position[1,2]

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Abstract—Western pine beetles were caught on unbaited sticky traps placed near a source of *exo*-brevicomin, frontalin, and myrcene. Size of trap, distance and direction from the source of attractant, and height from the ground were varied. Significant differences in trap catch were observed in relation to each of the variables. Traps close to the source of attractant caught more beetles than traps farther from the source. Traps downwind of the source of attractant caught more beetles than did upwind traps. More males than females were trapped close to the source of attractant.

Key Words—*Pinus ponderosa, Dendroctonus brevicomis,* western pine beetle, attractant, pheromone, behavior, traps, Coleoptera, Scolytidae.

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[2]Trade names and commercial enterprises or products are mentioned solely for information and do not constitute endorsement by the U.S. Department of Agriculture or University of California.

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INTRODUCTION

The western pine beetle (WPB), *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae), kills *Pinus ponderosa* Laws. in western United States and Canada, and *P. coulteri* D. Don in southern California (Miller and Keen 1960). This beetle aggregates on living trees in response to chemical stimuli (Person, 1931; Vité and Gara, 1962). *Exo*-brevicomin, frontalin, and myrcene are primarily responsible for this attraction (summarized in Wood, 1972), with the (+) enantiomer of *exo*-brevicomin and the (−) enantiomer of frontalin more attractive to the beetles than their antipodes (Wood et al. 1976). The identification of attractive pheromones and the concern for potential environmental hazards of persistent insecticides (Koerber, 1976) led to studies of these pheromones to suppress WPB populations in California (Bedard and Wood, 1974).

To aid our interpretation of the results of field assays of possible attractants and designing a trap to catch a large portion of responding beetles, we investigated effects of certain trap and attractant variables on the number and distribution of WPB trapped at and near a source of synthetic attractant.

Several studies have been made about the distribution of bark beetles near a source of attractant, and about the effects of trap size and location on the number of beetles trapped. Chapman (1962), and Gara et al. (1965), Goeden and Norris (1964), Rudinsky (1963), and Vité and Gara (1962) used barrier and sticky traps, olfactometers, or rotary nets to study flight response of several species of bark beetles to attractive logs and trees. Vité and Gara (1961, 1962) increased the number of beetles trapped at attractive logs by increasing the volume of air sampled with rotary nets or by increasing the size of barrier traps. Lanier et al. (1976) studied effects of size, design, and location of pheromone baited traps on the catch of *Scolytus multistriatus* (Marsham). They found that large traps caught more beetles than small traps. Increasing the visibility of traps by contrast with the background increased the number of beetles caught. Trap height on elm trees made a difference in trap catch, with the most effective height depending on whether the trees were healthy or diseased.

This paper reports the effects on trap catch of two trap sizes at two distances, two heights, and four directions from a source of attractant.

METHODS AND MATERIALS

Experimental Design. We set up two plots (A and B) 2.1 km apart in natural openings in a 60- to 120-year-old ponderosa pine forest at 1200 m elevation near Bass Lake, Sierra National Forest, Madera County,

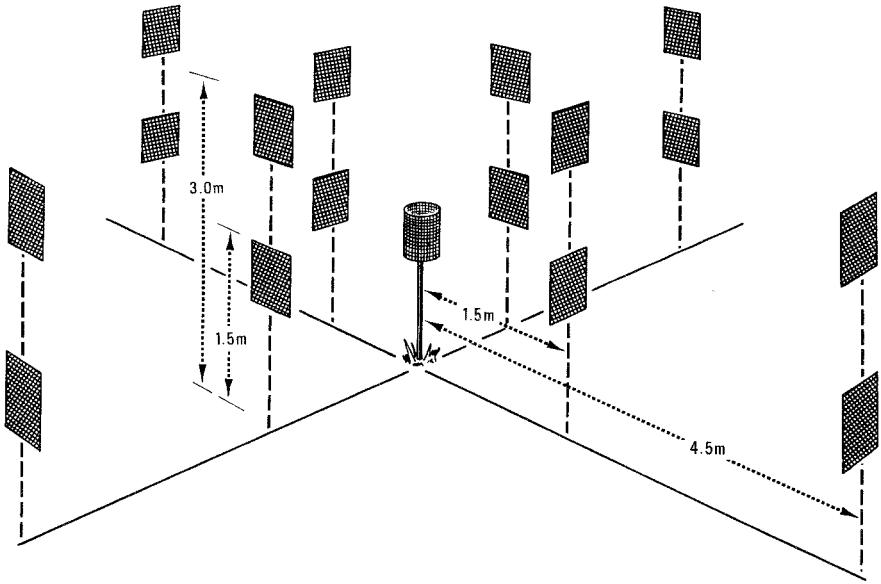

FIG. 1. Plot design for 16 traps surrounding a cylindrical trap where the attractive pheromones *exo*-brevicomin, frontalin, and myrcene were each released, Bass Lake, California, Aug. 27–29, Sept. 2, 1969.

California. In each plot (Figure 1) a cylindrical trap 30.5 cm high–× 20.3 cm diameter and 0.19 m^2 in area, the top of which was 1.5 m above the ground (Bedard and Browne, 1969), was baited with attractant and surrounded at the four cardinal points by 16 rectangular traps. The tops of these traps were 1.5 and 3.0 m above the ground at 1.5 and 4.5 m from the central trap. They were hung by wire hooks from 3-mm-diameter nylon cords, which were strung horizontally from eight 1.3-cm OD × 5-m-high metal pipes set vertically in the ground. The pipes were placed at plot corners away from traps to minimize the effect of even a small silhouette on the number of beetles caught. All traps were made of hardware cloth (0.95 cm mesh) and coated on both sides with melted Stickem Special® (Michel and Pelton, Emeryville, California). The 16 surrounding traps in each plot were either 0.19 m^2 (30.5 × 61 cm) or 0.37 m^2 (61 × 61 cm). The traps were alternated daily between plots A and B on 4 days as follows:

Dates	Plot A	Plot B
Aug. 27, 29, 1969	0.37 m^2	0.19 m^2
Aug. 28, Sept. 2, 1969	0.19 m^2	0.37 m^2

We alternated trap sizes to reduce the possible depletion of WPB in a plot by

the larger traps. This experimental design was incomplete in that both large and small traps did not appear in each plot on each day.

A baited cylindrical trap without surrounding traps was placed between the two plots, 0.3 km from plot A, and 1.8 km from plot B. The three locations were similar in forest type, exposure, and topography. They were chosen because in earlier tests the locations were comparable in number of WPB trapped at a source of synthetic attractant. The earlier tests consisted of 25 days of observation during an 8-week period immediately preceding the study reported here.

By separating the plots we risked testing under different environmental conditions and with different WPB populations. Had the plots been adjacent, however, the attractant in one plot might have influenced the flight behavior of beetles subsequently trapped in the other plot. The sources of attractant were placed at least 30 m from ponderosa pines to reduce the chance of WPB attacking trees (Bedard and Wood, 1974; Rudinsky, 1966). An attacked tree is an extraneous source of attractant which influences the behavior of beetles.

Each component (purity >99%) of the attractant—racemic *exo*-brevicomin, racemic frontalin, and myrcene—was evaporated separately from a silk thread wick inserted to the bottom of a glass vial (1.4 mm ID × 6 cm). The three vials were suspended vertically inside a 2.8-cm ID × 10.5-cm glass tube, open on both ends. A thread extension of $\frac{1}{4}$ mm above the top of the vial allowed approx. $\frac{1}{3}$ mg/hr of either *exo*-brevicomin or frontalin to evaporate. A thread extension of 1 mm allowed approx. 2 mg/hr of myrcene to evaporate. Evaporation rates were estimated gravimetrically. Attractant was placed in each plot in the afternoon, between 3:30 and 4:30 PM, and removed the next morning at 8:30 AM before most of the morning flight of WPB. Beetles were taken from traps and stored in vials of solvent (Chevron-325®) for later counting and sex determination (Tate and Bedard, 1967). The prevailing wind direction in plots A and B during the afternoon period of beetle flight was from west to east, as observed from the movement of threads tied to the cords supporting the surrounding traps.

Statistical Analysis. The underlying distributions of beetles caught on our traps were unknown. We used distribution-free (nonparametric) statistical methods, therefore, rather than methods sensitive to departures from assumptions about the underlying distributions. Because we did a large number of statistical tests, the probability of rejecting a true null hypothesis (type I error) may be large for the study as a whole. An alternative would have been to make each test extremely conservative. Because this study was exploratory, we accepted a larger probability of type I error for the study as a whole, in order to increase the probability that our tests would reject false null hypotheses (Tukey, 1962).

We used the Wilcoxon signed-rank statistic for matched pairs (Lehmann,

1975) to test for an effect of size of surrounding traps on the number of WPB caught at the source of attractant. The null hypothesis was that equal numbers of WPB were caught at the source. The one-sided alternative hypothesis was that, as size of surrounding traps increased from 0.19 to 0.37 m^2, the numbers of WPB caught at the source decreased. Data from the baited trap without surrounding traps were not included in the analysis. Because this treatment—baited trap without surrounding traps—occurred in only one of the three locations, it was impossible to separate treatment effects from plot effects.

We also used the Wilcoxon signed-rank statistic to test for effects of trap size, distance, and height on the numbers of WPB caught at the 16 surrounding trap positions (2 distances, 2 heights, 4 directions). For each trap size at each of the 16 positions, the four daily observations were added to give a single datum for analysis. Due to the daily alternation of trap size between plots, each datum is the sum of two observations from plot A and two from plot B. Implicit in this operation is the assumption that day and plot effects do not interact. If this assumption is true, then the data are comparable with respect to day and plot effects. Thus, the data for analysis were 32 numbers, one from each of two trap sizes at each of 16 positions. Each test compared the two levels of one trap variable (trap size, distance, or height) within a single level of a second trap variable by matching the data with

TABLE 1. SIGNIFICANCE PROBABILITIES OF 12 WILCOXON SIGNED-RANK TESTS[a]

	Trap variables compared		
	Size	Distance	Height
Levels of trap variables	0.19 m^2 vs. 0.37 m^2	1.5 m vs. 4.5 m	1.5 m vs. 3.0 m
Size			
0.19 m^2	*b*	2a.[c] $P = 0.0234$	3a. $P = 0.7734$
0.37 m^2	*b*	2b. $P = 0.0078$	3b. $P = 0.7422$
Distance			
1.5 m	1a. $P = 0.0039$	*b*	3c. $P = 0.0234$
4.5 m	1b. $P = 0.4883$	*b*	3d. $P = 0.0156$
Height			
1.5 m	1c. $P = 0.1914$	2c. $P = 0.0078$	*b*
3.0 m	1d. $P = 0.0117$	2d. $P = 0.0391$	*b*

[a]Effects of trap variables on the numbers of western pine beetles caught on traps of two sizes at two distances from a source of attractant and at two heights, Bass Lake, Calif., Aug. 27-29, Sept. 2, 1969. Attractant source was 1/3 mg/hr each of *exo*-brevicomin and frontalin, and 2 mg/hr of myrcene.

[b]Comparisons not applicable.

[c]Refers to an alternative hypothesis in the text.

respect to the third trap variable and with respect to direction (Table 1). For example, the variable "trap size" has two levels, 0.19 m^2 and 0.37 m^2. These were compared within a single level of the variable "distance" (e.g., 1.5 m from the source), by comparing the eight matched pairs of data from two levels of the variable "height" at four directions. For each test, the null hypothesis was that there was no difference in the numbers of WPB trapped due to the trap variable being compared. The 12 alternative hypotheses are (Table 1):

1. WPB caught on 0.37 m^2 traps exceeded WPB caught on 0.19 m^2 traps (one-sided alternative): (a) at 1.5 m from source of attractant, (b) at 4.5 m from source of attractant, (c) at 1.5 m above ground, and (d) at 3.0 m above ground.

2. WPB caught at 1.5 m and 4.5 m from the source of attractant were unequal (two-sided alternative): (a) for 0.19 m^2 traps, (b) for 0.37 m^2 traps, (c) at 1.5 m above ground, and (d) at 3.0 m above ground.

3. WPB caught 1.5 and 3.0 m above the ground were unequal (two-sided alternative): (a) for 0.19 m^2 traps, (b) for 0.37 m^2 traps, (c) at 1.5 m from source of attractant, and (d) at 4.5 m from source of attractant.

We used the classical chi-square goodness-of-fit statistic to test for differences in the spatial distributions of WPB caught as a result of the size of the surrounding traps. The null hypothesis was that the distributions, by height and distance, of WPB caught in plots with 0.19 m^2 traps and in plots with 0.37 m^2 traps were the same. The number trapped at any distance/height combination is the sum of the numbers caught at the four directions over four days.

The chi-square goodness-of-fit statistic was also used to test for difference due to direction of surrounding traps, relative to source of attractant. Under the null hypothesis, the total catch at each plot/day/distance/height combination is equally distributed among the four directions. Thus, a chi-square statistic with 3 degrees of freedom was calculated for each of the 32 plot/day/distance/height combinations. These statistics were not interpreted as statistical tests, but rather as measures of departure from the null hypothesis, providing a means of ranking the 32 plot/day/distance/height combinations. The 32 independent chi-square statistics, however, were summed to yield a chi-square statistic with 96 degrees of freedom. This statistic was used to test the null hypothesis of directional equality at all plot/day/distance/height combinations. We also determined the number of plot/day/distance/height combinations where the catch on the east trap exceeded that on the west trap, because the prevailing wind direction during the testing periods was from west to east. Under the null hypothesis of no difference, this number is binomially distributed as it is the number of successes in 32 independent trials where each trial results in a success with probability 0.5. We did a one-sided test of this hypothesis against the alternative that catch on the east trap was greater than catch on the west trap.

Multiple comparisons tests (Miller, 1966) were used to compare the sex ratios of WPB caught at the four distance/height combinations and at the source of attractant (Table 2). Two sets of comparisons were made: (1) where the surrounding traps were 0.19 m^2, and (2) where the surrounding traps were 0.37 m^2.

RESULTS

As the trap surface surrounding the source of attractant decreased, more WPB were trapped at that source (Table 2). The catch at the source when surrounding traps were small was significantly greater than the catch at the source when surrounding traps were large ($P = 0.0625$). (Although this is greater than 0.05, the result is judged significant because 0.0625 is the smallest significance probability possible with a sample size of four.) The lone, baited trap caught more WPB than the baited traps surrounded by traps of either size.

Results of the Wilcoxon signed-rank tests, which we used to

TABLE 2. MEAN DAILY CATCH AND SEX RATIO OF WESTERN PINE BEETLES AT A SOURCE OF ATTRACTANT[a]

No. traps	Trap size (m^2)	Distance (m) from attractant	Trap height (m)	Daily mean[b] per trap	Sex ratio ♂/♀[c]
1	0.19	Source	1.5	23.5 ± 8.8	1.85[a]
4	0.19	1.5	1.5	26.5 ± 12.8	0.80[b]
4	0.19	1.5	3.0	22.0 ± 10.5	1.35[ac]
4	0.19	4.5	1.5	13.4 ± 15.8	0.88[abc]
4	0.19	4.5	3.0	19.1 ± 14.8	0.87[bc]
			Total	347.5 ± 120.1	1.01
1	0.19	Source	1.5	11.3 ± 5.7	1.50[a]
4	0.37	1.5	1.5	35.4 ± 30.4	0.97[a]
4	0.37	1.5	3.0	28.8 ± 18.6	1.05[a]
4	0.37	4.5	1.5	11.4 ± 13.4	0.87[a]
4	0.37	4.5	3.0	20.9 ± 19.3	1.01[a]
			Total	397.3 ± 273.6	1.00
1[d]	0.19	Source	1.5	59.8 ± 21.6	1.17

[a]On traps of two sizes at two distances from a source of attractant and at two heights, Bass Lake, Calif., Aug. 27-29, Sept. 2, 1969. Attractant source was 1/3 mg/hr each of *exo*-brevicomin, and frontalin, and 2 mg/hr of myrcene.
[b]Variation is one standard deviation.
[c]Within the upper or lower portion of the table, sex ratios followed by the same letter are not different. Chi-square: $P \leq 0.05$, 1 df.
[d]Source of attractant without surrounding traps.

study the effects of trap size, distance, and height on the numbers of WPB caught on traps surrounding the source of attractant, were interpreted as follows (Table 1):

1. Effect of trap size: (a) More WPB were caught on 0.37 m^2 traps than on 0.19 m^2 traps at 1.5 m from the source of attractant ($P = 0.0039$). (b) Numbers caught on traps of two sizes at 4.5 m from the source of attractant did not differ ($P = 0.4883$). (c) Numbers caught on traps of two sizes at 1.5 m above the ground did not differ ($P = 0.1914$). (d) More WPB were caught on 0.37 m^2 than on 0.19 m^2 traps at 3.0 m above the ground ($P = 0.0117$).

2. Effect of trap distance: (a) Numbers caught on near and far traps differed for the 0.19-m^2 trap size ($P = 0.0234$). More WPB were caught on traps 1.5 m away than on traps 4.5 m away from the source of attractant. (b) Numbers caught on near and far traps differed for the 0.37-m^2 trap size ($P = 0.0078$). More WPB were caught on traps 1.5 m away than on traps 4.5 m away from the source of attractant. (c) Numbers caught on near and far traps differed at 1.5 m above the ground ($P = 0.0078$). More WPB were caught on traps 1.5 m away than on traps 4.5 m away from the source of attractant. (d) Numbers caught on near and far traps differed at 3.0 m above the ground ($P = 0.0391$). More WPB were caught on traps 1.5 m away than on traps 4.5 m away from the source of attractant.

3. Effect of trap height: (a) Numbers caught at either height on 0.19 m^2 traps did not differ ($P = 0.7734$). (b) Numbers caught at either height on 0.37 m^2 traps did not differ ($P = 0.7422$). (c) Numbers caught on high and low traps at 1.5 m from the source of attractant differed ($P = 0.0234$). More WPB were caught on traps 1.5 m high than on traps 3.0 m high. (d) Numbers caught on high and low traps at 4.5 m from the source of attractant differed ($P = 0.0156$). More WPB were caught on traps 3.0 m high than on traps 1.5 m high.

In summary, with respect to the number of WPB trapped:

1. Trap size made a difference at the closer distance and also at the higher position. More WPB were caught on the larger traps under each of these circumstances. There was an interaction between trap size and distance, and between trap size and height.

2. Trap distance from source of attractant made a difference. More WPB were caught closer to the source for traps of both sizes and at both heights.

3. Trap height made a difference only with respect to distance. More WPB were caught on the lower traps at the closer distance. At the farther distance, more were caught on the higher traps. There was an interaction between trap height and distance.

Doubling the size of the surrounding traps had a significant effect on the frequency distribution of the WPB catch ($P < 0.00001$) (Table 3). The contributions to the chi-square statistic, which are a measure of the

TABLE 3. CHI-SQUARE GOODNESS-OF-FIT COMPARISON OF TWO FREQUENCY DISTRIBUTIONS OF WESTERN PINE BEETLES CAUGHT AT A SOURCE OF ATTRACTANT[a]

Size of surrounding traps	Distance (m) from attractant	Height (m)	Obs.[b]	Exp.[c]	Contributions to χ^2
0.19 m^2	Source	1.5	94	65	13.09
	1.5	1.5	424	462	3.12
	1.5	3.0	352	379	1.97
	4.5	1.5	214	185	4.62
	4.5	3.0	306	299	0.16
Total			1390	1390	22.96
0.37 m^2	Source	1.5	45	74	11.45
	1.5	1.5	566	528	2.72
	1.5	3.0	461	434	1.72
	4.5	1.5	182	211	4.04
	4.5	3.0	335	342	0.14
Total			1589	1589	20.09
Total $\chi^2 = 43.05, P < 0.00001$, 4 df					

[a]On traps of two sizes at two distances from a source of attractant and at two heights, Bass Lake, Calif., Aug. 27-29, Sept. 2, 1969. Attractant source was 1/3 mg/hr each of *exo*-brevicomin and frontalin, and 2 mg/hr of myrcene.

[b]Observed frequencies = total number WPB trapped in 4 days.

[c]Expected frequencies, rounded to nearest whole number, were calculated as the product of the expected proportions and the total for the respective trap size, under the null hypothesis that distributions of beetles caught on 0.19 m^2 and 0.37 m^2 traps were the same. Expected proportions were estimated by pooling the two samples divided by the sum of the totals from the two trap sizes.

deviations of the observed from the expected frequencies, indicate that this effect was due primarily to a reduction in the number of WPB caught at the source of attractant when the trap size was doubled.

The frequency distribution of catch among the four directions shows considerable variation over the 32 plot/day/distance/height combinations. To see the spatial and temporal variation in the distribution of catch, the chi-square statistics were sorted by magnitude into four groups (Table 4). This table shows larger departures from directional equality in plot A than in plot B. In plot A, there was a tendency for the far traps to show larger departures from directional equality than the near traps; however, this was not apparent in plot B. There was no apparent trend among the 4 days, although day 3 had smaller departures from directional equality than the other days. There were no consistent differences between traps 1.5 and 3.0 m high in plot A. But in plot B the higher traps show larger departures from directional equality than the lower traps. The chi-square test of directional equality at all plot/day/distance/height combinations was highly significant ($\chi^2 = 589.081$, 96 df, $P < 0.00001$).

TABLE 4. DEPARTURE FROM EQUAL NUMBERS OF WESTERN PINE BEETLES CAUGHT AT FOUR DIRECTIONS,[a] BY PLOT/DAY/DISTANCE/HEIGHT, BASS LAKE, CALIFORNIA, AUG. 27-29, SEPT. 2, 1969

		Height (m)			
		Plot A		Plot B	
Day	Distance (m) from attractant	1.5	3.0	1.5	3.0
1	1.5	I	II	III	II
	4.5	I	I	IV	III
2	1.5	II	II	III	II
	4.5	I	I	IV	IV
3	1.5	II	IV	IV	II
	4.5	II	II	IV	II
4	1.5	II	I	IV	III
	4.5	I	I	IV	II

[a]Departure from directional equality is measured by magnitude of chi-square statistic calculated under the hypothesis of equal expected numbers at all directions. I indicates the eight largest chi-square values (range: 28.5-69.4); II, the next eight (range: 10.7-26.5); III, the next eight (range: 5.2-10.3); IV, the smallest eight chi-square values (range: 0.9-5.0).

Of the 32 plot/day/distance/height combinations, WPB catch on the east trap exceeded that on the west trap 31 times ($P < 0.00001$). The most frequent directional pattern is E>S>N>W; this occurs 11 times (not including a single tie: E>S = N>W). This observed frequency of 11 may be compared to an expected frequency of 1.33 under the hypothesis of directional equality.

When the surrounding traps were 0.19 m^2, the proportions of males caught at the source of attractant and at the trap position located 1.5 m away and 3.0 m high were significantly different from the proportions of males caught at 1.5 m away, 1.5 m high and at 4.5 m away, 3.0 m high (Table 2). There was a similar trend, not statistically significant, when the surrounding traps were 0.37 m^2.

DISCUSSION

A mechanism to explain distant orientation to an odor source in the field is positive optomotor anemotaxis, that is, upwind flight steered by visual perception of changing ground patterns (Kennedy, 1977). Attractive molecules can initiate anemotaxis (Kennedy, 1977). The distance from the odor source at which anemotaxis is initiated is probably related to the concentration of attractant which is influenced by windspeed and turbu-

lence (Aylor et al., 1976). In our plots with surrounding traps, the east traps downwind of the source of attractant caught more beetles than did the west traps. This difference could be explained by positive optomotor anemotaxis. Upwind orientation to a source of attractant has been observed for several species of bark beetles (Gara, 1963; McMullen and Atkins, 1962; Rudinsky 1963; Seybert and Gara, 1970; Vité and Gara, 1962). Chapman (1962) caught more *Trypodendron lineatum* (Oliv.) (Coleoptera: Scolytidae) on downwind than on upwind halves of traps placed over naturally infested attractive logs. Coster and Gara (1968) recovered marked *D. frontalis*, which had been released from four stations surrounding naturally infested attractive logs. Release stations were at right angles to each other and 30.5 m from the source of the attractant. Two stations were aligned with the prevailing wind and two were aligned perpendicular to the prevailing wind. Chi-square analysis revealed that beetles released from different stations were not equally successful in reaching the attractive bolts. Although the chi-square analysis cannot directly test whether these differences were due to wind direction, examination of the deviations of observed numbers from expected numbers (Table 2, p. 74, of Coster and Gara, 1968) reveals a pattern that is consistent with an upwind orientation of beetles to a source of attractant.

When close to a source of attractant, a flying insect's turning rate and/or angle increases (Kellogg et al., 1962; Traynier, 1968). This increased turning may be in response to a threshold concentration or to an abrupt change or a series of changes in concentration of attractants. In our study, catch per plot increased compared to the lone baited trap when we placed additional unbaited traps near the source of attractant. The number of WPB caught at the source of attractant appears to be related to the amount of surface area of surrounding traps. We speculate that the lower catch at the source resulted after many WPB, responding to the attractant, were caught on the surrounding traps as they flew toward, past, or turned near the source. Males were apparently more successful in reaching that source than females. It is possible that their turning rate is less than that of females, or that the threshold concentration (or change in concentration) of attractant, at which the turning rate increases, is higher. Also, males could be more responsive to the visual stimulus of the small trap at the source of the attractant.

The greater catch on traps 1.5 m than on traps 4.5 m from the source of attractant could be due to the increased trap surface area per unit volume of air, as well as to more frequent turning of the beetles closer to the source of attractant. Other workers have observed similar trapping patterns for bark beetles at and near naturally infested host material (Gara et al., 1965; Goeden and Norris, 1964; Rudinsky, 1963; Vité and Gara, 1962). In our study, however, the catch per unit of trap surface area decreased with increasing surface area. For the 0.19-m^2 traps, the catch was 81/0.19 m^2 and for the 0.37-m^2 traps, it was 48/0.19 m^2.

The fact that more beetles were caught at the higher traps at the far distance and at the lower traps at the close distance suggests that many of the beetles approached the area from a level higher than 1.5 m and flew down toward the source.

CONCLUSIONS

Large traps near the source could be expected to catch more beetles than small traps. Our study also indicates that more WPB were flying in the vicinity of a source of attractant than would be caught on a single small trap located at the source. The variation in catch with trap height and with distance and direction of the trap from the source of attractant, as well as with day and location of the test, suggests the need for careful interpretation of results from field assays that measure catch only at point sources of attractant with meager replication in space and time. These sources of variation should also be considered in designing tests to elucidate attractive behavior or evaluate relative attractiveness of compounds.

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ALLELOPATHIC INTERACTIONS AMONG ALGAE

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Abstract—Five species of green algae isolated from the Cleveland County, Oklahoma, area and a commercially obtained yellow-green alga were tested for allelopathic interactions. Axenic cultures of *Pandorina morum* Bory were stimulated in three sterile filtrates from nonaxenic cultures of *Scenedesmus incrassatulus* var. *mononae* G.M. Smith, with the oldest filtrate showing the least stimulation. By measuring the growth of axenic cultures inoculated into sterile filtrates of old cultures, instances of inhibition and stimulation were recorded in three screening experiments. *Cosmarium vexatum* West filtrate was inhibitory to the five other species, a characteristic probably important in its role of producing waterblooms in ponds and swamps. The allelopathic effects of filtrates of the five other species on *Botrydium becherianum* Vischer could be a key to its restriction to terrestrial sites. Instances of heat lability of the active substances were noted. These interactions, both allelopathic and stimulatory, probably play a significant role in the succession of algal blooms in nature.

Key Words—Algal succession, allelopathy, *Botrydium, Chlorella, Cosmarium, Pandorina, Pediastrum, Scenedesmus.*

INTRODUCTION

Algal allelopathy was first noted by Harder (1917), who recorded auto-inhibition in old cultures of *Nostoc punctiforme* (Kutz.) Hariot. More than a decade later, Akehurst (1931) postulated algal allelopathy as a factor in algal succession. Pratt and Fong (1940) established firm evidence of an algal inhibitor. They found old cultures of *Chlorella vulgaris* Beijerinck contained a substance which inhibited the growth of *Chlorella* and other algae. The substance, designated chlorellin, was later shown to possess antibiotic activity (Pratt et al., 1944).

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Lefèvre et al. (1952) demonstrated complex inhibitory and stimulatory effects among many species, particularly in the Chlorophyceae and the Cyanophyceae. Lefèvre (1964) distinguished between algal autoantagonism, and heteroantagonism, as noted by Lefèvre et al. (1952) in the death of algal cultures added to filtered water from blooms of Chlorophyceae and Cyanophyceae.

McVeigh and Brown (1954) noted stimulation or inhibition in mixed cultures of *Haematococcus pluvialis* Flotow em. Wille. and *Chlamydomonas chlamydogama* Bold, depending on the media used and the time in culture. Proctor (1957a) concluded that a fatlike extracellular substance produced by *Chlamydomonas reinhardi* Dang. and liberated upon death of the cells was responsible for elimination of *Haematococcus pluvialis* in mixed cultures. Rice (1954) concluded that antagonistic substances from freshwater phytoplankton are important in seasonal fluctuations of phytoplankton numbers as well as causing a definite succession of species. He earlier found that pond water containing a bloom of *Pandorina* was inhibitory to the growth of *Chlorella* and *Nitzchia.*

Instances of algal allelopathy are not limited to freshwater organisms. The diatomaceous phytoplankters of the Black Sea, *Skeletonema costatum* (Grev.) Cleve. and *Thalassionema nitzschioides* Grun. were found to be stimulatory or inhibitory to each other depending on the stage of growth (Kustenko, 1975). Pratt (1966) reported that the stimulatory or inhibitory effects of *Olisthodiscus luteus* on *Skeletonema costatum* accounted for the observed alternating dominance of the two species in Narragansett Bay.

Many genera of the Volvocaceae produce inhibitors which affect other genera in the family (Harris, 1971a). The inhibitor from culture filtrates of *Pandorina,* which affects most of the members of the family, is a low-molecular-weight, heat-labile compound which inhibits the light reactions of photosynthesis (Harris, 1971b, 1974).

The purpose of this study was to determine if allelopathic interactions occur between axenic cultures of algae isolated from the Norman, Oklahoma, area and relate any observed interactions to algal ecology.

METHODS AND MATERIALS

Cosmarium vexatum West, *Pediastrum boryanum* (Turp.) Menegh., *Pandorina morum* Bory, and *Scenedesmus incrassatulus* var. *mononae* G.M. Smith were collected and isolated from ponds in the Cleveland Co., Oklahoma, area by Lois Pfiester of the University of Oklahoma. *Chlorella ellipsoidea* Gerneck was collected and isolated from a damp wall of the University of Oklahoma greenhouse by James M. Wolfe. *Botrydium*

becherianum Vischer was obtained in culture from the Carolina Biological Supply, Burlington, North Carolina.

Axenic cultures were obtained by repeated ultrasonication and washing, followed by streaking on nutrient agar (Wiedeman, et al., 1964). Bacteria-free colonies were isolated from the agar surface and inoculated into test tubes containing autoclaved Carefoot's medium (Carefoot, 1968). After one month the isolates were tested for contamination by streaking a small aliquot on nutrient agar. Axenic stock cultures were maintained in Carefoot's medium in cotton-stoppered Erlenmeyer flasks. All cultures were grown in a growth chamber on a 14-hr photoperiod at 2200 ft-c and a temperature of 26.8°C and a 10-hr dark period at 21.0°C.

To determine if growth, as measured by absorbance readings, was closely correlated with growth as indicated by cell numbers, axenic cultures were grown in side-arm Erlenmeyer flasks with arms suitable for insertion in a spectrophotometer. Readings were made periodically at 400 nm, and samples were withdrawn for direct counting.

Initially, the growth of an axenic *Pandorina morum* culture in filter-sterilized filtrates from unialgal *Scenedesmus incrassatulus* cultures of three ages was examined. A 1-ml inoculum of a unialgal *Scenedesmus* culture was added to each of three 2-liter Erlenmeyer flasks containing 1 liter of Carefoot's medium. The cultures were maintained at the conditions mentioned for stock cultures. At 11, 19, and 55 days, cells were removed by filtering through Whatman No. 44 paper and the resulting filtrate filter-sterilized using 0.20-μm Gelman filters. Fifty milliliters of the filter-sterilized filtrates of each age were added to 50 ml of autoclaved double-strength Carefoot's medium in side-arm Erlenmeyer flasks. Controls containing 100 ml of autoclaved Carefoot's medium and double-strength controls containing 100 ml of autoclaved double-strength Carefoot's medium were prepared. All flasks were inoculated with 1 ml of an active axenic *Pandorina* culture. Two replicates were made for each flask and all flasks placed in a growth chamber under conditions used for stock cultures. Absorbance was read at 665 nm periodically for 15 days for each flask. Results for each two replicates were averaged and graphed.

Subsequently, a method modified from Lefèvre et al. (1952) and Harris (1971a), which employed old culture filtrates, was used to determine allelopathic interactions. One milliliter of an axenic culture was inoculated into 1 liter of Carefoot's medium. Flasks of each culture were placed in a growth chamber under the conditions previously described. After one month, the cultures were tested for contamination on nutrient agar and the media filtered twice through Whatman No. 44 paper to remove cells. The pH of each filtrate was adjusted to 5.8. After filtering through a 1.5-μm millipore filter, the filtrate was then filter-sterilized using a 0.20-μm Gelman filter apparatus, and the sterile filtrate stored at 5°C for no more than 1 week.

Subsequently each flask of filtrate was tested for sterility by streaking on nutrient agar, and each sterile filtrate was divided into three portions. One unamended portion was used directly, with no nutrients added. Another unamended portion was autoclaved for 20 min at 121°C. The third portion was mixed 1:1 with autoclaved double-strength Carefoot's medium to replenish any nutrients lost during growth of the old culture. This was termed the amended portion. A sterile Cornwall pipette was used to dispense each portion of each filtrate into 18 clear glass test tubes (Corning 9800) suitable for insertion in a Spectronic 20 spectrophotometer. Eighteen tubes each of autoclaved Carefoot's medium and 1.5-strength Carefoot's medium were used as controls.

Tubes were inoculated in triplicate with an actively growing culture from each of the six axenic stocks. At the time of inoculation, stock cultures were diluted with autoclaved Carefoot's to give an inoculum containing 50–100 cells or colonies per inoculation volume. *Botrydium becherianum* filtrate was not used in the first two screenings. For several weeks, each tube was periodically agitated for 10 sec with an automatic mixer and absorbance was read at 400 nm. At the conclusion of each screening, each tube was checked for contamination by streaking a small aliquot on nutrient agar, and at the conclusion of the second screening, narrow-range pH paper was used to check the pH of each tube.

Preparation of the filtrates for the third screening was similar except that both the amended and autoclaved portions were replenished directly with appropriate amounts of dry macronutrients so there would be no dilution of the filtrate. Micronutrients were added in solution, and inoculation was carried out as before. *Cosmarium* was not used as a test organism for the third screening. Controls of Carefoot's medium and double-strength Carefoot's medium were also run. Resultant absorbance readings were plotted against time, and changes in growth curves noted. Differences in growth curves at each sampling time were analyzed statistically by Student's *t* test.

RESULTS

Growth curves of *Scenedesmus incrassatulus* based on absorbance values paralleled those prepared from colony counts (Figure 1). Based on these two criteria, growth curves of the other species were similar. Thus, the rest of the results were based only on absorbance values.

Axenic cultures of *Pandorina morum* were strongly stimulated by sterile filtrates of nonaxenic *Scenedesmus incrassatulus* cultures (Figure 2). All three filtrates showed marked stimulation compared with the controls, the 55-day filtrate showing the least stimulation.

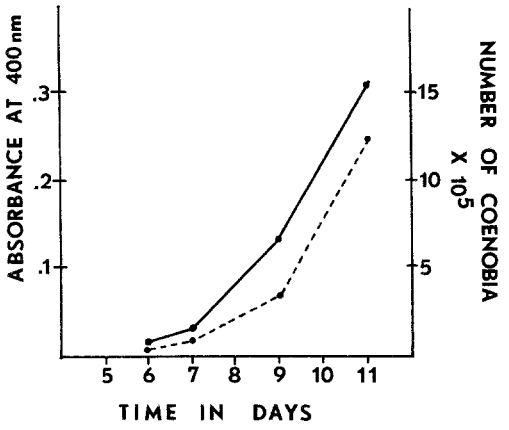

FIG. 1. Comparison of growth measurements for *Scenedesmus incrassatulus*. Solid line represents absorbance; broken line represents number of coenobia.

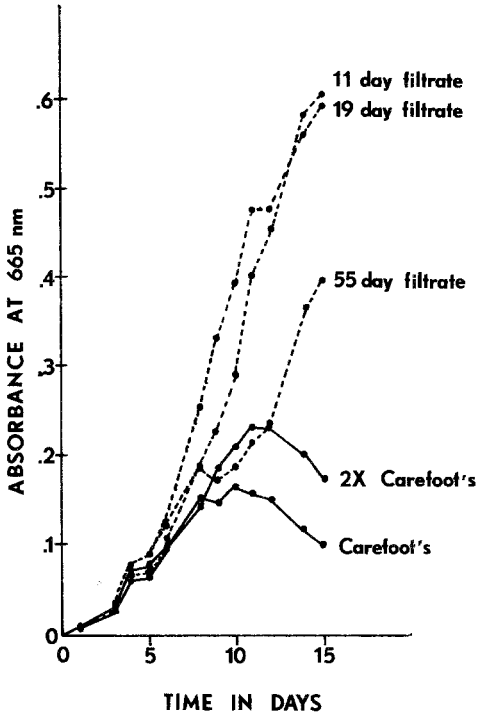

FIG. 2. Growth of *Pandorina morum* in sterile *Scenedesmus incrassatulus* filtrates.

TABLE I. ALGAL INTERACTIONS[a]

Test organism	Source of filtrate and screening number															
	Cosmarium vexatum			*Pediastrum boryanum*			*Scenedesmus incrassatulus*			*Chlorella ellipsoidea*			*Pandorina morum*			*Botrydium becherianum*
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	3
Botrydium becherianum	−*	−*	0	−*	−	−*	−*	−*	−*	−*	−	0	−*	−	−	−*
Pandorina morum	−*	− H	+* H	0	0	+*	−	0	+* H	−	−*	+*	−*	0	+*	+*
Scenedesmus incrassatulus	−	−*	0	+*	−*	−	0	m	+*	0	−*	+*	0	−* H	+* H	+*
Pediastrum boryanum	0	−*	0	+*	−	0	+	+	0	0	0	+	0 H	−*	+ H	+
Chlorella ellipsoidea	0	−	0	0	−*	0	0	−*	0	0	−*	+*	0	−*	+*	0
Cosmarium vexatum	+	+	X	+	+	X	0	+	X	0	0	X	0	0	X	X

[a]Symbols: −, inhibition; +, stimulation; 0, no effect; *, significant at 0.05 level of t statistic; H, active agent heat labile; m, missing; X, not tested.

Table 1 is a compilation of results from the three screenings employing filtrates from axenic cultures. A variety of interactions were evident, ranging from significant stimulation to significant inhibition, sometimes even between the same two species during the three screenings. *Cosmarium vexatum* generally significantly inhibited the growth of the five other species, but was autostimulatory. *Botrydium becherianum* was significantly inhibited by each of the six filtrates, including that from itself, in at least one of the three screenings. The *Botrydium* filtrate did not inhibit growth of any other species. Stimulation of some algal species by others was pronounced as in the case of *S. incrassatulus* by *B. becherianum* (Figure 3). Equally pronounced inhibition occurred in numerous instances.

The allelopathic agents in several of the filtrates were heat-labile (Table 1). Autoclaving of a particular filtrate did not eliminate its growth effect on all test organisms, however. This suggests that a particular filtrate sometimes had more than one allelopathic agent, that these agents affected different test species, and that the agents were not all heat labile in a given filtrate. *Cosmarium* had a pronounced effect on increasing the lag phase of growth of *Pandorina*, and this effect was lost on autoclaving the filtrate (Figure 4). On the other hand, the allelopathic agents produced by *Cosmarium* and effective against the other test species were not heat labile.

After 49 days of growth, pH values of the tubes from the second screening ranged from 4.8 to 6.7. This represented a change of approximately one unit from the initial value of 5.8. *Cosmarium vexatum*, *B. becherianum*, and *P. boryanum* showed a predominance of pH values less than 5.8 after the growth

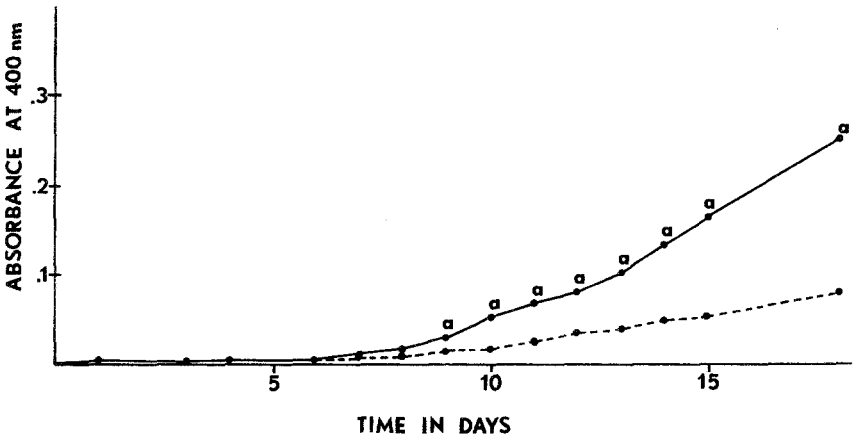

FIG. 3. Growth of *Scenedesmus incrassatulus* in *Botrydium becherianum* filtrate. Broken line represents 2× absorbance of Carefoot's controls; solid line represents absorbance of amended filtrate; *a* represents level of significance at $P < 0.05$ (Student's *t* test).

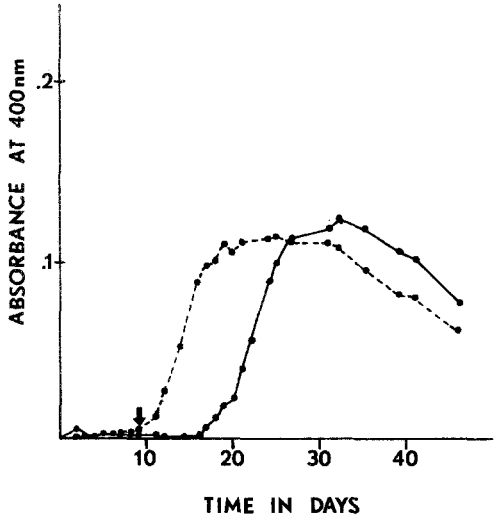

FIG. 4. Heat lability of allelopathic agents in *Cosmarium* filtrate as reflected by growth response of *Pandorina*. Solid line represents absorbance of unamended filtrate; broken line represents absorbance of autoclaved, unamended filtrate; arrow indicates start of log phase of growth in controls.

period. Sterile filtrates from old cultures of *S. incrassatulus* and *P. morum* were predominantly more acidic than the initial pH after any of the six test species grew in those filtrates.

DISCUSSION

The screening methods used in this study were devised to examine possible allelopathic interactions between a number of algal species. The use of unamended, amended (replenished with nutrients), and autoclaved sterile filtrates from old cultures eliminated the possibility that any interactions were the result solely of competition for nutrients, and provided data on the heat lability of the compounds involved. While mixed-culture experiments more closely approximate natural conditions, competitive effects of nutrient availability and shading cannot be separated from allelopathic interactions. The use of axenic cultures ensured that interactions were not the result of bacterial decomposition of the filtrates and that allelopathic agents were not produced by bacteria.

The preparation of old filtrates by filter-sterilization rather than by autoclaving was based on data of Harris (1971a), indicating the allelopathic substances produced by Volvocaceae are destroyed by heat. By monitoring

growth directly from the tubes with spectrophotometry, changes in growth patterns were detected without interruption of the system.

Age of algal cultures at the time filtrates were obtained for tests of allelopathic agents markedly affected the levels of growth responses in test algae (Figure 2). Lefèvre (1964) noted variation with time in the activity within a filtered medium. He believed this to be caused by the secretion of several active substances over time, with the action of a filtrate being determined by the type of active substance present in greatest amount at harvest time.

The variety of responses of one organism to the filtrate of another during the three screenings, ranging from significant stimulation to significant inhibition, probably resulted from such variables as the nature and size of the inoculum, amounts of active substances, and duration of the culture (Lefèvre, 1964). Pratt (1966) reported that the inhibitory or stimulatory effect of *Olisthodiscus* on *Skeletonema* depended on the concentration of the tannoid substance excreted by *Olisthodiscus*. Pratt and Fong (1940) and Pratt (1942) found chlorellin to be autostimulatory or inhibitory depending on concentration. Chlorellin was determined later to be a combination of at least three active compounds (Jørgensen and Steeman-Nielsen, 1961; Jørgensen, 1962).

The allelopathic effects of *C. vexatum* on the other test species is probably important ecologically. Lefèvre (1964) reported that waterblooms in ponds and swamps of fluctuating pH are sometimes caused by *Cosmarium*. If *Cosmarium* eliminated its competition by excreting a substance allelopathic to other phytoplankton species such as *Pandorina*, *Pediastrum*, and *Scenedesmus*, it would probably reproduce more rapidly and form a bloom.

Botrydium becherianum was adversely affected by filtrates of all species, including itself, and it significantly stimulated some species. *Botrydium* grows luxuriantly in liquid Carefoot's medium, yet in nature is largely terrestrial (Smith, 1950). The sensitivity of *Botrydium* to allelopathic substances produced by several species of phytoplankton and its stimulatory effect on those species are probably important factors restricting it to mainly terrestrial situations. Significantly, *Botrydium* filtrate did not affect *Chlorella ellipsoidea* which is a common terrestrial species.

Lefèvre et al. (1952) suggested that the active metabolites produced by blue-green algae which inhibit other species may be important in controlling species succession and species dominance within a given phytoplankton population. Keating (1977) found convincing evidence to the direct role of algal allelopathy in succession. The bloom sequence in eutrophic Linsley Pond over a period of three years was correlated with the effects of cell-free filtrates of dominant blue-green algae on their successors and predecessors. The effects of heat-labile allelopathic and stimulatory filtrates from blue-green algae isolated from Linsley corresponded with the rise and fall of bloom populations in situ.

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PRIMATE CHEMICAL COMMUNICATION, PART III[1] Synthesis of the Major Volatile Constituents of the Marmoset (*Saguinus fuscicollis*) Scent Mark

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Abstract—This paper presents the stereospecific synthesis of the long-chain butyrate esters (15 in number) which in addition to squalene comprise the major volatile constituents of the scent mark of the marmoset monkey, *Saguinus fuscicollis*.

Key Words—primate, chemical communication, marmoset monkeys, butyrates, synthesis, *Sanguinus fuscicollis*.

INTRODUCTION

In connection with our continuing studies concerning primate chemical communication, we recently reported on the isolation and identification of the major volatile constituents (1-16) present in the odoriferous scent mark of the marmoset monkey, a South American primate (Smith et al., 1976; Yarger et al., 1977). Although the importance of these substances to marmoset chemical communication is currently unknown, Epple (1974a,b) has demonstrated that the intact scent mark of the marmoset communicates to conspecifics a variety of information including the sex, social status, and identity of the donor monkey.

In order to assign rigorously the structures of the major constituents of

[1]For part II of this series, see Yarger, R.G., Smith, A.B., III, Preti, G., and Epple, G., 1977. *J. Chem. Ecol.* 3:45-56.
[2]Camille and Henry Dreyfus Teacher-Scholar, 1978-1983.

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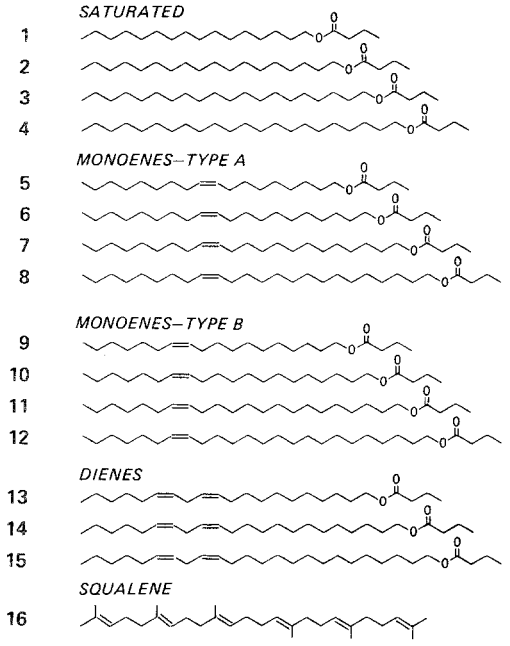
the scent mark, as well as to provide suitable quantities (e.g., 200–500 mg/each) of pure authentic samples of each ester for biological testing, an alternative source of 1–15 was sought. It is with these considerations in mind that we report here our stereospecific approach to these esters.

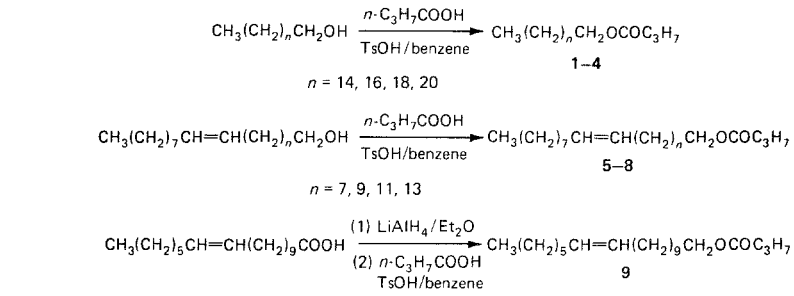
Initial examination of the major components of the scent mark revealed the existence of four major classes of esters, namely saturated butyrates (1–4), monosaturated butyrates (5–12) of two structural types (designated A and B) dependent on the location of the olefinic linkage, and diunsaturated butyrates (13–15). In addition, the configuration of each site of unsaturation was found to be cis. Finally, as often occurs with other mammalian fatty acids, each member of a class differed from the next by a two-carbon unit.



SYNTHETIC STUDIES

We initiated synthetic studies with the structurally simple saturated and monounsaturated butyrate esters (1–8). These esters were easily prepared in high-yield via acid-catalyzed esterification of the corresponding commercially available, albeit expensive, straight-chain alcohols with *n*-butyric acid. Similarly, ester 9 was prepared by esterification of (*Z*)-11-octadecen-1-ol which in turn was available in near-quantitative yield from the $LiAlH_4$ reduction of the corresponding commercially available unsaturated acid, (*Z*)-11-octadecenoic acid. In each case the spectroscopic properties, including the low resolution mass and 220-MHz NMR spectra as well as the high-resolution gas

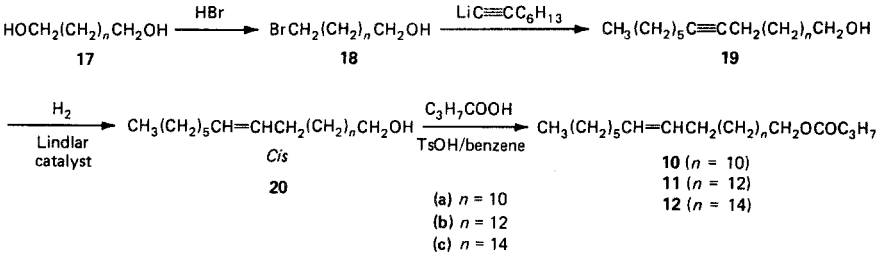
chromatographic retention properties (100-m, SF-96 glass capillary column), were identical to the esters isolated from the marmoset scent mark.



SCHEME 1

We next turned our attention to the preparation of monounsaturated esters 10–12. These esters differ only in the number of methylene units between the ester functionality and the olefinic bond. Our approach employed the synthetic strategy developed several years ago by Hendry and coworkers (1975). Specifically, an eight-carbon synthon containing a precursor of the required cis olefinic bond was added to a difunctionalized carbon chain of appropriate but variable length. A suitable eight-carbon synthon appeared to be the lithium salt of 1-octyne.

To this end, the commercially available diol (17a) was treated with concentrated hydrobromic acid at 85° C for 18 hr with simultaneous heptane extraction. This afforded a crystalline bromo alcohol (18a) melting at 30–31° C in 86% yield after chromatography and recrystallization. Similar treatment of diols 17b and 17c gave bromo alcohols 18b and 18c in 50 and 79% yield, respectively. The bromo alcohols (18a-c) were next transformed to ynols 19a-c via treatment in hexamethyl phosphorus triamide with the lithium

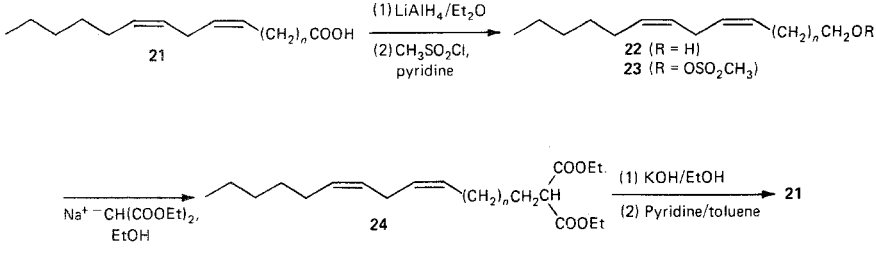


SCHEME 2

salt of 1-octyne, the latter readily prepared from 1-octyne and 1.1 equiv of *n*-butyl lithium. The crystalline ynols (19a-c) were obtained in 60–80% yield. At this point there remained only the stereospecific reduction of the acetylenic linkage to the required cis olefin, and esterification with *n*-butyric acid to

complete the preparation of butyrates 10–12. The former transformation was accomplished via hydrogenation at atmospheric pressure over 5% palladium on barium sulfate poisoned with a small amount of synthetic quinoline, while esterification was effected as described for butyrates 1–18 (*n*-butyric acid, TsOH, and benzene).

The fact that the above transformation led predominantly to the cis unsaturated butyrates was established by high-resolution capillary VPC (100-m, SF-96 glass capillary column). Analysis of various mixtures of oleyl butyrate (5) (C-22 Δ^9 cis) and elaidyl butyrate (C-22 Δ^9 trans) by VPC demonstrated that under our chromatographic conditions as little as 0.5% of elaidyl butyrate was easily detected when added to oleyl butyrate. Similar analysis of our synthetic samples demonstrated in each case that they were >95% configurationally pure.



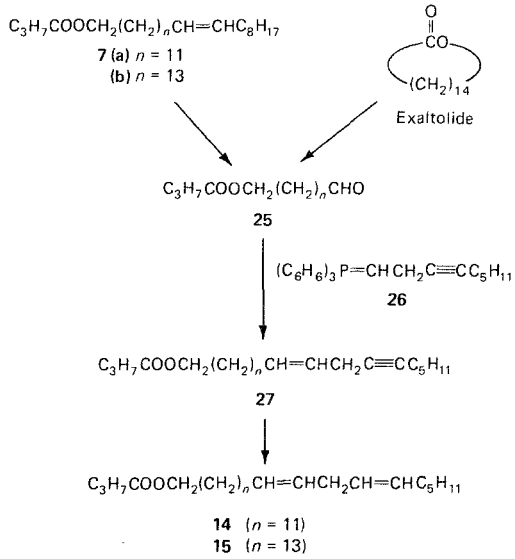
(a) $n = 7$
(b) $n = 9$
(c) $n = 11$
(d) $n = 13$

SCHEME 3

At this point there remained the preparation of the diunsaturated butyrates 13–15 of group four. The availability of (*Z,Z*)-11,14-eicosadienoic acid (21b), albeit expensive, would provide easy access to 13 via the straightforward reduction-esterification sequence employed in the preparation of 9. Butyrates 14 and 15, on the other hand, provided considerably more synthetic challenge. Two possible synthetic strategies appeared feasible. The first, but clearly the least elegant, would involve three consecutive two-carbon malonic ester-chain elongation-reduction sequences on the alcohol derived from readily available (i.e., inexpensive) cis-linoleic acid, followed at each stage by esterification with *n*-butyric acid. This approach by necessity provides alcohol 22b, thereby eliminating the need to acquire the expensive acid 21b.

Alternatively, careful examination of butyrates 14 and 15 suggested that if the appropriate long-chain aldehyde-ester fragments 25a and 25b were readily available, and if condensation with the previously unreported Wittig reagent (26) derived from 1-bromo-3-nonyne followed by subsequent hydro-

genation were in each case effected in a stereospecific cis manner, then an efficient synthetic route to 14 and 15 would be in hand. Interestingly, the aldehyde-ester fragment required for butyrate 14 is present in the previously prepared butyrate 7. Ozonolysis of 7 would provide ready access to aldehyde-ester 25a. Moreover, the C_{15} aldehyde-ester synthon 21b required for the preparation of butyrate 15 appeared to be reasonably accessible from exaltolide. In particular, hydrolysis of the macrocyclic lactone would provide



SCHEME 4

15-hydroxypentadecanoic acid which could in turn be esterified with *n*-butyric acid and subsequently transformed to the requisite aldehyde-ester (21b) by selective reduction of the carboxylic acid functionality with borane-THF (Brown and Korytnyk, 1960) followed by Collins oxidation (Ratcliffe and Rodehorst, 1970). Since the latter synthetic strategies provided the opportunity to explore more interesting chemistry, the decision was made to proceed initially with the preparation of aldehyde-ester (25).

To this end, ozonolysis of 7 followed by reductive work-up with triphenyl phosphine gave 13-oxotridecan-1-yl butyrate (25a) in 52% yield after column chromatography on silica gel. Treatment of this aldehyde with Wittig reagent 26 in THF followed by work-up and column chromatography on silica gel afforded the enyne 27a in 72% yield. Analysis of the product by high-resolution VPC (SF 96 capillary, 100 m × 0.66 mm ID, 200° C) indicated the presence of two compounds in a ratio of 87:13. This ratio corresponds very closely to the cis-trans ratio (86:14) for an analogous Wittig reaction, run

under identical conditions, reported in a comprehensive study by Anderson and Henrick (1975).

Next, the enyne butyrate mixture (27a) was hydrogenated using Lindlar catalyst. The NMR spectrum (220 Hz) of the product mixture indicated the complete disappearance of starting material (i.e., absence of the broad, two-proton singlet at 2.91 ppm corresponding to methylene protons: $=CHCH_2C\equiv$) and of any components possessing a triple bond (i.e., absence of any resonance at 2.16 ppm for methylene protons: $-CH_2CH_2C\equiv$). Signals for allylic methylene (2.03 ppm) and doubly allylic methylene (2.77 ppm) protons were present but integration showed their relative ratio to be 6.8:1.0. The desired diene (14) requires a ratio of only 2:1, thus indicating that overhydrogenation had taken place. Indeed, the high-resolution gas chromatogram displayed four peaks. The retention time of peak A (63.2 min, 47%), corresponded to that of a sample of the desired, naturally occurring diene butyrate 14, while that of peak B (64.6 min, 17%) corresponded to (*Z*)-13-docosenyl butyrate (7). Docosanyl butyrate (4) was not present. Proton NMR and VPC data suggest that peaks C and D (66.3 min, 16%, and 67.5 min, 19%, respectively) were other monoene isomers arising from the starting enyne mixture.

Due to the lack of stereospecificity in the Wittig condensation and the difficulties encountered in the subsequent hydrogenation, we returned to the malonic acid-chain elongation strategy. To this end, commercially available (*Z,Z*)-9,12-octadecadenoic acid (21a) was reduced in 96% yield to the corresponding alcohol (22a) with $LiAlH_4$. Conversion into mesylate 23a proceeded in 90% yield upon treatment of 22a at 0° C with methansulfonyl chloride in pyridine. The resultant mesylate was then homologated with the sodium salt of diethyl malonate. Hydrolysis of the derived diester afforded the corresponding diacid which, without purification, was decarboxylated employing the extremely mild conditions of Cordes et al. (1968); the diacid was heated at reflux for 2 hr in a solution of toluene and pyridine (5:1, v/v). The resultant homologated acid was purified at this point via silica gel chromatography. Reduction of the pure acid (21b) led to alcohol 22b in 90% yield, which was then ready both for conversion to butyrate 13, and further homolgation to alcohols 22c and 22d, respectively. Indeed, a similar series of transformations provided alcohols 22c and 22d in 37 and 39% overall yield from the respective acids (21b and 21c). Final conversion of alcohols 22b-d to butyrates 13-15 was effected in near-quantitative yield as described previously (see above).

Purification of the fifteen butyrates prior to spectroscopic comparison with the authentic butyrates (1-15) was accomplished where necessary by preparative high-pressure liquid chromatography on a silver-loaded macroporous cation exchange column (Warthen, 1976) with final purity being established using high-resolution gas chromatographic analysis on the

aforementioned 100-m SF-96 glass capillary column. In each case the butyrates were >95% configurationally pure and displayed 220 MHz NMR, IR, and VPC retention properties identical to the authentic butyrates.

METHODS AND MATERIALS

Vapor-phase chromatography was performed on a Perkin-Elmer model 990 gas chromatograph using the following column: 3% SF-96 open tubular glass capillary column dynamically coated, 100 m × 0.66 mm ID. The helium carrier gas flow rate was 10 ml/min, and the oven temperature ranged from 180 to 210°C. Compounds isolated were obtained either as colorless oils or white solids. Melting points were obtained on a Thomas Hoover capillary melting point apparatus and are uncorrected. Solutions were dried over $MgSO_4$ unless specified otherwise. IR spectra were obtained for CCl_4 solutions using a Perkin-Elmer model 237 spectrophotometer. NMR spectra were obtained in $CDCl_3$ solutions using a Varian HR-220 (220 MHz) spectrometer. Column chromatographies were performed using Hi-Flosil silica gel 60/200 mesh (Applied Science Laboratories, State College, Pennsylvania). High-pressure liquid chromatography (HPLC) was performed on a Perkin-Elmer model 601 HPLC using a 7-mm ID × 183-cm stainless-steel column packed with silver-loaded macroporous cation exchange resin (37–74 μm, AG MP-5, Bio Rad Laboratories, Richmond, California) eluting with methanol.

Butyrates 1–15: General Esterification Procedure. A solution of approx. 367 mg of cetyl alcohol, 2 ml of *n*-butyric acid, and 40 mg of *p*-toluenesulfonic acid in 40 ml of benzene was heated at reflux gently overnight with the azeotropic removal of H_2O using a micro Dean-Stark trap. The reaction was cooled, diluted with hexane, and washed twice with 5% aqueous NaOH, H_2O, and brine. The solution was dried, and the solvent was removed in vacuo to afford 467 mg (99%) of hexadecyl butyrate (1). Infrared and NMR data for butyrates 1–15 may be found in Yarger et al. (1977).

(Z)-11-Octadecen-1-ol. A solution of 200 mg of (*Z*)-11-octadecenoic acid in 2 ml anhydrous ether was added to a stirred suspension of 260 mg of $LiAlH_4$ in 40 ml of dry ether protected by a drying tube. The reaction mixture was heated at reflux for 3 hr, cooled to room temperature, and sodium sulfate decahydrate was added to consume the excess $LiAlH_4$. The solution was filtered, the residue rinsed with ether, the combined filtrates were evaporated in vacuo to afford 184 mg (97%) of the alcohol as an oil: IR 3340(m), 3005(w), 2935(s), 2855(s), 1460(m) cm^{-1}; NMR (220 MHz) δ 0.87 (t, J = 6Hz, 3H), 1.26 (s, br, 22H), 1.53 (m, 2H), 1.99 (m, 4H), 3.61 (t, J = 6 Hz, 2H), 5.30 (m, 2H).

12-Bromodecan-1-ol (18a). A mixture of 5.0 g of 1,12-dodecanediol and 25 ml of 48% aqueous hydrobromic acid was placed in an apparatus equipped to permit its continuous extraction with heptane. The reaction flask was

stirred and heated at 85° C; the extraction was allowed to proceed overnight. The heptane solution was cooled and diluted with ether. This solution was washed successively with H_2O, aqueous $NaHCO_3$, H_2O, 5% aqueous sodium thiosulfate, and H_2O and dried. The solvent was removed in vacuo and the resulting oil chromatographed on silica gel. Gradient elution with hexane–ether provided 5.65 g (86%) of the pure bromo-alcohol (18a). A sample was recrystallized from hexane: mp 30–31° C (lit. 29° C, Chuit et al., 1927); IR 3360(m), 2940(s), 2860(s), 1460(m), 1050(m) cm^{-1}; NMR (220 MHz) δ 1.27 (s, br, 16H), 1.55 (m, 2H), 1.84 (p, J = 6 Hz, 2H), 3.40 (t, J = 6 Hz, 2H), 3.62 (t, J = 6 Hz, 2H).

14-Bromotetradecan-1-ol (*18b*). Starting with 7.13 g of 1,14-tetradecanediol, the procedure used for the preparation of 18a provided 4.49 g (50%) of bromo-alcohol 18b: mp 40–41° C (lit. 46° C, Chuit et al., 1927); IR 3360(m), 2940(s), 2860(s), 1460(m), 1050(m) cm^{-1}; NMR (220 MHz) 1.25 (s, br, 20H), 1.55 (m, 2H), 1.84 (p, J = 12 Hz, 2H), 3.39 (t, J = 12 Hz, 2H), 3.62 (t, J = 12 Hz, 2H).

16-Bromohexadecan-1-ol (*18c*). Starting with 4.00 g of 1,16-hexadecanediol, the procedure used for the preparation of 18a provided 3.94 g (79%) of bromo-alcohol 18c: mp 52–53° C (lit. 53–54° C, Chuit and Hausser, 1929); IR 3360(m), 2940(s), 2860(s), 1460(m), 1050(m) cm^{-1}; NMR (220 MHz) δ1.25 (s, br, 24H), 1.55 (m, 2H), 1.83 (p, J = 6 Hz, 2H), 3.39 (t, J = 6 Hz, 2H), 3.62 (t, J = 6 Hz, 2H).

13-Eicosyn-1-ol (*19a*). To a stirred solution of 550 mg of 1-octyne in dry THF (distilled from $LiAlH_4$) maintained at −78° C under nitrogen was added 2 ml of *n*-butyllithium (2.5 M in hexane, 1 equiv). After 2 hr, a solution of 530 mg (0.4 equiv) of 12-bromodecan-1-ol (18a) in 1.5 ml of dry HMPA was added and the mixture maintained at −78° C an additional hour. The reaction was allowed to warm at room temperature and was stirred under nitrogen overnight. Water was added, and the mixture was partitioned between water and ether. The ether solution was washed five times with H_2O, once with brine, dried, and the solvent removed in vacuo. The residue was chromatographed on silica gel eluting with a hexane–ether gradient to afford 718 mg (95%) of the pure alkynol 19a. Recrystallization from hexane afforded an analytical sample: mp 37–38° C; IR 3680(w), 2940(s), 2850(s), 1460(m) cm^{-1}; NMR (220 MHz) δ 0.87 (t, J = 7 Hz, 3H), 1.25 (s, br, 26H), 1.48 (m, 2H), 2.12 (t, J = 7 Hz, 4H), 3.61 (t, J = 6 Hz, 2H).

Analysis: calculated for $C_{20}H_{38}O$: C, 81.56; H, 13.01; found: C, 81.79; H, 12.98.

15-Docosyn-1-ol (*19b*). Using the same procedure outlined for the preparation of 19a, 2.00 g of 14-bromotetradecan-1-ol (18b) afforded 1.57 g (71%) of ynol 19b: mp 46.9–47.4° C (hexane); IR 3680(m), 2940(s), 2850(s), 1460(m) cm^{-1}; NMR (220 MHz) δ 0.87 (t, J = 7 Hz, 3H), 1.25 (s, br, 30H), 1.45 (m, 2H), 2.11 (t, J = 7 Hz, 4H), 360 (t, J = 6 Hz, 2H).

Analysis: calculated for $C_{22}H_{42}O$: C, 81.91; H, 13.13; found: C, 81.96; H, 13.12.

17-Tetracosyn-1-ol (19c). Using the same procedure outlined for the preparation of 19a, 1.11 g of 16-bromohexadecan-1-ol (18c) afforded 854 mg (71%) of alkynol 19c: mp 53.0–53.5° C (hexane); IR 3680(w), 2940(s), 2850(s), 1460(m) cm^{-1}; NMR (220 MHz) δ 0.86 (t, J = 7 Hz, 3H), 1.25 (s, br, 34 H), 1.48 (m, 2H), 2.11 (t, br, J = 6 Hz, 4H), 3.60 (t, J = 6 Hz, 2H).

Analysis: calculated for $C_{24}H_{46}O$: C, 82.21; H, 13.23; found: C, 82.37; H, 13.19.

(Z)-13-Eicosen-1-ol (20a). A stirred mixture of 885 mg of 13-eicosyn-1-ol (19a), 24 mg of 5% palladium on barium sulfate (Research Inorganic Chemical Corp., Belleville, New Jersey), and 0.03 ml of synthetic quinoline in 9 ml of anhydrous methanol were hydrogenated at atmospheric pressure and room temperature until the uptake of hydrogen appeared to cease (approximately 1 equivalent of hydrogen absorbed). The mixture was filtered to remove the catalyst. The catalyst was rinsed repeatedly with ether, and the combined filtrates were concentrated in vacuo. The residue was taken up in ether and washed once with water, twice with 5% aqueous hydrochloric acid, twice with water, dried, and the solvent removed in vacuo to afford 885 mg (99%) of the enol 20a as an oil. An analytical sample was prepared by chromatography on silica gel eluting with hexane–ether: IR 3340(m), 3010(w), 2940(s), 2860(s), 1460(m) cm^{-1}; NMR (220 MHz) δ 0.87 (t, J = 7 Hz, 3H), 1.26 (s, br, 26 H), 1.53 (m, 2H), 2.00 (m, 4H), 3.62 (t, J = 6 Hz, 2H), 5.30 (m, 2H).

Analysis: calculated for $C_{20}H_{40}C$, 81.01; H, 13.60; found: C, 80.93; H, 13.66.

(Z)-Docosen-1-ol (20b). Using the same procedure described for the preparation of 20a, 1.56 g of 15-docosyn-1-ol (19b) provided 1.55 (99%) of enol 20b: IR 3340(m), 3010(w), 2940(s), 2860(s), 1460(m) cm^{-1}; NMR (220 MHz) δ 0.87 (t, J = 7 Hz, 3H), 1.25 (s, br, 30H), 1.53 (m, 2H), 1.98 (m, 4H), 3.62 (t, J = 6 Hz, 2H), 5.30 (m, 2H).

Analysis: calculated for $C_{22}H_{44}O$: C, 81.41; H, 13.66; found: C, 81.28; H, 13.62.

(Z)-17-Tetracosen-1-ol (20c). Using the same procedure described for the preparation of 20a, 793 mg of 17-tetracosyn-1-ol (19c) provided 759 mg (95%) of enol 20c: IR 3340(m), 3010(w), 2940(s), 2860(s), 1460(m) cm^{-1}; NMR (220 MHz), δ 0.87 (t, J = 6 Hz, 3H), 1.26 (s, br, 34H), 1.53 (m, 2H), 2.00 (m, 4H), 3.62 (t, J = 6 Hz, 2H), 5.31 (m, 2H).

Analysis: calculated for $C_{24}H_{48}O$: C, 81.74; H, 13.72; found: C, 81.92; H, 13.80.

13-Oxotridecan-1-yl Butyrate (25a). A solution of 742 mg of erucyl butyrate (7) in 20 ml of CH_2Cl_2 was ozonized at −78° C for 4 hr in a Supelco microozonolysis apparatus. The reaction mixture was then thoroughly flushed with N_2, 590 mg of triphenyl phosphine was added, and the reaction

was allowed to warm to room temperature. The solvent was removed in vacuo and the residue was chromatographed on 75 g of silica gel, eluting with a hexane–ether gradient to afford 278 mg (52%) of the aldehyde-ester: IR 2930(s), 2850(m), 2700(w), 1740 (s, br), 1455(w), 1160(m) cm^{-1}: NMR (220 MHz) δ 0.94 (t, J = 7 Hz, 3H), 1.27 (s, br, 18H), 1.64 (m, 4H), 2.28 (t, J = 6 Hz, 2H), 2.42 (t, J = 6 Hz, 2H), 4.06 (t, J = 6 Hz, 2H), 9.76 (s, 1H).

Analysis: calculated for $C_{17}H_{32}O_3$: C, 71.79; H, 11.34; found: C, 71.86; H, 11.36.

3-Nonyn-1-yl Triphenyl Phosphonium Bromide. A solution of 1.0 g. of 1-bromo-3-nonyne and 1.42 g of triphenylphosphine in 8 ml of chlorobenzene was heated at reflux for 24 hr. The solvent was removed in vacuo, and the residue was triturated with ethyl acetate at 0° to afford 1.44 g. (63%) of 3-nonyn-1-yl triphenyl phosphonium bromide. Recrystallization from ethyl acetate containing approx. 1% of methanol provided pure 3-nonyn-1-yl triphenylphosphonium bromide: mp 144–145° C.

Analysis: calculated for $C_{27}H_{30}BrP$: C, 69.68; H, 6.50; found: C, 69.60; H, 6.47.

13-Docosen-16-yn-1-yl Butyrate (27a). Ylide (26) was generated by adding 0.4 ml of a 2.5 M solution of *n*-butyllithium in hexane to a stirring suspension of 465 mg of 3-nonyn-1-yl triphenyl phosphonium bromide in 10 ml of dry tetrahydrofuran under nitrogen at 0° C. After stirring for 30 min at 0° C, a solution of 125 mg of 13-oxotridecan-1-yl butyrate in 2 ml of dry tetrahydrofuran was added. Stirring was continued for 1 hr while the reaction was allowed to warm to room temperature. The mixture was poured into water to quench the reaction and was extracted into hexane. The hexane solution was washed four times with water, once with saturated brine solution, dried, and concentrated in vacuo to a volume of about 5 ml. Precipitated triphenyl phosphonium oxide was removed by filtration and the filtrate was passed through 5 g. of silica gel eluting with 60 ml of 5% ether in hexane. Removal of the solvent in vacuo provided 159 mg (92%) of 13-docosen-16-yn-1-yl butyrate (27a) as an oil. An analytical sample was prepared by chromatography on silica gel eluting with hexane-ether: IR 3010(w), 2930 (s, br), 2850(m), 1740(m), 1460(w) cm^{-1}; NMR (220 MHz) δ (0.94 (m, 6H), 1.26 (s, br, 24H), 1.59–1.72 (m, 4H), 2.02 (m, br, 2H), 2.14 (t, J = 6 Hz, 2H), 2.28 (t, J = 7 Hz, 2H), 2.90 (s, br, 2H), 4.06 (t, J = 7 Hz, 2H), 5.42 (s, br, 2H).

Analysis: calculated for $C_{26}H_{46}O_2$: C, 79.94; H, 11.87; found: C, 79.72; H, 11.87.

(Z,Z)-13,16-Docosadien-1-yl Butyrate (14). Using the same procedure described for the preparation of 20a, 78 mg of 13-docosen-16-yn-l-yl butyrate (27a), 1.6 mg of palladium on barium sulfate, and 3.6 mg of synthetic quinoline in 0.6 ml of ethanol afforded 74 mg of a mixture of isomers (see text). The desired (*Z,Z*)-13,16-docosadien-1-yl butyrate (14) was the

predominant product (47%) and exhibited the same chromatographic and spectral properties as a sample of natural material.

1,1-Dicarbethoxynonadeca-10,13-diene (24a). A solution of 26.8 g of (*Z,Z*)-9,12-octadecadienoic acid (linoleic acid, Nu-Chek Prep, Inc., Elysian, Minnesota) in 50 ml of ether was added dropwise to a stirred suspension of 7.34 g of lithium aluminum hydride in 600 ml of ether under nitrogen at a rate which maintained a gentle reflux. The reaction was heated at reflux for 4 hr, cooled to room temperature, and the excess $LiAlH_4$ was consumed by adding sodium sulfate decahydrate. The solution was filtered and the residue was rinsed several times with ether. The combined ether solutions were washed with 5% sodium hydroxide and saturated brine solution and dried. Removal of the solvent in vacuo gave 24.3 g (96%) of (*Z,Z*)-9,12-octadecadien-1-ol (22a). This alcohol was converted directly to its mesylate by slowly adding 71.1 g of alcohol 22a to a stirring solution of 36.9 g of methanesulfonyl chloride in 800 ml of pyridine (dried by distilling from barium oxide) protected by a drying tube. After 3 hr the reaction was complete (TLC, silica gel, hexane–ether 1:1). The solution was poured into 800 ml of ice water and extracted twice with hexane. The combined hexane extracts were washed successively five times with water, once with saturated brine solution and dried. Removal of the solvent in vacuo afforded 78.5 g (85%) of (*Z,Z*)-9,12-octadecadien-1-yl mesylate (23a) which should be used immediately for the next reaction. To this end 8.55 g of sodium metal was dissolved by heating in 1000 ml of dry ethanol at reflux (distilled from sodium) under nitrogen atmosphere. The solution was cooled to room temperature, 81.2 g of diethyl malonate was quickly added, and the mixture was stirred for 30 min. The freshly prepared mesylate, 78.5 g, was then added dropwise at room temperature and the reaction heated at reflux for 4 hr. After cooling to 0°C, 1000 ml of water was added, and the reaction was made acidic with concentrated hydrochloric acid. The reaction was extracted three times with 300 ml of hexane. The combined hexane extractions were washed three times with water, once with saturated brine solution, and dried. Evaporation of the solvent in vacuo gave 83.8 g (90%) of 1,1-dicarbethoxynonadeca-10,13-diene (24a). An analytical sample was prepared by chromatography on silica gel using hexane–ether: IR 3010(w), 1750(m), 1730(s) cm^{-1}; NMR (220 MHz) δ 0.86 (t, br, J = 7 Hz, 3H), 1.27 (s, br, 24H), 1.85 (m, 2H), 2.01 (m, 4H), 2.74 (m, 2H), 3.27 (t, J = 6 Hz, 1H), 4.15 (q, J = 7 Hz, 4H), 5.30 (m, 4H).

Analysis: calculated for $C_{25}H_{44}O_4$: C, 73.48; H, 10.85; found: C, 73.45; H, 10.78.

(Z,Z)-11,14-Eicosadienyl Butyrate (13). A solution of 55.5 g of crude 1,1-dicarbethoxynonadeca-10,13-diene (24a) in 500 ml of 10% methanolic potassium hydroxide was heated at reflux for 1 hr. The reaction was cooled and slowly poured into 2000 ml of water, agitating to dissolve all solids. This aqueous solution was washed four times with ether. The aqueous solution was

cooled to ice temperature, acidified to pH 3 using concentrated hydrochloric acid, and extracted three times with ether. The combined ether layers were washed twice with water, once with saturated brine, dried, and the solvent removed in vacuo to provide 36.3 g (76%) of 1,1-dicarboxynonadeca-10,13-diene. A mixture of 31.8 g of this diacid with 64 ml of pyridine and 320 ml of toluene was heated vigorously at reflux for 2 hr. The solvents were removed in vacuo and the residue was taken up in hexane. The hexane solution was washed five times with water, once with saturated brine, dried, and the solvent was removed in vacuo. The residue was chromatographed on silica gel eluting with hexane-ether to provide 26.1 g (94%) of pure *Z,Z*-11,14-eicosadienoic acid (21b) which was identical in all respects (GC, IR, NMR) to an authentic sample (Nu-Check Prep, Inc.). This acid was reduced with $LiAlH_4$ to its alcohol (22b) using the same procedure described for the preparation of alcohol 22a. The (*Z,Z*)-11,14-eicosadien-1-ol (22b) was then esterified with butyric acid as outlined in the general esterification procedure to afford synthetic *Z,Z*-11,14-eicosadienyl butyrate (13) which was identical in all respects (IR, NMR, MS) to the naturally occurring butyrate.

1,1-Dicarbethoxyheneicosa-12,15-diene (*24b*). Using the procedure described for the preparation of 24a, 11,14-eicosadienoic acid (21b) was converted successively to the alcohol (22b), the mesylate (23b), and finally to the diester (24b) in an overall 78% yield. An analytical sample of 24b displayed the following spectroscopic properties: IR 3010(w), 1750(s) cm^{-1}; NMR (220 MHz) δ 0.86 (t, br, J = 7 Hz, 3H), 1.28 (s, br, 28H), 1.86 (m, 2H), 2.01 (m, 4H), 2.74 (m, 2H), 3.27 (t, J = 6 Hz, 1H), 4.15 (q, J = 7 Hz, 4H), 5.31 (m, 4H).

Analysis: calculated for $C_{27}H_{48}O_4$: C, 74.26; H, 11.08; found: C, 74.17; H, 11.27.

(*Z,Z*)-*13,16-Docosadienyl Butyrate* (*14*). Using the procedure described for the preparation of butyrate 13, 1,1-dicarbethoxyheneicosa-12,15-diene (24b) was hydrolyzed, decarboxylated, reduced, and esterified in an overall yield of 48% to provide synthetic *Z,Z*-13,16-docosadienyl butyrate (14) which was identical in all respects (IR, NMR, MS) to the naturally occurring material.

1,1-Dicarbethoxytricosa-14,17-diene (*24c*). Using the procedure described for the preparation of 24a, *Z,Z*-13,16-docosadienoic acid (21c) was converted successively to the alcohol 22c, the mesylate 23c and finally to the diester 24c in an overall 70% yield. An analytical sample of 24c displayed the following spectroscopic properties: IR 3010(w), 1750(m), 1730(s) cm^{-1}; NMR (220 MHz) δ 0.87 (t, br, J = 7 Hz, 3H), 1.27 (s, br, 32H), 1.84 (m, 2H), 2.00 (m, 4H), 2.74 (m, 2H), 3.27 (t, J = 6 Hz, 1H), 4.15 (q, J = 7 Hz, 4H), 5.30 (m, 4H).

Analysis: calculated for $C_{29}H_{52}O_4$: C, 74.95; H, 11.28; found: C, 75.05; H, 11.30.

(*Z,Z*)-*15,18-Tetracosadienyl Butyrate* (*15*). Using the procedure de-

scribed for the preparation of butyrate 13, 1,1-dicarbethoxytricosa-14,17-diene (24c) was hydrolyzed, decarboxylated, reduced, and esterified in an overall yield of 56% to provide synthetic *Z,Z*-15,18-tetracosadienyl butyrate (15) which was identical in all respects (IR, NMR, MS) to the naturally occurring material.

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AN EFFICIENT SYNTHESIS OF MUSCALURE FROM JOJOBA OIL OR OLEYL ALCOHOL

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Abstract—A four-step synthesis of (*Z*)-9-tricosene ("muscalure"), a component of the pheromone of the housefly, from jojoba oil (or three-step from oleyl alcohol) by 3-carbon (or 5-carbon) unit elongation was developed in overall high yield. The sequence of reactions and the purity of the products could be easily followed, with relatively good accuracy, by NMR technique.

Key Words—(*Z*)-9-tricosene, muscalure, housefly, pheromone, *Musca domestica*, jojoba oil, oleyl alcohol, sex attractant, Diptera.

INTRODUCTION

Several methods for the synthesis of muscalure, (*Z*)-9-tricosene, a sex attractant pheromone of the housefly (*Musca domestica* L.) have been published recently. These include a Wittig reaction on the appropriate aldehyde and phosphorane (Carlson et al., 1971; Bestmann et al., 1974), homologation of erucic acid (Cargill and Rosenblum, 1972; Richter and Mangold, 1973), homologation of 1-pentadecyne followed by partial hydrogenation (Eiter, 1972; Kovalev, 1977), mixed Kolbe electrolysis of oleic acid and heptanoic acid (Gribble and Sanstead, 1973), homologation of oleic acid, or oleyl alcohol derivative, with C_5 reagents (Ho and Wong, 1974; Julia, 1976; Abe, 1977) and metathesis of olefins (DiNunno and Flario, 1975; Kupper and Streck, 1976; Nakamura and Echigoya, 1977).

One can summarize these procedures as a combination of $C_{14} + C_9$, $C_{22} + C_1$, $C_{15} + C_8$, $C_{17} + C_6$, $C_{18} + C_5$, and $C_{14} + C_9$, or $C_{13} + C_{10}$ units, respectively.

Mansingh et al. (1972) have shown that a 7:3 mixture of (*Z*)-9-tricosene

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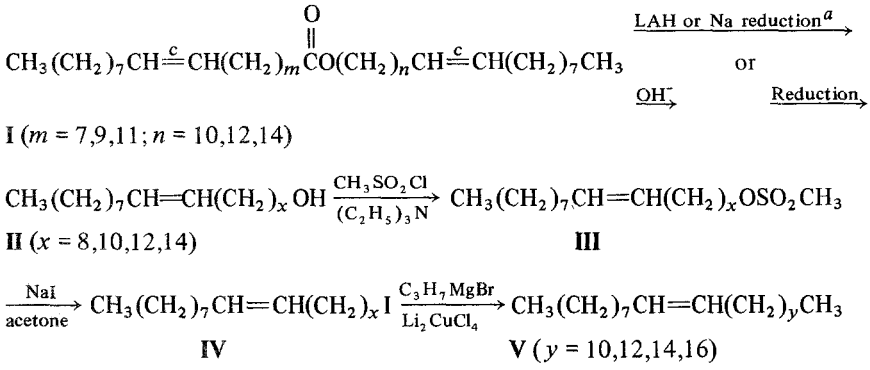
SCHEME 1



[a] Molaison et al., 1959.

and (*Z*)-9-heneicosene shows considerably higher useful biological activity than the pure (*Z*)-9-tricosene or the commercially available muscalure, although Carlson et al. (1974) and Richter et al. (1976) could not confirm this. On the other hand, higher homologs (C_{25}, C_{27}, C_{29}) and different isomers show lower activity (Carlson et al.,1971, 1974; Richter et al., 1976) but seem not to interfere with the biological activity of muscalure. Other components of the pheromone were identified recently (Uebel et al., 1976).

Combining all these facts, together with the potential importance of muscalure in pest control, we searched for an adequate supply from an economical starting material coupled with efficient synthesis.

TABLE 1. VARIABILITY OF JOJOBA ACIDS AND JOJOBA ALCOHOLS AFTER HYDROLYSIS (GLC%)

Methyl esters of acids	Ariz. desert	Tuscon suburbs	Calif. desert	Calif. oceanside
18:1	11	11	10	7
20:1	70	71	70	69
22:1	13	15	15	17
24:1	2	2	2	3
Alcohols				
20:0	0.8	0.6	0.8	2.1
20:1	46	46	42	30
22:0	2	2	2	6
22:1	42	41	47	47
24:0	0.2	0.2	0.1	0.4
24:1	7	8	8	13

Jojoba oil is a liquid wax (I) (see Scheme 1) and its composition is given in Table 1 (Miwa, 1971, 1972, 1974). The jojoba oil (50% of dried seeds) is extracted from the seeds of the desert bush jojoba [*Simmondsia chinensis* (Link)]. Its price might be cheaper than that of oleyl alcohol and oleic and erucic acids as its cultivation in southwest U.S. and Israel is at an advanced stage (Sherbrooke and Hasse, 1974; Maugh, 1977).

METHODS AND MATERIALS

General. Crude jojoba oil was purified by mixing with bleaching earth at 60° for 30 min and then filtering under vacuum. The crude product after each chemical transformation was used without any purification for the next step. Usual workup included pouring the reaction mixture into H_2O, extraction with petroleum ether (60–80), washing with saturated NaCl solution and drying over anhydrous Na_2SO_4. IR and NMR spectra showed product identity and purity. All NMR spectra showed the terminal CH_3 as triplet at δ 0.92–0.94, an intense signal at 1.2–1.4 for all aliphatic hydrogens, and signal at 1.98–2.05 for allylic hydrogens. Other signals are summarized in Table 3. Integration curves fitted for the different hydrogens. The mixture of iodides (IV) was analyzed on GC/MS and the olefins (V) were compared on GC (Packard 7400, on 6 ft × 2 mm ID glass column, 10% SP 2300 on acid wash chromosorb W at 190°) with authentic samples.[1]

Solvents. Petroleum ether (60–80) was dried over $CaCl_2$ and distilled. Ether was dried over $CaCl_2$, then on Na and distilled. Pyridine was kept on KOH prior to the reaction. Acetone was dried over $CaCl_2$. THF was kept on KOH, passed through basic alumina and dried over CaH_2. Benzene was dried over Na.

LAH Reduction of Jojoba Oil to Jojoba Alcohols (II). To a suspension of 8.35 g LAH (0.22 mol) in 1100 ml of dry ether a solution of 117 g of jojoba oil (0.2 mol) in 150 ml dry ether was added dropwise within 15 min. After 15–30 min of mild reflux, excess of LAH was destroyed by wet ether and then a saturated solution of Na_2SO_4 until a white precipitate of Li and Al hydroxides completely separated. The residue after evaporation (107 g, 92%) showed no carbonyl absorption in IR; $n_D^{25} = 1.4582$.

Mesylate of Jojoba Alcohols (III). To a solution of 58 g of jojoba alcohols (II) (0.2 mol) in 500 ml dry ether, 30.4 g of triethylamine (0.3 mol) was added. Then, 27.5 g of methanesulfonyl chloride (0.24 mol) was added dropwise while outside cooling kept the temperature at 0–5°. After stirring for 30 min at this temperature and regular workup, 67 g of III (90%) was isolated; $n_D^{25} = 1.4651$.

[1]Thanks to Dr. A. Yaron and Miss K. Fried for these measurements.

Jojoba Iodides (IV). A mixture of 32.6 g of mesylate III (0.088 mol) in 200 ml dry acetone and 19.8 g NaI (0.132 mol) was stirred and refluxed for 2.5 hr. After regular workup, 32.4 g of iodide (91%) was isolated; $n_D^{25} = 1.4885$.

Muscalure and Other Olefins in a Mixture (V). A Grignard reagent of propyl bromide was prepared from 4.86 g Mg (0.2 g-atom) in 10 ml dry THF and 24.6 g C_3H_7Br (0.2 mol) in 100 ml C_6H_6, all under N_2. The reaction mixture was cooled to 0°, 40.6 g of iodides IV (0.1 mol) in 70 ml C_6H_6 was added dropwise, and only slight change in temperature was detected. Then 25 ml of Li_2CuCl_4 solution in THF (0.1 M) was added dropwise while the inside temperature was kept at 0–5°. After 2 hr at this temperature and warming to room temperature, the reaction mixture was poured into a NH_4Cl solution, to give 30.6 g of olefins (95%); $n_D^{25} = 1.4580$. By fractional distillation, one can enrich the C_{23} fraction, which is distilled at 125–130°/0.01 mm and contains >85% of (*Z*)-9-tricosene. When oleyl alcohol (technical grade, ~75% purity) was used (*Z*)-9-tricosene (95% pure) was distilled at 152–155°/0.05 mm. The yield of isolated distilled product was 60% based on oleyl iodide; $n_D^{25} = 1.4525$.

RESULTS AND DISCUSSION

Careful examination of the composition of the American jojoba oil reveals that by reduction of the ester function one can get a mixture of four long-chain alcohols C_{18}, C_{20}, C_{22}, C_{24} in the rough ratio of 5:50–58:27–32:5–8. On the other hand, hydrolysis of the ester and reduction of the acidic fraction, by using it as an acid mixture, gives a better ratio for C_{20}, namely: $C_{18}:C_{20}:C_{22}:C_{24} \approx 10\text{–}11:70:13\text{–}15:2$. Thus, by C_3 unit elongation of the alcohols, which already contain the cis double bond in the right position, one can get a rough mixture of four olefins, C_{21}, C_{23}, C_{25}, C_{27}, in the same ratio as above. This gives a mixture of C_{23} and C_{21} in the ratio of 10–11 to 1 or 7 to 1. The overall synthesis is outlined thus in the scheme.

All the above reactions proceed smoothly, in high yields (over 90% each), in a short time, and more importantly, the double bond is not isomerized. Thus, the LAH reduction of 100–150 g of oil is usually completed within 30 min, but quenching of excess of LAH should not be carried out with ethyl acetate. This is because it was found that transesterification occurs and acetate esters of the long-chain alcohols are obtained up to 10% (found by GC/MC[2] and NMR):

[2]Measurements were conducted on a DuPont 491 BR, on 6 ft × 2.1 mm ID glass column, 3% OV 101 on Chromo G; temperature programed from 150° to 280° at 6°/min. Thanks to J.A. Schmit from DuPont Lab., Wilmington, Delaware, for these measurements.

$$CH_3(CH_2)_7CH{=}CH(CH_2)_mCH_2OLi + CH_3\overset{\overset{O}{\|}}{C}OC_2H_5 \longrightarrow$$

$$CH_3(CH_2)_7CH{=}CH(CH_2)_mCH_2O\overset{\overset{O}{\|}}{C}CH_3 + C_2H_5OLi$$

The use of mesylate is preferred to the tosylate, which forms an unworkable emulsion. It is also important to note that pyridine is not the best base and solvent for the preparation of the mesylate, as it reacts as a nucleophile with the mesylate to give up to 25% of alkyl pyridinium salts as by-product:

$$CH_3(CH_2)_7CH{=}CH(CH_2)_xOSO_2CH_3 + \text{N}\bigcirc \longrightarrow$$

III

$$CH_3(CH_2)_7CH{=}CH(CH_2)_x{-}\overset{\oplus}{\text{N}}\bigcirc\; CH_3SO_3^{\ominus}$$

VI

It was found that the mesylate reacts slowly with pyridine at room temperature (~90%, after 9 days), but much faster at 50–60° (>95% reaction after 45 min). The relatively high yield of alkyl pyridinium salt obtained despite the low temperature in the reaction mixture (outside ice cooling) suggests local heating during addition of CH_3SO_2Cl to the pyridine solution of II. The fact that the alkyl pyridinium salt VI contains a long hydrophobic chain causes this product to remain in the organic phase and to separate as a semisolid phase when it is dissolved in ether. If it is transferred to the next step (III→IV) it then separates as semisolid material, thus giving two phases of iodides: the upper one, clean oil, containing only IV, and lower one, semisolid material, containing iodides IV and alkyl pyridinium salts (OH^- or I^- in VI, instead of $CH_3SO_3^{\ominus}$). So, triethylamine in ether or CH_2Cl_2 is a better system for preparation of III.

Different halides were checked for preparation of IV or its analogs (Table 2). The best conditions are those mentioned in Scheme 1.

The last step of elongation of the long chain with a C_3 unit is easily achieved by using Li_2CuCl_4-catalyzed coupling of IV with C_3H_7MgBr (Tamura and Kochi, 1974). Confirmation of the correct chain length was obtained by comparison with pure olefins prepared from oleyl iodide and the appropriate alkyl halide Grignard (C_3, C_5, C_7). The oleyl iodide was synthesized from oleyl alcohol by the same procedure outlined in Scheme 1.

When we tried to add a C_1 unit by reacting CH_3MgI with IV, a very low yield of olefin was obtained, if at all. Dimethyl lithium

TABLE 2. REACTION OF OLEYL AND JOJOBA ALCOHOL MESYLATE WITH DIFFERENT HALIDES[a]

Starting mesylate	Amount (mmol)	Halide		Solvent[b]	Time (hr)	Yield[c]
		MX	mmol			
Jojoba	13.5	NaBr	25.7	Acetone	5	<5
	13.5	KBr	25.8	Acetone	5	<5
Oleyl	1.94	KBr	30.1	THF	5	83
	1.94	$MgBr_2$ (anhydrous)	28.8	THF	5	76
	2.3	$MgBr_2$ (anhydrous)	29.3	Ethyl ether	4	29
	2.0	$MgBr_2 \cdot 6H_2O$	29.4	THF	5	[d]
	2.0	$CaBr_2$ (anhydrous)	30.4	THF	4.5	91
	160	$CaBr_2$ (anhydrous)	320	Acetone	4.5	78
	2.0	NaI	30	Acetone	1	88
	1.88	NaI	31.6	Acetone	3	92
Jojoba	88	NaI	132	Acetone	2.5	91
Oleyl	2.2	KI	32.5	THF	5	79

[a]This part of the work was conducted by Mrs. N. Kornberg as an undergraduate project.
[b]All solvents were dried prior to the reaction.
[c]Yields are based on isolated crude halide as measured by NMR.
[d]Oleyl alcohol was obtained.

cuprate [$(CH_3)_2CuLi$] was found to be a much better reagent, but even in this case the yield never exceeded 60-65%.[3]

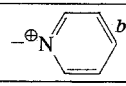
When the mesylate III was tried in reaction with C_3H_7MgBr or CH_3MgI at $-10°$ to 0°, only exchange occurred to yield the corresponding bromide or iodide. At $-75°$ some coupling occurred to yield olefin V and the iodide IV.[2]

All the reactions were followed by NMR by using the $-CH_2-Y$ group ($Y = C_5H_5N^+$, OMes, OCOR, Cl, Br, I, OH) to identify the mixture and to calculate its exact composition in relatively accurate measurement (±2% of absolute amount) (Table 3). This was confirmed by preparing known mixtures, where small amounts of "impurities" of one or two components could be detected in amounts as little as 2-3% of the mixture.

Acknowledgments—The author wishes to thank Mrs. Z. Sidon and Miss M. Marbach for technical assistance.

[3]For recent review on carbon-carbon bond formation via organocopper reagents, see Posner (1975), Ashby and Lin (1977), Eiter et al. (1978).
[4]For tosylate reactions, see Fouquet (1974), Schlosser (1974), Normant (1977), and Krauss (1977).

TABLE 3. CHEMICAL SHIFTS OF α-METHYLENE (*t*, J = 6 cps) IN DIFFERENT LONG-CHAIN DERIVATIVES: $CH_3(CH_2)_7CH{=}CH(CH_2)_{x-1}\underline{CH_2}Y$

Y	$-\overset{\oplus}{N}$ 	OSO_2CH_3[c]	OCOR	OH	Cl	Br	I
δ[a]	4.88	4.10	3.95	3.45	3.38	3.26	3.08

[a] δ, ppm downfield from TMS as internal standard in CCl_4 solution. The olefinic protons appear at 5.20. Spectra were run on a Varian XL-100.
[b] The aromatic hydrogens appear at 9.18 (2H, *t*, J = 6 cps, C_2—H); 8.40 (1H, *t*, J = 6 cps, C_4—H); 8.04 (2H, *t*, J = 6 cps, C_3—H). For similar systems see J.A. Elvidge and L.M. Jackman, *J. Chem. Soc.* 1961:860. The $\underline{CH_3}SO_3^-$ group appears as a sharp singlet at 2.68.
[c] The methyl group appears as a sharp singlet at 2.88.

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SPECIFICITY OF TRAIL MARKERS OF FOREST AND EASTERN TENT CATERPILLARS

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Abstract—Exploratory trails deposited on paper strips by the forest tent caterpillar (FTC), *Malacosoma disstria* Hubner, and the eastern tent caterpillar (ETC), *M. americanum* (Fabricius), as well as extracts of these trails, readily elicited interspecific trail-following behavior. In 2-choice tests involving simple Y mazes constructed from these paper strips, the caterpillars of both species preferred by approximately 3:1 the trails of the FTC. Studies involving whole colonies of the ETC maintained under near-natural conditions in the laboratory, however, indicated that the trails deposited by successful foragers of the ETC as they returned to their tent from feeding sites were more attractive than the exploratory trails of either the ETC or FTC. The pronounced interspecific response of these congeners to each other's trails suggests that they utilize either qualitatively similar or identical trail-marking chemicals. Both species preferred their own trails to those of *Archips cerasivoranus* (Fitch) (Tortricidae), providing the first evidence that more distantly related lepidopterous larvae utilize distinct trails.

Key Words—Lepidoptera, Lasiocampidae, *Malacosoma americanum, Malacosoma disstria,* Tortricidae, *Archips cerasivoranus*, trail marker, pheromone, interspecific response, tent caterpillar.

INTRODUCTION

The phytophagous larvae of *Malacosoma disstria* Hubner, the forest tent caterpillar (FTC), and *M. americanum* (Fabricius), the eastern tent caterpillar (ETC), deposit conspicuous silk trails as they forage over the branches of host trees. The trails of the ETC lead from a communal tent constructed in the branches of the host tree to remote feeding sites. Recent studies of this species

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have shown that one or more trail chemicals rather than physical properties of the silk strand elicit the trail-following response (Fitzgerald, 1976; Fitzgerald and Gallagher, 1976). The trail-following behavior of the FTC has not been previously investigated. This species does not construct a tent but the larvae feed and rest gregariously and form a diffuse network of trails as they forage. The caterpillars occur sympatrically over much of their range (Stehr and Cook, 1968). The FTC may develop on the same rosaceous host species to which the ETC is limited, but it has a broader range of host plants and is usually found on other species. In the spring of 1976, however, we found that caterpillars of both species had dispersed from their colonies and were feeding and resting side by side on infested trees in St. Lawrence County, New York. Observations in this area suggested that the two species were utilizing each other's trails and prompted laboratory studies to determine the specificity of the trail markers utilized by these congeners.

METHODS AND MATERIALS

Egg masses or young colonies of the ETC were collected from two different locations in Cortland County, New York. Egg masses of the FTC were collected from St. Lawrence County and newly eclosed larvae from the SUNY Cortland Campus. Caterpillars were maintained on black cherry leaves in the laboratory at 20 ± 1°C under a 16:8-hr light-dark regime.

Colonies of the ETC were established on tripods constructed from wooden dowels to simulate conditions under which the tents are naturally formed. Small contingents of FTC were added to these colonies, and daily observations of the interactions of the two species were made.

To obtain trails for interspecific response studies, 40 fourth instar larvae of each species were allowed to circle on separate 9-cm diameter by 10-mm wide rings cut from filter paper. The rings were suspended above the substrate by pins to prevent the caterpillars from walking off. After 2 hr the caterpillars were removed, and the rings were cut into 25-mm long strips. The response of each species to the other's trail marker and to blank (control) strips was tested by placing individual second instar larvae at the start of each strip and recording the time taken to reach the other end.

Another study was conducted to determine if the FTC was responsive to an extract of the silk trail of the ETC. Silk was obtained by allowing 10 fourth instar ETC to circle overnight in a petri plate. Twenty-four milligrams of the silk spun on the glass by the ETC during this period were collected and extracted in 3 ml of methylene chloride. The extract was concentrated to 0.2 ml then laid out in 5 μl quantities along 20-mm lines on paper cards. Lines were indicated by pencil dots placed at each end. The responses of second instar FTC to these trails and solvent control trails were observed. Response

of the ETC to an extract of the FTC trail was similarly tested. In this case, extract was prepared from trails deposited by 20 fourth instar FTC allowed to circle on a suspended filter paper ring. After 75 min, the caterpillars were removed and the filter paper was cut into small pieces which were then extracted. Unused filter paper was extracted to obtain a control solution.

Tests were also conducted to determine the response of each species when allowed to choose between its own and the other species trail. To obtain trails for this test, 10 caterpillars of each species were placed at the bottom of vertically suspended 4 mm × 30 cm strips of black construction paper and allowed to walk to the top. The caterpillars moved slowly and deposited silk as they proceeded up the strips. The strips were then cut into 25-mm long sections and used to form the alternate arms of a Y maze (Figure 1). The stem of the maze (runway) was prepared by allowing 5 of each species to walk up a

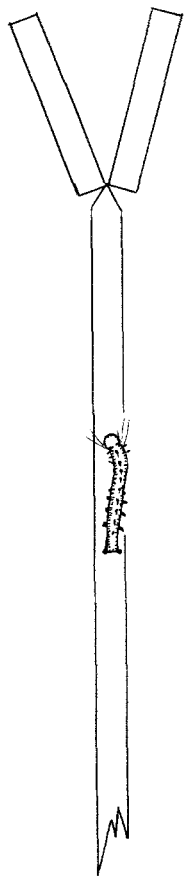

FIG. 1. Paper Y maze used to determine the specificity of tent caterpillar trails.

single 10-cm strip. Preference tests were conducted by starting individual larvae at the base of the runway and allowing them to choose between the two arms. Since test larvae reinforced the trails as they passed over them, strips serving as the alternate arms of the maze were used only once but runways were reused. The species tested was alternated for each test. The angle between the arms was adjusted to assure that the head of the larva swept across the bases of both arms before the insect proceeded and the position of the trails was alternated after every two tests to preclude a positional bias. All larvae used in these tests, either to establish trails or as experimental subjects, were fed 4–6 hr prior to testing.

RESULTS

Behavior of Caterpillars in Mixed Colonies. Two colonies of fourth instar ETC to which 10 fourth instar FTC were added were observed over 5-day periods. The FTC established resting sites on the surface of the ETC tents soon after introduction but thereafter occasionally rested together in small groups on the branches or leaves of the host tree between their feeding periods. The FTC were sometimes joined by ETC larvae as they rested on the surface of the tent, but for the most part the ETC larvae rested separately inside of their tent between forages. The FTC foraged as a group either independent of the ETC or more commonly along with them, the two species often feeding side by side. The caterpillars utilized common trails as they moved over the branches of the host tree and over the surface of the tent. After feeding, the FTC frequently returned to the tent along with the ETC. The FTC followed the trails constructed by the ETC on their tents which led to the tent entrance. The FTC larvae, however, never went inside of the tents. The FTC formed molting mats on the host tree but returned to the tent following ecdysis. The activity of the ETC on the tent apparently prevented molting on the surface of the structure. No interspecific agonism was observed.

Interspecific Responses to Trail Markers. ETC larvae readily followed trails of fourth instar FTC deposited on filter paper. The caterpillars crossed 25-mm long trail sections in 32 ± 5.4 sec ($\pm$SE) ($n = 10$), but refused to cross filter-paper controls. FTC larvae also crossed the trails of fourth instar ETC similarly prepared in an average of 47 ± 7.5 sec ($n = 10$). Control strips elicited no response.

Both species followed solvent extracts of the other's trail. The ETC crossed 20-mm long FTC extract trails in an average of 47 ± 8.5 sec ($n = 10$), and the FTC crossed the ETC extract trail in an average of 31 ± 4.9 sec ($n = 10$). Neither species showed any response to solvent control trails during 3-min trials.

Unlike preceding instars, the last instar larvae (sixth) of both the ETC

and FTC did not deposit silk while circling on filter paper rings or while moving over the branches of the host tree. Since it was previously reported that the earlier instars of the ETC can deposit a chemical trail factor without simultaneously depositing a silk strand (Fitzgerald, 1976), tests were undertaken to determine if sixth instars marked trails in this manner. Seven sixth instar larvae of both species were allowed to circle overnight on filter paper rings enclosed in separate petri dishes. Filter paper so treated, and a solvent extract of the paper, elicited both intra- and interspecific following responses from second instar larvae of both species, indicating that the sixth instar larvae of both species utilize chemical trail markers.

Choice Tests. Preliminary testing of the two species of caterpillars showed that construction-paper Y mazes could be used with confidence to demonstrate the presence or absence of a chemical trail. For these initial tests, one arm of the maze consisted of a blank strip of construction paper; the other arm and the runway were prepared from strips previously crossed by conspecifics. The caterpillars started at the base of the runway, moved readily down the runway, then typically hesitated at the choice point and swung their heads across the bases of each of the alternate arms before proceeding. In all of 10 separate tests for both species, the caterpillars moved onto the treated arm, turned when they reached the end of the strip, then moved back down the runway. Interspecific choice tests were then conducted by allowing individuals of each species to choose between alternate arms bearing either the trail of conspecifics or the trails of the other species. In three separate groups of tests, involving colonies of caterpillars obtained from two different field locations, approximately 75% of the caterpillars of both species preferred the trails deposited by the FTC (Table 1).

A marked preference of ETC larvae for FTC trails was also observed in a follow-up study. Trails of hungry FTC were established on the surface of ETC tents near the onset of the host colony's activity period by placing 5–10 fourth instar larvae at the upper end of a randomly selected tripod leg and allowing them to walk down to the tent's surface. In each of three separate tests the ETC detected these trails soon after the onset of their activity period and small contingents moved up the tripod legs used to introduce the FTC. Trails deposited on tripod legs by hungry ETC larvae that were removed from the surface of the tent, then reintroduced in the identical manner as the FTC, elicited no detectable response from their tentmates. Since it was previously shown that trails reinforced by ETC larvae as they return to their tent from feeding sites are significantly more attractive to their tentmates than the exploratory trails of hungry caterpillars, an additional series of tests was conducted to determine if the ETC would prefer the trails of successful foragers over those of the FTC.

Groups of 10 ETC were fed, then immediately placed at the bottom of

TABLE 1. INTERSPECIFIC RESPONSE OF *Malacosoma americanum* AND *M. disstria* CATERPILLARS TO TRAILS DEPOSITED ON PAPER STRIPS.

Replicate[a]	Species tested	Number of larvae tested	Trail selected		Percentage of larvae choosing *M. disstria* trail	χ^{2b}
			M. americanum	*M. disstria*		
1	*M. americanum*	22	6	16	73	4.54
	M. disstria	21	7	14	67	2.34
2	*M. americanum*	17	4	13	76	4.76
	M. disstria	17	5	12	71	2.88
3	*M. americanum*	19	5	14	74	4.76
	M. disstria	20	2	18	90	12.8
Totals	*M. americanum*	58	15	43	74	13.5
	M. disstria	58	14	44	76	15.5

[a]Each replicate tests different colonies.
[b]Probability that χ^2 will exceed 3.84 due to chance alone is 5%.

paper strips and allowed to walk to the top. Subsequent testing showed, however, that these caterpillars deposited trails of only nominal strength. Tests were therefore conducted under more natural conditions. A colony of fourth instar ETC was isolated from its food supply at the onset of an activity period causing the hungry caterpillars to lay down exploratory trails over the previously unsilked legs of the tripod supporting their tent. When the caterpillars were regularly moving up and down the legs, indicating that new exploratory trails had been established, a foliated branch and a branch manually stripped of its leaves were placed along side the tent and 10 caterpillars were allowed to move onto each. As soon as the caterpillars were collected, the branches were moved away from the tent. A spot of light from a high-intensity lamp was directed onto the surface of the tent to restrict further activity by the remainder of the colony. Tent caterpillars respond to light so directed by aggregating motionlessly under it. When the caterpillars that had been transferred to the foliated branch had finished feeding, that branch and the defoliated branch were placed at the ends of different tripod legs, randomly selected, and the caterpillars were allowed to return to the tent. Simultaneously, 10 FTC not fed for 6 hr were placed at the end of the remaining tripod leg and allowed to walk down to the tent. The fed ETC larvae became inactive after contacting their resting tentmates or entered the tent directly. The FTC, however, wandered over the tent and had to be removed shortly after reaching the surface to prevent them from laying trails on either of the other two tripod legs. When all three groups of caterpillars had been returned, new foliated branches were placed at the end of each tripod leg, the spotlight was turned off, and the response of the hungry ETC to each of the tripod legs was observed. The results of three separate tests appear in Table 2. In two of these tests the ETC strongly preferred the trails deposited by their fed tentmates over those of the FTC. In the remaining test, the caterpillars selected the trails deposited by their fed tentmates and those of the FTC in approximately equal numbers. This test, however, is less representative than the other two, since the tripod leg used to introduce the FTC led directly to the tent entrance. During the course of the test, a number of the ETC went back into the tent, then reemerged later. Upon reemergence, these caterpillars immediately encountered the FTC trail and many moved directly up the arm bearing this trail rather than first circling on the tent surface where contact with all trails would have occurred. The lack of significant difference in the response of ETC larvae to the trails of their fed tentmates and the FTC trails in spite of this bias, and the marked preference shown in the other tests, indicates that the trails of the fed ETC provide a stronger stimulus than that of the FTC. In all three tests, trails deposited by the unfed ETC elicited little or no response from their tentmates.

Intergeneric Specificity of Trail Markers. The response of these

TABLE 2. RESPONSE OF *Malacosoma americanum* CATERPILLARS TO TRAILS OF FED TENTMATES, UNFED TENTMATES, AND *M. disstria* DEPOSITED ON THE LEGS OF THE TRIPOD SUPPORTING THEIR TENT

Replicate	Number of larvae responding	Trail selected (%)			
		M. americanum			
		Unfed	Fed	*M. disstria*	χ^{2a}
1	63	0	79	21	21.7
2	80	5	46	49	0.05
3	38	0	71	29	6.74

[a]Tests fed *M. americanum* and *M. disstria* only. Probability that χ^2 will exceed 3.84 due to chance alone is 5%.

congeners to each other's trails raised the possibility that caterpillars in general might utilize relatively nonspecific trail-marking chemicals. No other studies had yet been reported that showed that any degree of trail specificity existed among the Lepidoptera. To further investigate this possibility, we obtained a colony of the more distantly related *Archips cerasivoranus* (Fitch) (Tortricidae). The larvae of this species feed gregariously on the leaves of cherry and form a conspicuous web enclosing the branches of the host. The caterpillar is not a trail follower in the strict sense but the larvae travel short distances beyond the boundary of their web to extend the structure and occasionally move over longer distances to new feeding sites. The caterpillars deposit silk as they proceed and are hesitant to move over unsilked substrates.

Third instar *Archips* were made to walk up a vertically suspended strip of construction paper in the manner previously described for *Malacosoma*. The caterpillars deposited copious quantities of silk on the surface of the strip as they proceeded. When individual *Archips* were later placed on these trails, they confined their activity to them, turning each time they reached the end of the strips. When the strips were arranged in the form of a Y maze, and the caterpillars allowed to choose between alternate arms of 25-mm long sections of blank paper or prepared trail, all turned onto the silked strip ($n = 10$). Choice tests were then conducted to determine if *Archips* and *Malacosoma* trails elicited interspecific following responses. For these tests, alternate arms of the maze were cut from long strips over which 10 ETC, 10 FTC, or 10 *Archips* had walked. Runways 10 cm long were prepared by letting 5 *Archips* and 5 of the species of *Malacosoma* to be tested walk up a common strip. The results of the study reported in Table 3 show that the insects preferred their own trails. Both *Archips* and *Malacosoma*, however, followed each other's trails when placed directly on them, indicating that the trails share some common properties.

TABLE 3. INTERSPECIFIC RESPONSES OF *Archips cerasivoranus, Malacosoma americanum*, AND *M. disstria* TO TRAILS DEPOSITED ON PAPER STRIPS

Species tested	Number of larvae tested	Trail selected (%)		
		M. americanum	*M. disstria*	*A. cerasivoranus*
M. americanum	10	100	[a]	0
M. disstria	10	[a]	90	10
A. cerasivoranus	10	[a]	0	100
A. cerasivoranus	15	0	[a]	100

[a]Not tested.

DISCUSSION

The pronounced interspecific response elicited by the trails of the tent caterpillars suggests that the insects employ similar or identical trail marking chemicals. The potentially deleterious consequences of utilizing a trail marker to which a sympatric congener is responsive are probably not realized in these species, since partitioning of preferred hosts largely precludes competitive interactions.

The employment of a dual-trail system by the ETC to distinguish exploratory trails from those that lead directly to new food finds contrasts with the behavior of many trail-following ants which utilize visual cues to maintain contact with their nest and deposit trail-marking pheromones only after locating food (Wilson, 1971). Due to the small number of FTC egg masses available to us during the course of this study, and the less tractable behavior of this species, we did not determine whether the FTC also employs different trail types. The FTC is a more nomadic species than the ETC, however, and may have no need for more than simple trails.

Little is presently known of the chemical nature of the trail markers of tent caterpillars. Behavioral evidence is compatible with the possibility that the two species share a single marker which is secreted in varying quantities, but qualitatively different compounds or different mixtures of the same substances may be involved. Studies are now underway to determine the site of secretion, source, and chemical identity of the trail marker(s) of the ETC.

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PHEROMONES OF HAMSTER VAGINAL DISCHARGE
Attraction to Femtogram Amounts of Dimethyl Disulfide and to Mixtures of Volatile Components[1]

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Abstract—The responses of normal male hamsters to the odors of several components of estrous female hamster vaginal discharge (FHVD) were quantified with a two-bottle preference test in order to clarify the role of dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) in the attraction of males to females. These two compounds were originally isolated and identified in two separate, behaviorally active fractions of FHVD, one containing DMDS and the second containing DMTS. However, only authentic DMDS proved to be attractive to males; DMTS was inactive in the amounts tested (50 ng to 10 μg). When smaller amounts of highly purified samples of DMTS (0.03–3.32 ng) are assayed, measurable amounts of activity are obtained. This activity of authentic DMTS is not due to any intrinsic activity of its own but rather can be ascribed to the activity of the residual DMDS (0.07%) that it contains. Highly purified samples of authentic DMDS (containing 0.03% DMTS) were again found to be attractive to males. Doses as small as 500 fg were significantly active when compared to controls. Arguments are presented suggesting that the behaviorally effective dose of DMDS is several orders of magnitude less than 500 fg. Deliberate mixtures of DMDS and DMTS failed to show any synergistic effects. In fact, at the two lowest doses examined (0.1 and 0.46 ng total sulfides), the response obtained with the mixture was smaller than that expected if DMDS were the only odorant in the solution. Therefore, DMTS seems to suppress the activity of DMDS.

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Key Words—Olfaction, female hamster vaginal discharge, hamster sex attractant, dimethyl disulfide, dimethyl trisulfide.

INTRODUCTION

Estrous female golden hamsters produce a viscous vaginal discharge which is attractive to male hamsters in a variety of experimental situations (cf. Johnston, 1977). We have been engaged for some time in an investigation of the chemical identity and behavioral import of the volatile constituents of this discharge. We found that distillation of an aqueous suspension of pooled discharge resulted in an active distillate containing a complex mixture of volatile compounds. Examination of the distillate with conventional gas chromatography (GC) revealed more than 85 resolvable chromatographic peaks (Singer et al., 1976). When the distillate was separated chromatographically into narrow fractions, each representing only one or a few contiguous peaks on the chromatogram, two nonadjacent fractions were found to be attractive to male hamsters. Dimethyl disulfide (DMDS) was identified as a minor component of the first active fraction and dimethyl trisulfide (DMTS) was identified in the second (fraction 2). Approximately 5 ng of DMDS and 2 ng of DMTS were isolated from each 20-mg collection of fresh vaginal discharge. Subsequent bioassay of authentic samples of these two compounds revealed that DMDS had attractant activity qualitatively similar to that obtained with estrous vaginal discharge. Quantitatively DMDS appeared to account for about half of the ability of the discharge to attract males to the odor source. DMTS on the other hand was inactive in the amounts assayed (50 ng to 10 μg; Singer et al., 1976).

There are at least three reasonable explanations for the failure of authentic DMTS to attract males, given its presence in the second active fraction: (1) There is an active but as yet unidentified compound in fraction 2 which accounts for the activity originally observed. (2) DMTS is in fact attractive to males but has a very sharp response cut off at high doses. Perhaps 50 ng or more falls within this region and thus DMTS appears to be inactive. (3) DMTS has no intrinsic activity of its own but is partially converted into DMDS in the exit port of the GC by a known thermal disproportionation reaction (Pickering et al., 1967). Possibly this DMDS, either alone or in combination with the remaining DMTS, accounts for the activity of volatile fraction 2.

In this study we explore these two latter possibilities by examining the behavioral responses of male hamsters to small doses of highly purified samples of DMDS, DMTS, and their binary mixtures. The resulting data clarify the role of higher-order sulfides in the hamster attraction system and resolve the apparent discrepancy observed between the biological activity of

fraction 2, which is known to contain DMTS, and the previous failure of authentic DMTS to elicit male attraction.

METHODS AND MATERIALS

Animals and Bioassay. Male hamsters [Lak:LVG(SYR)] were obtained from the Lakeview Hamster Colony (Newfield, New Jersey) and maintained on a 14 hr:10 hr light-dark cycle. The animals were housed singly in plastic cages (42 × 21 × 20 cm) with solid plastic covers in an all male colony room and fed thrice weekly with rat and guinea pig chow, carrots, and sunflower seeds. Males were initially screened for sexual responsiveness in a single pairing with a receptive female. All the males used made intromission attempts within 7 min; none was allowed to ejaculate. The assay for attraction activity was the two-jar preference test described previously (Singer et al., 1976; O'Connell et al., 1978). The screw tops of two 30-ml ointment jars were attached beneath the floor of the home cage of each male, one near each end. Five 6-mm holes drilled through the jar top and the floor of the cage allowed the volatile compounds that were to be tested to diffuse from the sample jar, up through the 2-cm layer of Sanicell bedding on the floor of the cage and into the cage space (approx. 16 liter). Each bioassay was preceded by a 3-min period during which both jars contained the solvent used to disperse the test sample. None of the animals used in this study responded to these control odors or to the sounds and movements associated with manipulation of the odor jars. At the end of the control period one of the jars, usually the one farthest from the animal, was removed and replaced with a jar containing the sample to be evaluated. The duration of the response (burrowing and sniffing in the bedding over an odor port) and the latency to the onset of the response were recorded. Two minutes after the beginning of the response the sample was removed, and the animal was observed for another minute (maximum response duration = 180 sec). When no response occurred, the test was terminated in 7 min. Separate sets of 12 males each were used for each of the experiments reported here. Within each series of tests every male received each of the samples to be evaluated in a counterbalanced order. A separate odor sample was prepared for each test male. Testing was conducted during the dark periods with each male receiving only one test every 2–3 days. Test samples were thawed and brought to room temperature (21 ± 1° C) just prior to assay. In each case the data reported are the averages from a set of 12 males.

Chemical Purification and Sample Preparation. Commercial samples of DMDS and DMTS routinely contain significant amounts of impurities which are assumed to arise from the disproportionation of organic sulfides (Pickering et al., 1967). All GC analyses and purifications of the sulfides were done on a 4-mm ID × 0.8-m glass column packed with 1% squalene on

Chromosorb G (HP) 80/100 mesh. The flow rate through the column and the electron capture detector was 55 ml/min of a mixture of argon and methane (95:5). DMDS was purified by preparative GC at a column temperature of 57° C and contained less than the minimum detectable amount of DMTS (0.04%). DMTS was purified by distillation (bp 59–61° C, 15 torr) through a 10-cm Vigreux column and then by preparative GC at 25° C. Higher chromatographic temperatures were found to convert significant amounts of the trisulfide into the disulfide. The purified DMTS contained 0.07% DMDS. Purified samples of both the disulfide and the trisulfide could be stored at −20° C for periods as long as two months without a measurable change in the level of cross-contamination.

Stock solutions of both purified DMDS (2.5 and 0.1 mg/ml) and DMTS (1.0 and 0.04 mg/ml) were prepared in propylene glycol. The final solutions for the various assays were prepared by diluting aliquots of the stock solutions in propylene glycol. The mixtures of DMDS and DMTS were prepared so that the range of concentrations examined overlapped those observed in fresh vaginal discharge and extended two orders of magnitude to lower concentrations. The total amount of sulfide in the mixture varied more than 100-fold, but the ratio between the two components was fixed at the value measured in fresh discharge (5 parts DMDS to 2 parts DMTS). Whole volatiles from hamster vaginal discharge were collected by adsorption on a porous polymer resin (Singer et al., 1976) and transferred to a capillary tube (1.5 mm OD) by heating the polymer to 220° C for 3 min in a stream of nitrogen (3 ml/min) that had been passed over water (purified by distillation from alkaline permanganate) saturated with sodium chloride. The whole volatile material eluted from the resin was condensed along with approximately 2 mg of water in the capillary tube cooled with dry ice. Condensed volatiles and water were rinsed from the tube with an amount of propylene glycol sufficient to make a solution containing 30 female equivalents/ml.

Stock solutions and some of the more concentrated assay solutions were analyzed to verify the final concentrations of the sulfides. The sulfides were extracted from the propylene glycol solutions by shaking a 0.25-ml aliquot with an equal volume of pentane (distilled from concentrated sulfuric acid) and separating the pentane extract after chilling the mixture in dry ice. The pentane solutions were then analyzed by GC at a column temperature of 25° C.

Samples of the sulfides for behavioral assay were prepared on filter paper circles (2.3-cm diameter) placed in individual 30-ml ointment jars which could be affixed beneath the bottom of the test cages. In order to minimize the volatilization of the samples during preparation of the individual jars, each filter paper was first loaded with 0.04 ml of purified water and frozen on dry ice. Aliquots (0.04 ml) of the test solution were applied, and the jars were capped and returned to dry-ice temperatures until assayed.

Vaginal discharge was collected on pieces of filter paper (1 × 3 cm) from normally cycling females during the early dark phase of cycle day 1 and stored at −20° C until needed. A single collection from each receptive female averaged 20 mg of vaginal discharge on each of the filter papers. This value defined one female equivalent (FE) of vaginal discharge.

RESULTS

Responses to Mixtures of DMDS and DMTS. As expected from previous studies (Singer et al., 1976; Macrides et al., 1977; O'Connell et al., 1978), nanogram quantities of DMDS were again attractive to normal male hamsters. However, unlike our previous study (Singer et al., 1976) in which relatively large amounts (50 ng to 10 μg) of material were assayed, we found, in this study, that small doses of authentic DMTS (containing 0.07% DMDS) were attractive to males in the range of 0.03–3.32 ng (Table 1). The responses observed here with small amounts of DMTS and the earlier failure to provoke significant attraction with large amounts of DMTS lends credence to the notion that DMTS has a sharp response cut off at high doses. Furthermore, the probability that there is an unidentified active compound in active fraction 2 of the whole volatiles is greatly reduced.

Alternative explanations for the observed activity of DMTS rest on the fact that the assayed sample of authentic DMTS contains a small amount of DMDS as a contaminant. Therefore, the observed activity of the sample could arise in two ways. In one, DMTS may have no intrinsic activity but may interact with the DMDS contaminant so that the resultant mixture produces measurable amounts of attraction in our assay. In this case one would expect that deliberate mixtures of DMDS and DMTS would elicit responses significantly larger than the sum of those obtained with the unmixed components. In

TABLE 1. MEAN DURATION (± STANDARD ERROR) OF SNIFFING AND DIGGING BEHAVIORAL RESPONSE OF 12 NORMAL MALES EXPOSED TO INDICATED DOSES OF HIGHLY PURIFIED SAMPLES OF DIMETHYL DISULFIDE AND DIMETHYL TRISULFIDE

Dimethyl disulfide		Dimethyl trisulfide	
Measured dose (ng)	Mean duration (sec)	Measured dose (ng)	Mean duration (sec)
0.07	28 ± 16	0.03	45 ± 21
0.33	69 ± 22	0.13	22 ± 16
1.66	26 ± 9	0.66	35 ± 20
8.30	45 ± 20	3.32	17 ± 10

the second, the total observed activity could be accounted for solely by the activity of the minute amounts of the DMDS contaminant. In this case one should be able to demonstrate significant responses to subnanogram amounts of DMDS. We have examined the first of these alternatives by comparing the activity obtained with deliberate mixture of DMDS and DMTS with the activity obtained when the individual compounds are assayed alone.

The results of assaying various concentrations of a deliberate mixture of DMDS and DMTS are summarized in Figure 1. In each case the ratio of the two components of the mixture was held constant at 2:5 (DMDS/DMTS) to correspond to the ratio measured in vaginal discharge. At the three lowest concentrations, the mixture was less attractive than the sum of the attraction scores observed when each individual component was assayed alone (the data for the individual compounds and for the mixture were obtained in the same experiment). At the highest concentration the response to the mixture was approximately equal to that expected from the sum of the individual scores. Therefore it appears that addition of DMTS to DMDS does not enhance the activity of the resulting mixture beyond what would be expected from a summation of the individual responses. As a matter of fact, at the two lower concentrations it appears that the addition of DMTS actually suppresses the activity of the mixture to below that expected from DMDS alone.

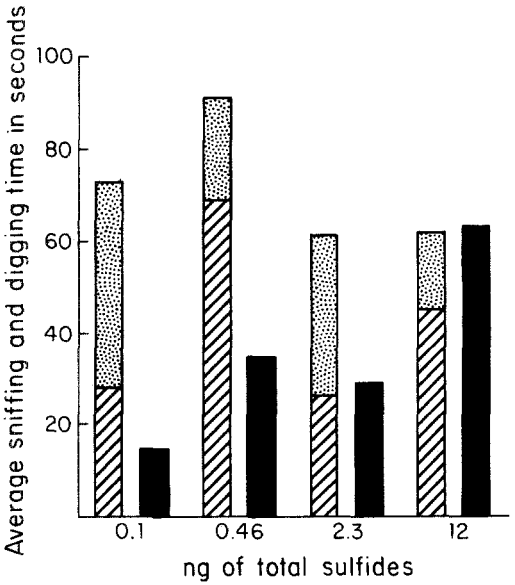

FIG. 1. Mean behavioral responses of 12 normal male hamsters to DMDS (hatched areas), DMTS (stippled areas), and their mixtures (solid bars). The abscissa lists the total amount of DMDS and DMTS in the mixture. The values for the individual compounds are given in Table 1. Maximum possible response duration is 180 sec. Propylene glycol was the solvent.

Responses to a Concentration Series of DMDS. Since the addition of DMTS to DMDS does not enhance the attractiveness of the resulting mixture, the activity observed with low doses of authentic DMTS might be due entirely to the activity of the DMDS contaminant. A simple calculation reveals that the lowest concentration of DMTS assayed (0.03 ng) should contain 21 femtograms (fg) of DMDS (21×10^{-15} g). In order to determine whether femtogram quantities of DMDS are, in fact, attractive to males, a concentration series was made up in propylene glycol. It spanned 5 log steps of dilution, from a high of 1 female collection (5 ng) to a low of 1×10^{-5} female collections (50 fg). The mean attraction scores of a second set of 12 normal males for the six concentrations of DMDS and for a solvent blank (propylene glycol) are shown in Figure 2.

The overall differences between the various treatments was evaluated with the Friedman two-way analysis of variance by ranks test (Siegel, 1956) and was found to be statistically significant ($P < 0.001$). The Wilcoxon matched-pairs signed-ranks test (Siegel, 1956) was then used to determine which concentrations of DMDS elicited responses significantly greater than those elicited by the solvent. The behavioral data fit a typical compressive dose-response function. DMDS elicits a response approximately twice as large as that obtained with the control at a concentration of 50 fg, is significantly more attractive than the control at 500 fg, and reaches an

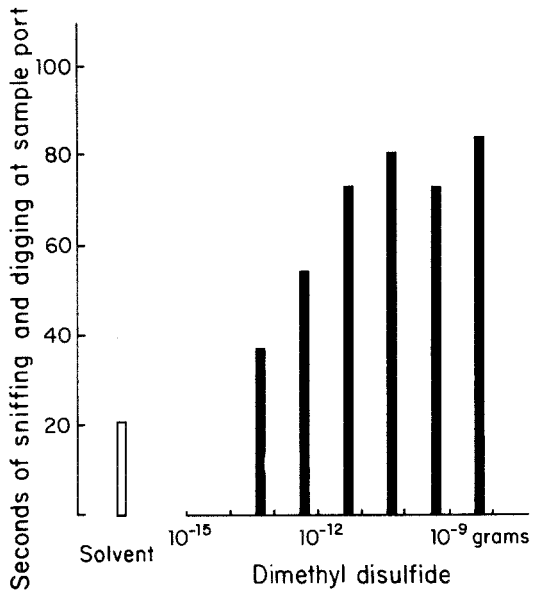

FIG. 2. Mean behavioral response to 12 normal male hamsters to various doses of the hamster sex attractant DMDS. The doses of DMDS assayed ranged in five equal log steps from 50 fg to 5 ng. Maximum possible response duration is 180 sec. Propylene glycol was the solvent.

asymptote of attraction at a concentration of 50 picograms (pg). A linear extrapolation of the dose-response function to concentrations below 50 fg reveals that a sample containing 6 fg of DMDS should elicit a behavioral response equal to that obtained with the solvent. It is reasonable to conclude therefore, that all the behavioral responses obtained with authentic samples of DMTS can be ascribed to the extreme potency of the DMDS contaminant and that DMTS is not attractive to normal males. The only behavioral effect of DMTS seems to be its ability to suppress the activity of DMDS.

DISCUSSION

The data obtained from these behavioral assays of highly purified samples of authentic DMDS, DMTS, and their binary mixtures reveal that DMTS does not operate as a sex attractant pheromone. All of the behavioral responses obtained thus far with DMTS can be ascribed to the presence of a small amount of DMDS present as a contaminant. Further we have determined that approximately 3% of the DMTS present in unprocessed vaginal discharge is converted into DMDS in the exit tube of the GC. Therefore the activity originally described in the second active fraction (Singer et al., 1976) is the result of the thermal disproportionation of DMTS into DMDS during the chromatographic isolation of the fraction.

The behavioral responses obtained with the deliberate mixture of DMDS and DMTS were, in general, smaller than would be expected from the summation of the individual responses obtained with each component. At the two lowest amounts examined (0.1 and 0.46 ng), the response to the mixture was actually less than the response obtained with DMDS alone. At 2.32 ng the response to the mixture was approximately equal to the DMDS response. The suppression of response intensity when mixtures of compounds are assayed is relatively common in psychophysical experiments (Berglund et al., 1971, 1973; Berglund, 1974) and has been shown to be a function of the shape of the dose-response curves of the individual components (Bartoshuk and Cleveland, 1977). When the individual dose-response curves show compression (equal increments in the dose elicit progressively smaller increments in response) as they do with both DMDS and DMTS (see Table 1 and Figure 2), the responses to their mixtures are usually suppressed. At the highest concentration tested (11.62 ng) the response to the mixture of DMDS and DMTS was approximately equal to that expected from the sum of the individual responses. This approach to additivity at higher concentrations is also seen in human psychophysical judgments of binary mixtures of odorants (Cain, 1975).

One of our long-term concerns has been the estimatation of the potency of DMDS relative to a standard amount of vaginal discharge. We had hoped that such a comparison would allow us to assess whether DMDS alone is

TABLE 2. NUMBER OF ANIMALS RESPONDING WITH SNIFFING AND DIGGING BEHAVIOR PATTERN TO GRADED AMOUNTS OF HAMSTER VAGINAL DISCHARGE AND ITS COMPONENTS, AND MEAN DURATION AND LATENCY OF BEHAVIOR AVERAGED OVER 12 NORMAL MALES

Test material (FE)	Positive responses (No.)[a]		Mean duration ± SEM (sec)	Mean latency ± SEM (sec)
	Test jar	Control jar		
Vaginal discharge				
3	12[b]	0	123 ± 17	109 ± 15
2	12[b]	0	53 ± 11	157 ± 31
1	5[c]	0	20 ± 9	338 ± 35
Whole volatiles				
0.5	8[d]	0	48 ± 15	292 ± 35
Dimethyl disulfide				
0.5	5[c]	0	30 ± 12	303 ± 44
Nonvolatile residue				
1	1	0	1	45

[a] Binomial test.
[b] $P < 0.001$.
[c] $P < 0.05$.
[d] $P < 0.005$.

responsible for the attractiveness of vaginal discharge. We had found that DMDS could account for about half of the activity of the discharge, and we suggested the possibility that another as yet unidentified compound might account for the remainder of the activity (Singer et al., 1976). However, if one measures the activity of various doses of vaginal discharge, the whole volatile fraction, DMDS, and the nonvolatile vaginal discharge residue (Table 2), it is apparent that such comparisons are inconclusive. The difficulties arise primarily because of the important role played by the solvent system in determining the measured activity of an olfactory pheromone (Regnier and Goodwin, 1977). For example, 0.5 female equivalents (FE) of either authentic DMDS or of the whole volatile fraction elicit more sniffing and digging than does 1 FE of vaginal discharge. Each of the test materials assayed (Table 2) has its own unique solvent system. Vaginal discharge is a polydisperse mucoid material containing water, salts, proteins, sloughed epithelial cells, and a large number of volatile compounds including DMDS and DMTS. The whole volatile fraction tested is similar to vaginal discharge but has had the nonvolatile material removed and is dissolved in propylene glycol. The authentic DMDS assayed is a simple mixture of the sex attractant pheromone, a small amount of DMTS (0.04%), and propylene glycol. One can conclude therefore, that the enhanced activity of fractions of vaginal discharge compared to unprocessed discharge is due primarily to differences

in the rate of release of active materials, which is itself controlled by the properties of the different solvent systems employed. A secondary factor is the possibility of positive responses to the odor of the solvent. This has occurred only once in this series of experiments (Figure 2).

It is clear that even the most effective doses of DMDS fail to elicit behavioral responses as strong as those obtained with 3 FEs of vaginal discharge (Table 2). This may indicate that the natural product releases an optimum dose of DMDS for time periods which are longer than those which can be obtained with the authentic material in propylene glycol.

The male hamster is extraordinarily sensitive to the presence of DMDS. For example, if 50 fg of DMDS were to volatilize directly and completely into the 16-liter volume of the animal's cage, then each ml of inspired air would contain approximately 20,000 molecules of DMDS (2.2×10^{-16} M). There are several significant factors which collectively force us to the conclusion that the behaviorally effective dose of DMDS is several orders of magnitude below 50 fg. In our bioassay DMDS is presented (1) as a dilute solution in propylene glycol and water, and (2) is spotted on a piece of cellulose filter paper. Each of these factors has been shown to reduce the volatilization of behaviorally active substances by as much as 50% (Regnier and Goodwin, 1977). (3) Once present in the air space of the odor jar, DMDS must diffuse up through holes in the cage floor and then through a 2-cm layer of Sanicell bedding before it reaches the head space of the cage where it can be sensed by the animal. The extended surface area of the cage and its bedding and the potential for adsorption to them should further reduce the effective concentration of DMDS reaching the vicinity of the animal. (4) Since the animal's cage is not air tight, an additional fraction of the volatilized DMDS will diffuse out of the cage before it can be detected by the animal. (5) Given the average respiratory rate of the hamster (74/min) and its average tidal volume (0.83 ml), one can compute the total inspiratory volume during the 7-min, duration of a typical bioassay (Guyton, 1947). During this period the animal will sample 430 ml of potentially odorized air which represents only 2.7% of the total cage volume. (6) Finally the response latency of the male hamster varies from 0.5 to 7 min, with response durations which are often longer than 2 min. This indicates that DMDS is released gradually over a period of at least 7 min. Moreover the fact that the male unerringly orients to the correct odor port indicates that the concentration of DMDS is not uniform throughout the cage volume.

The extreme sensitivity of the male hamster to DMDS is without precedent in the field of mammalian pheromones. For parallels in pheromonal phenomena it is necessary to look to work on insect sex attractants (cf. Kaissling, 1971). It is likely that the male hamster's behavioral response to its attractant pheromone is of comparable sensitivity to that observed in the response of male moths to their attractant pheromones. The

simple fact that male hamsters can detect very small amounts of DMDS lends further support to the notion that this compound functions primarily to attract male hamsters to scent marks of vaginal discharge over long distances. It may also explain how such marks can remain attractive to males for such considerable lengths of time (i.e., 50 days or more; Johnston, 1977). For example, if 50 fg of DMDS were released from a dried scent mark each minute it would take 70 days to exhaust completely the 5 ng of DMDS present in the average 20-mg collection of vaginal discharge (Singer et al., 1976).

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MATURATION AND SENESCENCE OF AN INSECT CHEMOSENSORY RESPONSE[1]

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Abstract—Electroantennogram responses of male and female *Pseudaletia unipuncta* to the two major components of the presumed pheromone associated with the male anterior abdominal scent brushes vary with age. There is a postemergence period of maturation of responsiveness, followed by senescence. The age of maximum responsiveness differs according to the sex and to the compound tested.

Key Words—Electroantennogram, acceptors, *Pseudaletia unipuncta*, maturation, senescence, age, benzaldehyde, benzyl alcohol, male pheromone, Lepidoptera. Noctuidae.

INTRODUCTION

The maturation and senescence of insect chemoreceptors is a subject which has received scant attention in the literature. This lack of study is rather surprising as age-related sensitivity could result in a considerable variation in the electroantennogram (EAG) responses obtained when one is screening potential olfactory-stimulating compounds. Such variability in antennal receptor responsiveness could lead to erroneous conclusions regarding the

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relative activity of stimulatory compounds. Payne et al. (1970) noted that in *Trichoplusia ni*, female sex pheromone stimuli of similar concentration resulted in the generation of EAGs of a similar amplitude regardless of the age of the exposed male moths. A similar phenomenon was reported in *Argyrotaenia velutinana* (Roelofs and Comeau, 1971b), although there appeared to be a small decrease in EAG activity 4 days after emergence.

The development of the chemosensory response in the male *Manduca sexta* to the sex pheromone of its species has been investigated by Schweitzer et al. (1976). They found an increase in pupal antennal responsiveness relative to an assumed constant adult response.

The major components of the presumed male pheromone of *Pseudoletia unipuncta* are benzaldehyde and benzyl alcohol, and they are perceived by receptors on the antenna of both sexes (Grant et al., 1972). While determining a dose-response curve for this pheromone receptor system, certain anomalies appeared which led us to believe that the magnitude of EAG response to a constant pheromone concentration was age dependent.

METHODS AND MATERIALS

A colony of *Pseudaletia unipuncta* was maintained using a method similar to that described by Shorey and Hale (1965). All stages were maintained at a temperature of 25° ± 2° C under a 12:12-hr light-dark cycle. They were sexed as pupae and thereafter maintained separately by sex. Actively flying moths were used for all tests.

The EAG recording system used in this study was similar to that described by Roelofs and Comeau (1971a) and Roelofs (1977), with the exception that a unity gain voltage follower was used in place of the 100× amplifier.

Odorous compounds were applied to the antenna in 1-sec puffs. Ultra-zero grade bottled air (Matheson Co.) was used as the carrier at a rate of 150 ml/min. The odor delivery system was similar to that described by Albert et al. (1974). An odor-evacuation system was set up immediately behind the preparation; this prevented a buildup of pheromone in the vicinity of the test antenna and provided a flow of clean air, at approximately 1 cm/sec, over the preparation between test exposures.

Saturation concentration has been defined by Boeckh (1969). It is that concentration of odorant molecules beyond which a further increase in stimulus strength causes no further increase in EAG amplitude. Preliminary studies of the EAG dose-response curve for this species, and this odor delivery system (Seabrook, unpublished), indicate that 10^4 μg at source of either benzaldehyde or benzyl alcohol exceeds the saturation concentration.

The benzaldehyde and benzyl alcohol (both 98+% pure by GLC) were dissolved in ether at a concentration of 10^4 μg of test compound per 100 μl

solvent. One-hundred microliter aliquots of the test solution were applied to 1.5 × 1.5-cm pieces of Whatman #1 filter paper and evaporated to dryness. The treated filter papers were placed in glass tubes (6 mm inside diameter × 65 mm long), which were then wrapped in Parafilm until used later that same day. The control stimulus was a similar piece of filter paper, treated with 100 μl of ether and evaporated to dryness. Each stimulus cartridge was used no more than three times.

The antennal preparation was initially exposed to a control stimulus. This was followed by a stimulus using one of the two test compounds and subsequently by the other compound. The order of presentation of test stimuli was random. There was a 3-min recovery period between stimuli. Preliminary studies indicated complete recovery of the EAG response during this period. After the above sequence of stimuli the antenna was discarded.

The stimulus EAG voltage was calculated by subtraction of the control voltage from the voltage generated by a given compound. As there was no apparent reduction in the EAG voltage over the duration of the test, a more sophisticated treatment of the data (Roelofs and Comeau, 1971b) was not necessary. Three to five replicates were carried out for each test compound at each age of moth.

RESULTS

EAG responses to the tested compounds indicate that both the male and female antennae are more sensitive to benzaldehyde than to benzyl alcohol on the day of peak sensitivity. The male is approximately twice as sensitive on day 3 (Figure 1) and the female 1.5 times as sensitive on day 8 (Figure 2) to benzaldehyde as to benzyl alcohol.

The sensitivity of the male antenna to both compounds differs with age (Figure 1). On the day of emergence the male EAG response to benzaldehyde is only 0.7 mV. It increases to a peak of 6.7 mV on day three and then decreases ($F = 63$; Student-Newman-Keuls, $P < 0.05$). The voltage decreases on day 5 to approximately 5 mV and remains at about that level until day 9, after which it steadily decreases until day 13. A similar, although less pronounced, response pattern is seen with respect to benzyl alcohol, for which the peak response of 3.2 mV was reached on days 1-5 ($F = 4.4$; Student-Newman-Keuls $P < 0.05$) (Figure 1). Likewise, an age-dependent response is found in female antennae. The EAG response to benzaldehyde rises from 0.3mV on the day of emergence to a peak of 4.0 mV on days 8-9, and then decreases steadily to 1.1 mV on day 13 ($F = 6.3$; Student-Newman-Keuls, $P = 0.05$) (Figure 2). A similar effect is observed with respect to the female EAG response to benzyl alcohol with the peak response on days 9-11 ($F = 6.8$; Student-Newman-Keuls, $P < 0.05$).

The male antenna is more sensitive to benzaldehyde than is the female

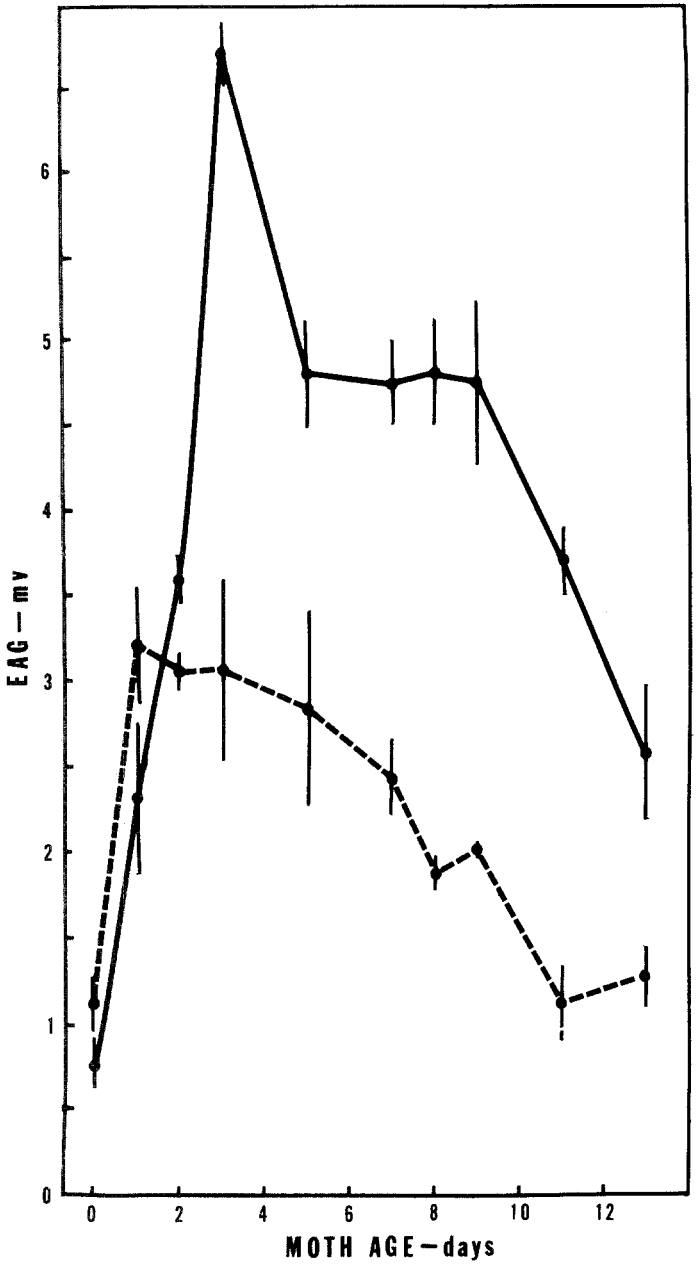

FIG. 1. The EAG responsiveness of the male *P. unipuncta* antenna to a 10^4-μg source concentration of benzaldehyde (———), and benzyl alcohol (--------), as a function of age. Vertical bars represent standard errors.

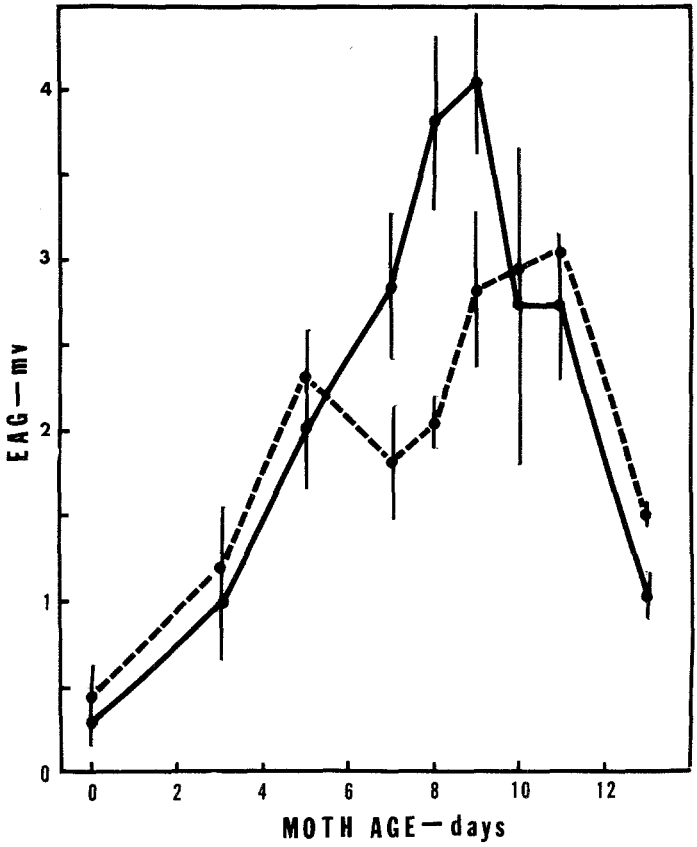

FIG. 2. The EAG responsiveness of the female *P. unipuncta* antenna to a 10^4-μg source concentration of benzaldehyde (———), and benzyl alcohol (--------), as a function of age. Vertical bars represent standard errors.

antenna and reaches its peak of sensitivity at a much younger age (day 3 vs. day 8-9). In the case of benzyl alcohol, both male and female antennae are approximately equal in peak sensitivity, although they reach that peak at considerably different ages (day 1 vs. day 9).

EAG Waveform. The EAG generated by *P. unipuncta* to large amounts of benzaldehyde and benzyl alcohol has an unusual waveform. Despite the 1-sec period of stimulation, depolarization lasts an extremely long time, the voltage continuing to drop for up to 20 sec before any indication of repolarization appears. This effect is more pronounced at higher amounts (10^4 μg) than at lower amounts (10^2 μg) where the voltage stops dropping after approximately 5 sec. At amounts of 10^2 μg the waveform approximates a "normal" EAG with a rapid recovery phase at the end of stimulation (Boeckh et al., 1965).

In the case of benzaldehyde, but not in the case of benzyl alcohol, the depolarization is preceded by a hyperpolarization lasting approximately 1 sec at amounts of 10^4 μg and up to 3 sec at lower amounts (10^2 μg).

DISCUSSION

It is apparent from Figures 1 and 2 that both the adult male and female antennal responses to both benzaldehyde and benzyl alcohol change with age. This implies that the olfactory neurons in the adult moth's antenna are not physiologically stable but vary with age in a pattern of maturation and senescence. Apparently this is not the case in the EAG response of males of *Trichoplusia ni* (Payne et al., 1970) to the female sex pheromone of that species, although behavioral responses of male *T. ni* to its sex pheromone vary with age (Shorey and Gaston, 1964). *Argyrotaenia velutinana* (Roelofs and Comeau, 1971b) shows no change of EAG amplitude with age. In *Manduca sexta* (Schweitzer et al., 1976) there is a maturation of the EAG response of the male antenna to the female pheromone from first responses on day 17 after pupation to day 1 of the adult stage, and to *trans*-2-hexenal from day 14 after pupation to day 1 of the adult stage. Unfortunately, no data are available on the subsequent adult responses.

The functional units on the dendritic membrane which interact with the odor molecules are the acceptors (Kaissling, 1969). It has been proposed that the odorant molecule binds to active sites on the acceptor, based on a stereochemical fit (Roelofs and Comeau, 1971a). Kaissling (1971) has suggested that the acceptors are initially cleared of the stimulatory molecules by a process referred to as early inactivation. These molecules are subsequently enzymatically degraded (Kasang and Kaissling, 1971). The question of the permanence of dendritic acceptors has not been considered.

The EAG, developed by Schneider (1957), is thought to be the summed responses of receptor potential of the activated sensory neurons of the antenna (Boechk, 1969). The strengths and weaknesses of this technique have been discussed by Roelofs (1977). Dickens and Payne (1977) have suggested that the magnitude of the EAG is directly related to the relative number of acceptors occupied. Therefore, EAGs generated at saturation concentrations of pheromone should result from activation of all the available acceptors. If such is the case, then any increase or decrease in the EAG voltage at concentrations higher than the saturation concentration of pheromone must be based on an increase or decrease in the number of available acceptors.

We propose that the maturation and senescence of the EAG response to the male pheromone in *P. unipuncta* may be based upon an initial increase and subsequent decrease in the number of available acceptors. We further propose that acceptors are not stable during the adult life of the insect but are

being formed and broken down and that the maturation and senescence of the adult EAG response, the rate of which varies for the individual compounds tested, is related to the relative rates of formation and degradation of the different acceptors types.

It is interesting to note that the male and female EAG responses to benzaldehyde and to benzyl alcohol do not vary with age at the same rate (Figures 1 and 2). If the argument holds that at saturation concentrations all available acceptors are occupied, and if the aldehyde and alcohol are occupying the same acceptors, then one would expect a similar rate of increase in the EAG voltage with age. As this is not the case, we assume that *P. unipuncta* uses separate acceptors to bind benzaldehyde and benzyl alcohol.

Grant et al. (1972) have reported that in *P. unipuncta* EAGs generated in response to the male scent brush compounds do not differ on the basis of sex tested or compounds tested. Similar results have been reported for other moth species (Birch, 1971; Grant, 1971). With the exception of the 2-day-old moth, our findings are contrary to the above. There is a pronounced sexual difference in the amplitude of the EAG response to benzaldehyde, males being twice as responsive as females, although there are no sexual differences in the EAG response to benzyl alcohol if one disregards moth age.

We also find that the antenna, male or female, is considerably more sensitive to benzaldehyde than to benzyl alcohol, thus indicating a chemical specificity, contrary to findings indicating a lack of chemical specificity (Birch, 1971; Grant, 1971; Grant et al., 1972).

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BIOASSAY APPARATUS FOR RODENT OLFACTORY PREFERENCES UNDER LABORATORY AND FIELD CONDITIONS

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Abstract—A bioassay apparatus is described which automatically registers frequency and the total time that rodents investigate sample odors. The apparatus provides free access for wild rodents, and its rugged weatherproof construction allows outdoor use for extended time periods. The photocell light source is covered by an infrared filter and it can be operated continuously or for preset time intervals. When the apparatus was utilized for rat sex attractant research, many of the odorants preferred by tame laboratory rats were also attractive to wild rodents under field conditions.

Key Words—Olfactory preferences, attractants, rodent behavior.

INTRODUCTION

Although the presence of pheromone communication in rodents has been firmly established (Carr et al., 1965), almost no work has been done with wild animals in their natural habitat. A number of investigations have shown that the preputial glands of the mature white rat and mouse produce odors attractive to the animals of the opposite sex and remain under hormonal control (Bronson and Caroom, 1971; Orsulak and Gawienowski, 1972; Gawienowski et al., 1975, 1976). Recently we found that aliphatic acetates act as attractants for female laboratory rats (Stacewicz-Sapuntzakis and Gawienowski, 1977). Volatile aliphatic acetates are present in the rat and mouse preputial glands along with other compounds (Spener et al., 1969; Stacewicz-Sapuntzakis and Gawienowski, 1977) and their occurrence is related to the level of androgens (Sansone-Bazzano et al., 1972).

We extend our laboratory studies of preputial attractants to wild rodents

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in their natural conditions. The task required construction of a fully automated nonobtrusive apparatus monitoring animal behavior toward samples without the presence of the experimenter.

METHODS AND MATERIALS

Testing Apparatus. The testing apparatus was designed similarly to that employed for laboratory bioassays (Stacewicz-Sapuntzakis and Gawienowski, 1977) but adapted for the outdoors. Animals could freely reach the inside of a circular cage made of sheet metal, 46 cm in diameter and 23 cm high, painted black and supported at three points 6 cm from the ground (Figure 1). The cage wall had openings at two locations 150° apart for 5 × 5-cm tunnels, placed 2.5 cm above the lower edge of the enclosure. The tunnels were 10 cm deep, made of black painted metal, and closed with metal inserts with holes drilled for optional air flow from a tank. The inserts were converted to sample holders by the attachment of a scintillation vial cap with an air flow opening. Samples of the odorants could be placed in a scintillation vial with the bottom cut off and attached by the screw cap in the tunnels. The animals could insert their heads into the tunnels but were prevented from licking the samples by wire screen placed 2.5 cm from the entrance. Presence of animals investigating the samples was detected by placing a photocell 1.5 cm from the entrance (0.5 cm from the bottom of the tunnel). Each tunnel had a light source (8L7, Sigma Instruments inc., Braintree, Massachusetts) covered by an infrared filter which cut off all visible light. The filter was made from a plastic layer of infrared filter from Edmund Scientific Co. (Barrington, New Jersey), by thinning it to approximately 0.05-0.075 mm in the center. On the opposite wall of each tunnel we installed a photo-receiver (8P7, Sigma Instruments).

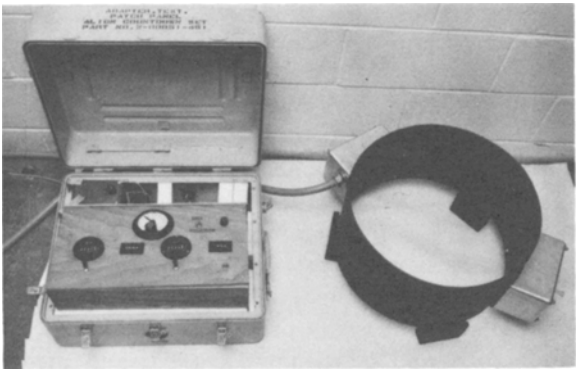

FIG. 1. Bioassay apparatus without the cover.

The tunnels and electronic equipment were covered on the outside by sturdy weatherproof shields, and the whole cage was protected from the top by a round metal cover attached with springs.

The recording equipment was placed behind a panel in a weatherproof metal box. The photocells were connected to elapsed time indicators (reset model 636, 10,000-sec limit from Cramer, Old Saybrook, Connecticut) and event counters (reset model Veeder-Root, 4 digits). They were wired to an automatic reset interval timer (type 241, time range 15 min, from Cramer) equipped with two modes of operation—present interval or continuous (Figure 2). In the continuous mode the apparatus would register the animals indefinitely. The control box also contained amplifiers (8 A, from Sigma

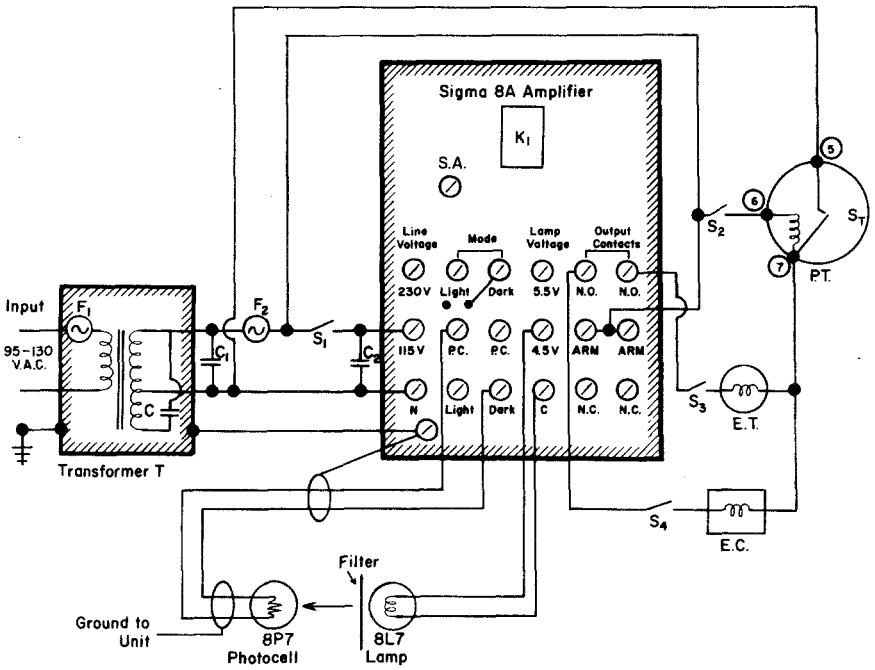

FIG. 2. Schematic diagram of photocell circuit. Transformer T—SOLA constant voltage transformer, 95-130 V AC input, 118 V AC output at 0.5 A. C_1 and C_2—0.015 μF at 600 V DC. F_1 and F_2—fuses 0.5 A. S_1—amplifier power switch. S_2—mode switch: open, continuous; closed, timed. S_3—timed indicator switch. S_4—event counter switch. P.T.—preset interval timer. S_T—latch start switch of preset interval timer (unlatches at the end of preset time). E.T.—elapsed time indicator. E.C.—event counter. K_1—relay of 8-A amplifier with output contacts (N.O.) normally opened, (N.C.) normally closed, ARM—armature. Photocell and lamp cable—Belden #8722. (For further explanation see text.)

Instruments) for the photocells, a master switch, two switches for the time indicators, a fuse, and a constant voltage transformer (SOLA, 118 V, 0.5 A).

Cable leading from the bioassay cage to the control box was protected by the metal covering. The whole apparatus required one standard electrical outlet to operate.

Application. For our preliminary studies under field conditions, the equipment was placed in a corner of a shelter storing horse food supplies, close to the local stables. The shelter had been plagued by a large number of wild rats and mice as judged by losses of feed supplies, numerous droppings and sightings of the rodents even in the daytime. Investigated compounds, diluted with ethanol, were placed inside one of the sample holders. The concentrations of the test compounds were at the lowest levels detectable to laboratory personnel. The opposite tunnel occasionally was not used, or served as a control containing only the solvent sample (ethanol). Samples were usually left in the tunnels for 24 hr (from 3 PM until 3 PM next day), and then the number of approaches and the total investigation time for each tunnel were recorded.

We used a number of compounds which were effective in attracting Sprague-Dawley rats in the laboratory bioassay system (Table 1). Laboratory

TABLE 1. RESPONSE OF MALE AND FEMALE LABORATORY RATS TO THE ODORANTS USED IN FIELD BIOASSAYS[a]

Compound	Time of investigation		Frequency of approach	
	♂	♀	♂	♀
Arachidonyl acetate	++	+++		+++
Pentyl acetate		+++		++
Dimethyl sulfite	+++		+++	
Diacetone alcohol	+++	++		
Hexanal	++	+	++	++
Decanoic acid	+++			
2-Acetyl-pyridine		+++		+++
Acetal	+++	+++	++	+
4-Methyl-3-pentene-2-one	+	+	+	
Decanol		+++		+
Dimethyl disulfide	+++	+++	+++	++
Anisole	+++	+++	+++	++
Hexyl mercaptan	+++		+	
Pentanol	+++		+++	
Hexanol	+++	+++	+++	+++

[a]The sign + marks attraction, lack of a sign indicates indifference toward the compound. Probabilities were determined using F values obtained from the two-way analysis of variance: +, $P < 0.1$; ++, $P < 0.05$; and +++, $P < 0.01$.

TABLE 2. WILD RODENT RESPONSE TO VARIOUS ODORANTS UNDER FIELD CONDITIONS

Sample composition[a]	Frequency of approach	
	Sample	Control
Arachidonyl acetate (10 μl of 50% soln)	396	[b]
Pentyl acetate (10 μl of 50% soln)	325	[b]
Dimethyl sulfite (10 μl of 10% soln)	248	[b]
Diacetone alcohol (10 μl of 5% soln)	9	[b]
Hexanal (0.5 ml of 2% soln)	415	107
Decanoic acid (10 μl of 50% soln)	391	229
2-Acetyl-pyridine (0.5 ml of 0.2% soln)	381	127
Acetal (0.5 ml of 2% soln)	139	7
4-Methyl-3-pentene-2-one (10 μl of 5% soln)	95	12
Decanol (0.5 ml of 2% soln)	94	4
Dimethyl disulfide (10 μl of 10% soln)	60	0
Anisole (10 μl of 0.5% soln)	19	4
Hexyl mercaptan (0.5 ml of 0.02% soln)	18	1
Pentanol (10 μl of 10% soln)	15	10
Hexanol (10 μl of 10% soln)	8	4

[a] All the solutions were made with ethanol. Equal amounts of ethanol were placed in the control tunnel.
[b] The second photocell was not in use.

bioassays were conducted in a similar apparatus, 61 cm in diameter, and totally enclosed (Stacewicz-Sapuntzakis and Gawienowski, 1977). The adult rats of both sexes were individually placed in the testing area for 5 min, and special precautions were taken to eliminate contamination by conspecific odor.

The choice of compounds was originally suggested by gas chromatography and mass spectroscopy studies of rat preputial gland volatiles (Stacewicz-Sapuntzakis and Gawienowski, 1977). We also included dimethyl disulfide, which is suspected to be a sex attractant for male hamsters (Singer et al., 1976); Gawienowski and Stacewicz-Sapuntzakis, 1978).

The elapsed time indicator had a too limited time span for overnight trials of the most attractive compounds. Therefore we report only the number of approaches to the sample as compared with a solvent control (Table 2). The frequency of approach to the solvent control probably depends to a certain extent on the attractiveness of the sampled compound in the opposite tunnel. If the animals remain longer in the circular testing arena, the natural alternating pattern (Schultz and Topp, 1973) will usually result in a higher number of approaches to the control tunnel.

RESULTS AND DISCUSSION

Many compounds proved attractive to wild rodents. Among the compounds that appeared to be most effective under field conditions, hexanal and arachidonyl acetate were significantly attractive to the laboratory rats of both sexes. Decanoic acid and dimethyl sulfite were preferred by the male laboratory rats, while pentyl acetate and 2-acetyl-pyridine evoked a significant response in laboratory female rats. A good, although less dramatic, attraction was found under preliminary field conditions for acetal, 4-methyl-3-pentene-2-one, decanol, dimethyl disulfide, anisole, and hexyl mercaptan (Table 2).

The field results with dimethyl disulfide confirm our previous conclusions (Gawienowski and Stacewicz-Sapuntzakis, 1978) that attraction to this compound is not limited to male hamsters (Singer et al., 1976), but appears to be attractive to both sexes of other rodent species.

A few compounds which were appealing to the tame laboratory rats (diacetone alcohol, pentanol, and hexanol) did not evoke a favorable response in the field trials. Further tests are necessary, involving the simultaneous presentation of two or more possible attractants, preferably in separate bioassay cages. The relative position of samples and the distance between them should be frequently changed to avoid conditioning of animals.

Although in our initial field tests we did not distinguish the sex or species of visiting rodents, the problem could be avoided if the apparatus were placed in an enclosed area with a controlled population of animals. A discreetly situated observer could note multiple approaches of each individual, its sex and other characteristics, if the cover of the apparatus were removed.

This bioassay apparatus can provide useful information on olfactory preferences both in laboratory and field experiments. Under field conditions the evaluation of area repellants or attractants for toxic baits in wildlife management could be performed by use of this apparatus with relative ease.

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MARKING PHEROMONES OF *Alpinobombus* MALES[1]

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Abstract—The male marking pheromone blends which emanate from the cephalic part of the labial gland, have been analyzed in four species of the bumble bee genus *Alpinobombus*, viz., *A. alpinus*, *A. balteatus*, *A. hyperboreus*, and *A. polaris*, by combined capillary gas chromatography, mass spectrometry, and thin-layer chromatography. In all, 36 specimens were analyzed. *A. alpinus* and *A. polaris*, which are similar morphologically, both showed an acetogenic composition of the pheromone blend. The dominant compound in *A. alpinus* proved to be a hexadecenol, whereas *A. polaris* had a hexadecadienol and a hexadecenol in the proportions 5:2 as major components. *A. balteatus* and *A. hyperboreus*, another species pair as regards their morphology, differed more in their chemical makeup. Both mevalogenins and acetogenins were found in their marking secretions. *A. balteatus* is unique among all other male bumble bee species analyzed, 29 in total, by having C_{12}- and C_{14}-butyrates in the secretion, which dominated together with tetradecyl acetate and geranylcitronellol. In *A. hyperboreus* the main marking compounds are an octadecenol and 2,3-dihydro-6-*trans*-farnesol. This species also contains citronellol and geranylcitronellol.

Key Words—Hymenoptera, Apoidea, Alpinobombus, bumble bees, male sexual behavior, marking pheromone blends, attractant, labial gland, chemotaxonomy.

INTRODUCTION

This work forms part of a study of the marking secretions of male bumble bees, summarizing the north European species in particular. The genera reported on hitherto are *Bombus* Latr. (Bergström et al., 1973) and

[1]Hymenoptera, Apoidea, Bombinae.

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Pyrobombus D.-T. (Svensson and Bergström, 1977). Reports on various species from other genera were given by Calam (1969) and Kullenberg et al. (1970). Three species of *Pyrobombus* were studied in detail (Bergström and Svensson, 1973a,b) and the composition of chemical substances in the marking secretions was found to be species specific. The secretions contain mixtures of fatty acid derivatives and isoprenoid compounds. Furthermore, species and, to some extent, genera may be characterized as "acetogenic," "mevalogenic," or "mixed," depending on the proportions between these compounds. In some species a single component is quantitatively dominating, whereas others possess several large components.

The marking secretion, emanating from the cephalic part of the labial gland (Kullenberg et al., 1973; Svensson and Bergstöm, 1977; Cederberg, 1977), is used by males when performing a route-flight behavior. The males alight on various objects (e.g., twigs, leaves, mosses, and grass) along their route-flight circuits at the beginning of the daily route flight and mark these with the secretion. Several males of the same species may use the same object for marking. Later in the day the males mark the objects less frequently, just stopping briefly at these places while in the air and then continuing to the next marked object. These stopping places for marking and for approach usually remain the same during the day and also to a large extent during the life time of the individual. The characteristics of the behavior performed by the different species in the route-flight circuit have been shown to be species specific. Some of these characteristics are: height above the ground, choice of objects, approach behavior, and habitat selection for the route-flight circuits. Papers dealing with male nuptial flight of European species include those of Bringer (1973), Frank (1941), Free (1971), Darwin (1886), Haas (1946, 1949), Krüger (1951), Kullenberg (1956, 1973, 1975) and Svensson (1977b, 1978) and, for Nearctic species, Plath (1934) and Stiles (1976).

The behavior briefly described above is not valid for all bumble bee males. Species belonging to the genera *Bombias*, *Confusibombus* and *Mendacibombus*, where the males have very large eyes, perform a precopulatory behavior (Schremmer, 1972; Haas, 1976) which is similar to that of some solitary apoids and sphecids (Alcock, 1975; Velthuis and Camargo, 1975).

Free (1971), Haas (1968) and Kullenberg (1973) have made observations establishing that conspecific males and females react in a fixed member to the smell of the exposed labial gland secretion. In the male sex this response can be characterized as a stimulus for approach behavior and in the virgin queens as a sexual attractant and recognition factor. We therefore consider it appropriate to classify the marking secretions as pheromones. Moreover, since it was shown that the places marked will attract virgin queens for initiation of the mating, the specificity of the marking secretions and behavior

are assumed to function among the reproductive isolation mechanisms of the species. Consequently, closely allied species might be distinguished by the specificity of their behavior and marking secretion of the males (cf. Bergström et al., 1973; Svensson, 1973, 1977a, 1978).

In this paper results of the chemical analysis of marking pheromone blends of four species of the genus *Alpinobombus* Skor. are reported. The field work was performed at Abisko, Torne Lappmark, in northern Sweden, and the chemical analyses at the Ecological Station of Uppsala University on the island of Öland in the southern Baltic. Studies on the behavior of the males in the field will be reported separately.

NOTES ON THE TAXONOMY, DISTRIBUTION, AND BIOLOGY OF *Alpinobombus* SPECIES

Alpinobombus is a small homogeneous genus with only six species recognized throughout the world. As some of the species exhibit a holarctic distribution and some species are very similar morphologically, there has been much taxonomic confusion. Summarizing the works by Franklin (1912), Løken (1973), Milliron (1973), Pittioni (1938), Richards (1931), and Richards (1973), the genus should comprise the following species:

A. alpinus (Linné 1754)

A. hyperboreus (Schönherr 1809)

A. polaris (Curtis 1831); alpiniformis (Richards 1931); arcticus (Kirby 1821); kincaidii (Cockerall 1898)

A. balteatus (Dahlbom 1832)

A. kirbyellus (Curtis 1835)

A. strenuus (Cresson 1836)

Within the genus the species are morphologically closely connected in pairs. The pairs are *A. alpinus*—*A. polaris*, *A. balteatus*—*A. kirbyellus*, and *A. hyperboreus*—*A. strenuus*.

The distribution of the species is commented on by the authors mentioned above, and by Pittioni (1942) and Skorikov (1931). *A. kirbyellus* and *A. strenuus* are strictly Nearctic species and in Europe four species are distributed: *A. alpinus*, *A. balteatus*, *A. hyperboreus*, and *A. polaris*. *A. alpinus* is restricted to Europe, whereas the other three species are circumpolar. In Fennoscandia the four species are generally confined to alpine habitats, except *A. balteatus* which is also found in subalpine habitats and in adjacent coniferous forests (Løken, 1973; Svensson and Lundberg, 1977). Moreover, *A. hyperboreus* and *A. polaris* seem to have developed a more alpine/arctic adaptation then *A. alpinus* and *A. balteatus*.

Sakagami (1976) reviewed the fragmentary known biology of the

Alpinobombus species and further references are given by Løken (1973) and Richards (1973). The generic classification of bumble bees proposed by Tkalců (1972, 1974, and in several other papers) is followed.

METHODS AND MATERIALS

Collection and Preparation of Bees. The bees were collected in the Abisko area, Torne Lappmark, northern Sweden. Data on collection, biological material prepared and analytical technique used for each sample are given in Table 1. For further information on the preparation of the bees, see Bergström and Svensson (1973a) and Svensson and Bergström (1977). The specimens analyzed are kept at the Department of Entomology, Uppsala University.

Chemical Analysis. Material for the chemical work was usually (see Table 1) obtained by extracting whole heads in hexane for 2 days, during which time the extract was kept at +20° C, or for 30 days at −20° C. In one case (*A. balteatus*) labial glands were dissected out and placed directly in the precolumn of the gas chromatograph. In all species, except for *A. polaris*, whole heads were also analyzed in a fresh state. We have found that the extraction of whole heads gives results as good as direct degassing in gas chromatography. Irrelevant substances from interior parts of the bumble bee head or from other sources such as food plants amount to very minute quantities, and extracts could easily be transported from Abisko to Öland for analysis.

The chemical analyses were made by combined CGC/MS (capillary gas chromatography-mass spectrometry) using a modified splitter-free inlet system, the original of which has been described earlier (Bergström, 1973; Ställberg-Stenhagen, 1972; Stenhagen et al., 1973). In this way, volatile compounds were transferred directly into the column by heating the precolumn to 150° C for 5 min. The precolumn consists of a stainless steel syringe, housing a small glass tube (45 × 6 mm OD) in which biological material, extracts, or adsorbants can be placed. The syringe can be inserted through the rubber septum of the inlet system and the material for analysis is heated with a precolumn heater while purged with carrier gas.

The glass capillary columns (25 m long) used in this study have SE-30 and OV-101 as stationary phases, and a theoretical plate number of 76,000 and 66,000, respectively. Gas chromatographic retention values were obtained by relating the position of the peaks of the gas chromatogram to that of the closest saturated, straight-chain hydrocarbons, which were given the number $N_C \times 100$. The temperature of the GC oven was raised by programming it from +25° or +50° to 220° C at 8°/min. The sharp vertical lines of the gas chromatograms presented indicate mass spectral recordings. Chemical

TABLE 1. COLLECTION DATA AND CHEMICAL ANALYSES OF THE *Alpinobombus* SPECIES STUDIED[a]

Species	Collection no. (at 1 PM)	Locality		Date of coll.	Feeding on/collected in	Material analyzed	Number of individuals	Analytical method
A. alpinus	1	Abisko, Njulla	900 m	12/8 1971	*Saxifraga aizoides*	f.h.	1	MS
	74	Abisko, Njulla	650 m	16/8 1972	*Solidago virgaurea*	e.	1	CGC, TLC
	4	Abisko, Njulla	975 m	13/8 1977	Foraging flight	e.	1	MS
	5	Abisko, Njulla	975 m	13/8 1977	Foraging flight	e.	1	MS
	9	Slåttatjåkka	1000 m	14/8 1977	Route-flight circuit	e.	1	MS
A. balteatus	1	Abisko, Njulla	900 m	12/8 1971	*Astragalus alpinus*	f.h.	3	MS
	16	Abisko, Njulla	900 m	13/7 1972	*Astragalus alpinus*	e.	1	CGC, TLC
	44	Abisko, Njulla	900 m	24/7 1972	*Astragalus alpinus*	e.	2	CGC
	45	Abisko, Njulla	900 m	24/7 1972	*Astragalus alpinus*	e.	1	CGC
	70	Abisko, Njulla	900 m	14/8 1972	*Saussurea alpina*	e.	1	CGC
	71	Abisko, Njulla	650 m	16/8 1972	*Solidago virgaurea*	e.	1	CGC
	72	Abisko, Njulla	900 m	16/8 1972	*S. virgaurea*, *S. alpina*	e.	3	CGC
	129	Abisko, Njulla	350 m	30/7 1973	*Chamaenrion angustifolium*	e.	2	MS, CGC
	131	Abisko, Njulla	400 m	31/7 1973	*Vicia cracca*	e.	1	MS, CGC
	2	Abisko, Njulla	650 m	10/8 1975	*Geranium silvaticum*	f.l.g.	1	MS
	18	Abisko, Njulla	400 m	26/8 1975	*Trifolium pratense*	e.	2	MS
A. hyperboreus	1	Abisko, Njulla	900 m	12/8 1971	*Astragalus alpinus*	f.h.	1	MS
	2	Abisko, Njulla	900 m	12/8 1971	*Astragalus alpinus*	f.h.	1	CGC
	56	Abisko, Njulla	900 m	6/8 1972	*Astragalus alpinus*	e.	1	CGC, TLC
	141	Kärketjårro	750 m	4/8 1973	Route-flight circuit	e.	1	MS, CGC
	142	Kärketjårro	750 m	4/8 1973	Route-flight circuit	e.	1	MS, CGC
	143	Kärketjårro	750 m	4/8 1973	Route-flight circuit	e.	1	MS, CGC
	2	Abisko, Njulla	975 m	13/8 1977	*Silene aucalis*/route-flight circuit	e.	3	MS
A. polaris	3	Abisko, Njulla	975 m	13/8 1977	Route-flight circuit	e.	1	MS
	6	Slåttatjåkka	1100 m	14/8 1977	*Vaccinium vitis-idaea*	e.	1	MS
	7	Slåttatjåkka	1100 m	14/8 1977	*Polygonum viviparum*	e.	1	MS
	8	Slåttatjåkka	1000 m	14/8 1977	Route-flight circuit	e.	1	MS

identifications, or partial identifications, were made by comparing mass spectra and capillary gas chromatographic retention values with those of available reference compounds.

RESULTS

A. alpinus. The main compound in the volatile marking secretion of this species was found to be a straight-chain hexadecenol. It corresponds to component 2 in the gas chromatogram given in Figure 1. In this study double-bond positions and cis/trans isomerism were not determined in any of the fatty acid derivatives where they occur. The reason is the rarity of all the present species except *A. balteatus*. Compounds from the different species with identical chain length, number of double bonds, and functional groups have been tabulated in Table 2. The compounds not fully identified may not be identical, although this is the most plausible conclusion on biogenetic grounds. As shown by Figure 1, hexadecenol is quantitatively very dominating in the secretion. The gas chromatographic retention value on the OV-101 column was found to be 1847 (1800 for *n*-octadecane). The corresponding value for a synthetic hexadec-9-*cis*-enol is 1846. Other compounds identified in *A. alpinus* are: a hexadecadienol (1), a hexadecenyl acetate (3), and the straight-chain hydrocarbons heneicosene (4), heneicosane (5), tricosene (6), and tricosane (7); the numbers in parenthesis refer to

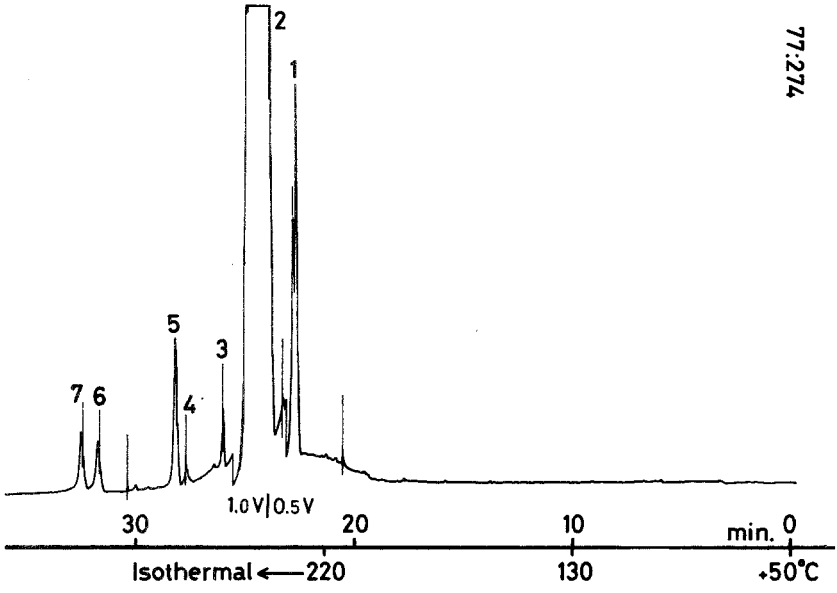

FIG. 1. Capillary gas chromatogram of a portion of an extract in hexane of one male head of *A. alpinus*. Analytical conditions given in Methods and Materials.

TABLE 2. VOLATILE COMPONENTS OF THE CEPHALIC LABIAL GLAND SECRETION OF FOUR SPECIES OF *Alpinobombus*[a]

Species	MEVALOGENINS				ACETOGENINS														
	Citronellol (156)	Dihydrofarnesal (222)	Dihydrofarnesol (224)	Geranylcitronellol (292)	Dodecanol (186)	Tetradecanol (214)	Hexadecadienol (238)	Hexadecenol (240)	Oktadecadienol (266)	Oktadecenol (268)	Dodecyl acetate (228)	Tetradecyl acetate (256)	Hexadecenyl acetate (282)	Dodecyl butyrate (256)	Tetradecyl butyrate (284)	Heneicosene (294)	Heneicosane (296)	Tricosene (322)	Tricosane (324)	Pentacosane (352)
A. alpinus							1	②					3			4	5	6	7	
A. polaris							①	2									3	4	5	
A. hyperboreus	1	2	3	7					4	⑤							6	8	9	
A. balteatus				8	1	3					2	⑤		4	6	7		9	10	11

[a]The numbers correspond to components marked in figures 3-6. Summarizing all the analyses performed of each species, main components are encircled and large components underlined. Molecular weights in parentheses.

component numbers given in the gas chromatogram in Figure 1 and in Table 2. The proportion between hexadecenol and hexadecadienol is approximately 20:1.

A. polaris. Figure 2 shows a gas chromatogram of the volatile material from *A. polaris*. Although the composition of the marking secretion of this species shows many similarities with that of *A. alpinus*, the main compound here is a hexadecadienol (1). It may be identical with the hexadecadienol present in smaller amounts in *A. alpinus* (1).

The second compound in order of magnitude in *A. polaris* is a hexadecenol (2), probably identical with the main compound of *A. alpinus*. The proportion between components 1 and 2 of *A. polaris* is approximately 5:2. Beside these two alcohols, heneicosane (3) and tricosane (4) were identified in the secretion in smaller amounts.

A. hyperboreus. A straight-chain octadecenol is the main substance in the *A. hyperboreus* marking secretion (component 5 in Figure 3). The retention value of this component (OV-101 column) is 2047 as compared to 2046 for synthetic *cis*-9-octadecen-1-ol. The next largest component was identified as a dihydrofarnesol (3), presumably *trans*-2,3-dihydro-6-farnesol. This compound has earlier been identified by us in marking secretions of the bumble bees *B. terrestris* and *B. jonellus* (Bergström et al., 1973; Bergström and Svensson, 1973b; Svensson and Bergström, 1977). The retention value (OV-101) is 1672 for this component. The same value was

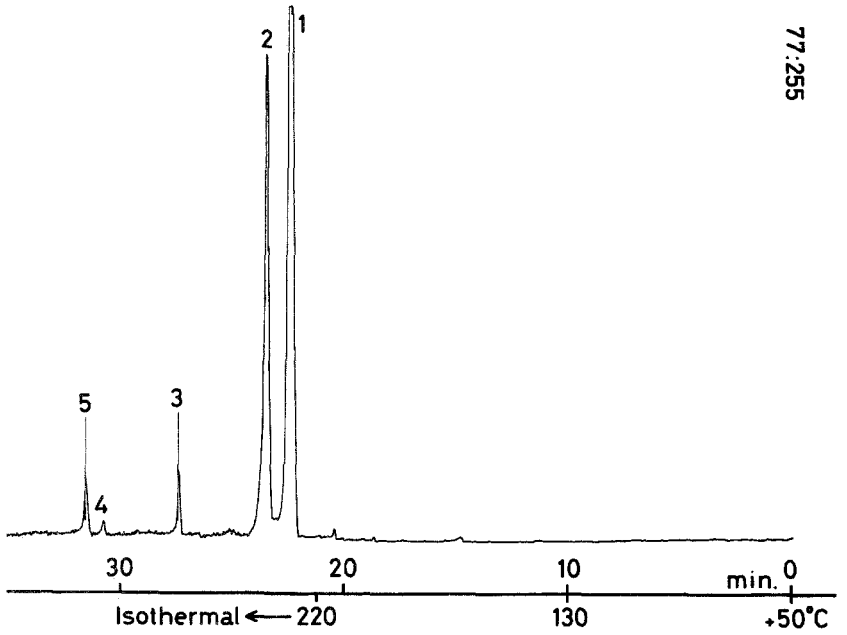

FIG. 2. Capillary gas chromatogram of a portion of an extract in hexane of one male head of *A. polaris*.

measured for synthetic *trans*-2,3-dihydro-6-farnesol. It is present in the secretion in proportions varying from 1:1 to 1:2 relative to the main compound.

Smaller components which have been identified are citronellol (1), retention value 1225 for the natural compound and 1223 for a reference sample on an OV-101 column, a dihydrofarnesal (presumably *trans*-2,3-dihydro-6-farnesal) (3), and geranylcitronellol (7). The latter compound was found to have a gas chromatographic retention value of 2133. The same value was obtained with a synthetic sample of all-*trans*-geranylcitronellol (Ahlquist and Ställberg-Stenhagen, 1971). These three compounds make up the mevalogenic part of the secretion and have large differences in volatility. Fatty acid derivatives occurring in small amounts are: an octadecadienol (4), heneicosane (6), a tricosene (8), and tricosane (9).

A. balteatus. The marking secretion of this species was found to be characterized by several homologous straight-chain alcohols, acetates, and butyrates. The latter class of compounds has not earlier been found in our analyses of bumble bee secretions. The main compound is tetradecyl acetate, component 5 in the gas chromatogram shown in Figure 4. Other large components identified are dodecyl butyrate (4) and tetradecyl butyrate (6). The retention values of these three compounds were: 1798, 1774, and 1974,

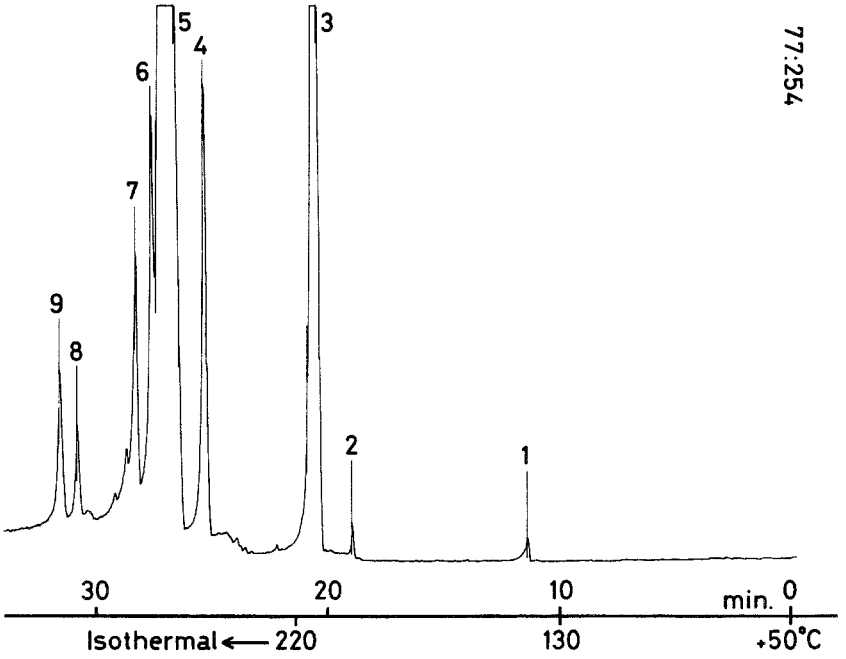

FIG. 3. Capillary gas chromatogram of a portion of an extract of three male heads of *A. hyperboreus*.

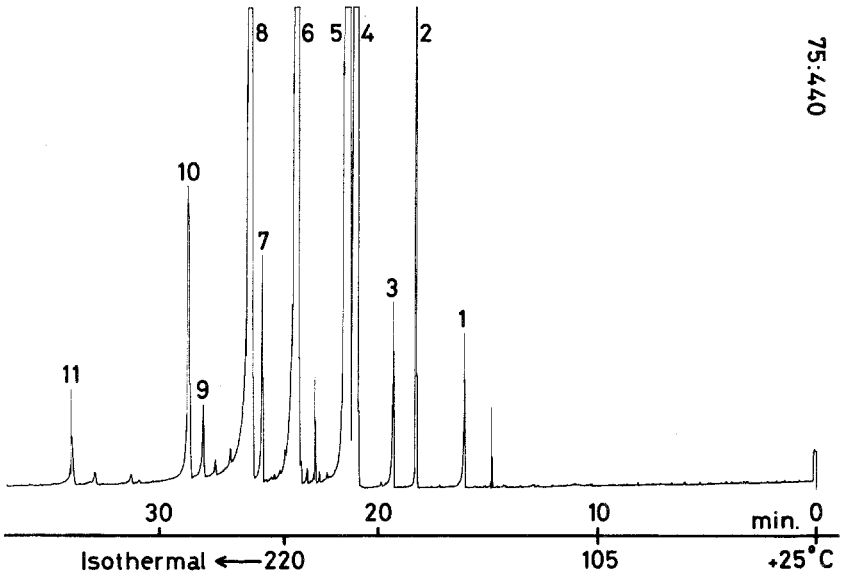

FIG. 4. Capillary gas chromatogram of volatile compounds from part of one labial gland from a single male of *A. balteatus*.

respectively. Synthetic reference compounds, run under the same conditions (Silicone OV-101), gave 1797, 1774, and 1974, respectively.

Besides these major components, the following substances have been found to be present in smaller amounts (compare Table 2): dodecanol (1), dodecyl acetate (2), tetradecanol (3), heneicosane (7), tricosene (9), tricosane (10), and pentacosane (11). Geranylcitronellol (8) has also been found, retention value 2133, which was also obtained for the synthetic reference compound. The amount of this diterpene alcohol varies in different preparations. Usually the amounts present in the secretion are smaller than shown by the gas chromatogram in Figure 4. The average proportions between the components 4, 5, 6, and 8 are 2:4:2:1. In this species there is an unusual variation in the color of the coat as regards the degree of melanism. We found no corresponding variation in the chemical composition of the marking secretion.

DISCUSSION

The results of the chemical analysis of the male marking secretions of the four species reported here fall into the general pattern found by earlier analyses of other species of *Bombus* s.l. and *Psithyrus* (29 and 6 species, respectively) (Kullenberg et al., 1970, Bergström and Svensson, 1973 a,b; Svensson and Bergström, 1977). However, *A. balteatus* stands out by having two butyrates, (C_{12} and C_{14} butyrates) as large components of the secretion. They have not earlier been found in any bumble bee species, but were found to be present in the Dufour gland secretion of *Melitta haemorrhoidalis* and in the male cephalic secretion of its nest parasite, *Nomada flavopicta* (Tengö and Bergström, 1976).

Another fact which is immediately clear from the results reported here is that there are considerable similarities between the compositions of the secretions of *A. alpinus* and *A. polaris* (Table 2). They both possess a hexadecadienol and a hexadecenol, each of which may be identical in the two species. A major difference lies in the quantitative proportions between these two alcohols. The hexadecenol is the completely dominant compound in *A. alpinus*, in the proportion 20:1, whereas in *A. polaris* the proportion between them is about 2:5. As known from other insects, despite the great similarity of the two species, the specifity of the sexual pheromones could be maintained by differences in the proportions between the compounds (Minks et al., 1973). *A. polaris* lacks hexadecenyl acetate altogether, and both species seem to lack mevalogenic compounds.

A. hyperboreus and *A. balteatus* have one similarity in the fact that they both contain appreciable amounts of geranylcitronellol. They also both contain straight-chain alcohols, albeit of different chain length and degree of

saturation. The presence of esters (acetates and butyrates) in *A. balteatus* and the mono- and sesquiterpenes in *A. hyperboreus* constitutes a characteristic difference between the two species. The secretions of both species are very rich in components.

As in all other species of bumble bees studied by us, straight long-chain hydrocarbons are present in the secretion. They are mainly saturated C_{21} and C_{23} alkanes.

Summarizing the results of the chemical analysis from a biosynthetic point of view, we note that there is no major chemical group of volatile compounds in the four *Alpinobombus* species. Such a group was found in *Pyrobombus*, where, with one exception, the dominant compound consisted of mevalogenins (Svensson and Bergström, 1977). The exact function of the different compounds in the secretion is not yet known. Some may be releasers of behavior and some may decrease the volatility of the releaser substances. Our knowledge of the chemical composition of male bumble bee marking pheromones has phylogenetic and evolutionary aspects as well as taxonomic ones, not to mention the ethological and ecological interpretations.

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IDENTIFICATION AND FIELD EVALUATION OF THE COMPOUNDS COMPRISING THE SEX PHEROMONE OF THE FEMALE BOLL WEEVIL[1,2,3]

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Abstract—The terpenoid compounds (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol (I), (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol (II), and β-caryophyllene were isolated from frass of the female boll weevil (*Anthonomus grandis* Boheman). In laboratory bioassays, a mixture of these components attracted primarily males, whereas the male pheromone, grandlure, attracted primarily females. The addition to the cotton bud hydrocarbons, α-pinene, myrcene, and *l*-limonene, improved the response by males so that the potency of the mixture was comparable to that of grandlure for females. In field tests, I + II + hydrocarbons attracted both sexes, but grandlure alone and grandlure + hydrocarbons were more effective.

Key Words: Sex pheromone components, olfaction, behavior, female boll weevil, Coleoptera, Curculionidae, *Anthonomus grandis*.

INTRODUCTION

Keller et al. (1964) had reported that trapped volatiles from the male boll weevil, *Anthonomus grandis* Boheman, attracted females in the laboratory. Tumlinson et al. (1969) later identified this pheromone as a mixture of two

[1] Coleoptera: Curculionidae.
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[3] Mention of a commercial or proprietary product in this paper does not constitute endorsement of this product by USDA.
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terpene alcohols and two terpene aldehydes [I = (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; II = *Z*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III = (*Z*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; IV = (*E*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde]. Field activity of the synthesized mixture, which was named grandlure, was confirmed. Primarily females responded to grandlure, but under some conditions both sexes responded to it in the field (McKibben et al., 1971; Hardee et al., 1972). In laboratory bioassays, however, male boll weevils were not attracted to females significantly more than to other males (Hardee et al., 1967). Likewise Tumlinson et al. (1968) reported that neither males or females were responsive to extracts or steam distillates of females.

However, Cross and Mitchell (1966) reported that males did respond to females from distances of less than about 5 cm, and they speculated that the female emitted a weak secondary pheromone. Cross (personal communication) also observed that male weevils became excited as if a female weevil was nearby, when in fact the female was on the opposite side of a cotton leaf. They therefore speculated that this observed activity of the male was initiated as a result of olfactory perception.

McKibben et al. (1977) recently reported that steam distillation of frass (100,000 insect-day equivalents) of the female boll weevil, *Anthonomus grandis* Boheman, yielded an extract that was more attractive to males than to females in the laboratory. Extracts purified by TLC were attractive to males only. The active components were thought to be alcohols and hydrocarbons. Apparently the failures of the earlier attempts to find evidence for a female pheromone (Hardee et al., 1967; Tumlinson et al., 1968) could be attributed to insufficient concentrations of test materials.

We now report the identification of three pheromone compounds from females, their effects on males in laboratory bioassays, and the results of several field evaluations.

METHODS AND MATERIALS

Handling of Insects and Frass. Laboratory-reared boll weevils (Gast and Davich, 1966) were separated by sex within 2–3 days after emergence. Females were maintained for 2 weeks in 30 × 30 × 30-cm stainless-steel cages at 18° C and fed fresh cotton squares (buds) supplemented with artificial diet plugs. Frass accumulating under the cages was collected twice daily and stored in stoppered flasks at −20° C. Collections were continued for 2 weeks because males biosynthesize pheromone for as long as a month (Hedin et al., 1974).

Laboratory Bioassay. The laboratory bioassay procedure was that developed by Hardee et al. (1967) in which 20 boll weevils of the desired sex were placed in an arena consisting of an inverted 15-cm glass funnel fitted with

a platform and side arms. The candidate compounds or live, sexed insects to be evaluated were placed in one of the side arms, and air was pulled through the system from the side arms so that attracted insects in the arena would respond by moving to the odor source. Insects used for the bioassay were separated by sex on the day of emergence and fed fresh cotton squares daily. Insects that were 4–6 days old were used for the bioassay. Tests were replicated four times on at least three separate days. An index of attraction was calculated as defined by Tumlinson et al. (1968).

Test Compounds and Cotton Bud Essential Oil. The four boll weevil pheromone components [I = (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; II = (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III = (*Z*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; IV = (*E*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde] were obtained from Chemical Samples, Co. (Columbus, Ohio) and formulated in the ratio I:II:III:IV/30:40:15:15 in Carbowax® 1000 unless otherwise stated. The components were all of 95% purity or better as analyzed by GLC. Compound I is a 1:1 mixture of optical isomers plus minor positional isomers. The predominant impurity of compound II is the *E* isomer. Three major impurities, all resulting from oxidation of compounds III and IV, are 3,3-dimethylcyclohexanecarboxaldehyde, (*Z*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetic acid (and the *E* isomer), and (*Z*)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexanemethanol (and the *E* isomer) (Hedin et al., 1976). The hydrocarbons, β-caryophyllene, α-pinene, myrcene, *l*-limonene, and α-phellandrene, were obtained from Pfaltz and Bauer, Inc. (Stamford, Connecticut), and purified by GLC to 95% or better on a 0.2 × 305-cm stainless-steel column packed with 10% SP-2401® on 80/100 Gas Chrom Q®. The cotton bud essential oil was obtained in a yield of approx. 100 ppm by steam distillation of buds. The hydrocarbon fraction was separated from the polar fraction by column chromatography on Florisil with pentane. The polar fraction was eluted with methylene chloride.

Isolation. The frass volatiles were collected by steam distillation of the frass for 1 hr and subsequent extraction of the distillate with methylene chloride. The concentrated frass essential oil (100,000 female-day equivalents) was fractionated by chromatography on a silica gel-G TLC plate with 50% methylene chloride in pentane. As a result, the hydrocarbons which migrated with the solvent front were separated from five fractions of polar compounds including the alcohols. Each fraction was evaluated separately and in combinations of two or more by the previously described laboratory bioassay (Hardee et al., 1967). Recombination of fractions 2 (alcohols) and 6 (hydrocarbons) in a 1:1 mixture was required for attractancy of males.

Fraction 2 was rechromatographed by SG-TLC with methylene chloride-pentane, 1:1, to yield two major adjacent bands that were located by UV light and visualized by end-spraying the plate with 3% vanillin in 0.5% ethanolic H_2SO_4. Each band was eluted with methylene chloride, and the contents were

then isolated for bioassay and identification by fractionation on a SP-2401 GLC column (see previous section for details).

Fraction 6 was rechromatographed by TLC on $AgNO_3$-impregnated (15%) silica gel with methylene chloride–pentane, 1:9, to yield several bands that were visualized by end-exposing the plate to I_2 vapors. The band that was found to possess biological activity was isolated for identification by fractionation on the SP-2401 GLC column. All of the hydrocarbon fractions were evaluated in combination with fraction 2 (alcohols) by the laboratory bioassay as a guide to the isolation work.

Gas Chromatographic, Mass Spectral, and Nuclear Magnetic Resonance Analysis. The previously described SP-2401 column was utilized both for separation and analysis work. The unit was fitted with an exit splitter for collection. An estimate of the daily production of each pheromone component was made from GLC analysis of a female frass sample collected from a known number of females during 14 days. An internal standard, α-terpineol, was added to the frass oil to aid in the GLC quantitation.

The essential oil and fractions were also analyzed by introduction into a Hewlett Packard 5930 Quadrupole® mass spectrometer from a 76m × 0.8-mm stainless-steel capillary gas chromatographic column coated with OV-17®. Carrier gas flow was 8.0 ml/min of He. The GLC unit was programed from 100 to 180° C at 2° C/min. The final temperature was maintained for 20 min. Mass spectra were obtained at 70 eV. Proton magnetic resonance spectra of the hydrocarbons were obtained in $CDCl_3$ with a 100 MHz Thompson Packard® spectrometer. The hydrocarbons were collected by GLC trapping from the previously described SP-2401 column at the stated conditions.

Field Evaluations in Florida and Winston County, Mississippi. From February 1 to 4, 1977, a randomized test was conducted at the Florida Experiment Station, Homestead, Florida. A second test was conducted in Winston Co., Mississippi, from June 6 to 8, 1977. Laboratory-reared insects of mixed sexes were released into an open field and allowed to respond to standard Leggett traps (Leggett and Cross, 1971) baited with grandlure, plant and synthetic hydrocarbons, the male boll weevil pheromone alcohols, and various combinations of these materials. The test was replicated five times. In these tests, 3 mg of grandlure or pheromone alcohols were employed at the specified ratios in 0.5 ml of water–polyethylene glycol–glycerol–methanol (12.5:12.5:25:50) applied to a dental wick. A second wick filled with 0.5 ml of heptane containing 100 mg of the specified hydrocarbon was attached to the first wick with a rubber band and placed in the Leggett trap. While previous studies had shown that 3 mg of grandlure was effective for at least one week (Hardee et al., 1972), optimum levels for hydrocarbon deployment had not been established. Thus the 100-mg level was chosen for the early season, lower

temperature test, and this level was repeated in the Winston County test. For comparison, a much lower concentration (3 mg) was chosen for the Carolina tests (see next section).

Field Evaluations in North and South Carolina. From July 28 to September 6, 1977, in Florence County, South Carolina, tests were conducted in 10 to 12-hectare fields containing low and high populations of boll weevils. The low-population condition was achieved as a result of heavy applications of insecticide to control *Heliothis* spp. No laboratory insects were released. Grandlure (Tumlinson et al., 1969) was compared with the synthetic female pheromone mixture and with an empty trap (Leggett). Also, in a test conducted in Cleveland County, North Carolina, from October 4 to 11, 1977, grandlure with and without a synthetic hydrocarbon formulation (equal weight quantities of caryophyllene, pinene, myrcene, and limonene) was compared by baiting traps located inside and adjacent to the test field. There were three replications. Formulations were as described in the previous section.

RESULTS

Identification of Pheromone Compounds. GLC of the entire frass essential oil on SP-2401 gave a chromatogram consisting of 12 major maxima. This chromatogram was essentially identical to that included as a figure in McKibben et al. (1977). TLC of the frass oil yielded six fractions. A 1:1 mixture of fractions 2 (alcohols) and 6 (hydrocarbons) was required for attractancy of males in the laboratory (Table 1). Upon rechromatography of fraction 2 on SG-TLC, two adjacent bands were located that were unattractive individually to males, but elicited some attractivity upon combination. Each was chromatographed by GLC on the SP-2401 column until a single peak was obtained. These components (isolated I and II) were not attractive individually, but again elicited intermediate attractivity upon combination (Table 1). The retention volumes of isolated I and II were those of peaks 6 and 7 in the figure of McKibben et al., (1977). MS analysis of crude TLC-2 introduced from a 75-m capillary OV-17 revealed a number of components. The two major peaks at I_k values of 1363 and 1383, respectively, were found to be (+)-*cis*-2-isopropenyl-1-methyl-cyclobutaneethanol (I) and (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol (II), the alcohols of the male boll weevil pheromone (Tumlinson et al., 1969). Subsequent GLC-MS of the individually isolated components on the same verified the above analysis by revealing that isolate I (peak 6) was compound I and isolate II (peak 7) was compound II. The GLC, TLC, and MS properties were identical to those of the synthetic compounds.

TABLE 1. LABORATORY BIOASSAYS OF PHEROMONE CANDIDATES

Test	Index of attraction[a] Males	Females
Grandlure,[b] 8 μg	2.6 ± 0.6	31.9 ± 2.3
Cotton bud hydrocarbons, 6 μg	− 0.8 ± 0.4	0.6 ± 0.2
Cotton essential oil, 6 μg	− 0.6 ± 0.2	2.5 ± 1.1
Female frass oil, 20 μg	27.0 ± 4.0	10.0 ± 2.3
TLC-2 + TLC-6[c]	19.0 ± 3.0	1.0 ± 0.5
Isolated I	2.3 ± 1.0	0.9 ± 0.4
Isolated II	4.6 ± 0.5	0.8 ± 0.2
Isolated I + isolated II	9.0 ± 1.3	3.0 ± 0.3
Isolated β-caryophyllene	4.3 ± 0.4	3.6 ± 0.2
Isolated α-humulene	3.1 ± 0.2	1.7 ± 0.3
Isolated I + II + β-caryophyllene	15.9 ± 0.9	4.3 ± 0.8
Isolated I + II + β-caryophyllene + α-humulene	18.1 ± 0.5	2.9 ± 0.4
Synthetic I, 10 μg[d]	− 2.5 ± 0.6	1.3 ± 0.5
Synthetic II, 10 μg[d]	10.6 ± 1.0	3.1 ± 0.4
I, 2.25 μg + II, 10 μg	10.6 ± 1.8	5.0 ± 0.5
I, 10 μg + cotton bud hydrocarbons, 6 μg	20.0 ± 2.5	
II, 10 μg + cotton bud hydrocarbons, 6 μg	23.3 ± 3.6	5.0 ± 1.5
I, 2.25 μg + II, 10 μg + cotton bud hydrocarbons, 6 μg	32.7 ± 2.1	6.0 ± 1.4
I, 2.25 μg + II, 10 μg + β-caryophyllene, 2 μg	15.8 ± 1.9	3.1 ± 0.9
I, 2.25 μg + II, 10 μg + β-caryophyllene, pinene, and myrcene (1:1:1), 2 μg	27.0 ± 3.9	1.0 ± 0.3
Grandlure, 8 μg + β-caryophyllene, pinene, and mycene (1:1:1) 2 μg[e]	34.5 ± 1.8	30.7 ± 3.8
Grandlure, 8 g + β-caryophyllene, pinene, myrcene, and limonene (1:1:1), 2 μg	30.6 ± 2.5	31.9 ± 4.1

[a] Index of attraction = $\frac{\text{No. weevils responding to attractant} - \text{no. responding to control}}{\text{No. weevils released} - \text{no. responding to control}}$.

[b] The male boll weevil pheromone I–II–III–IV (30:40:15:15) in methanol (Hardee et al., 1972).

[c] Isolate from 1000 female-day equivalents of frass.

[d] (+)-cis-2-Isopropenyl-1-methylcyclobutaneethanol (I) and (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol II; ratio of 2.25:10 based on ratio present in female frass.

[e] Grandlure, 8 μg + β-caryophyllene, 2 μg: 16.5 ± 2.7; G + α-pinene, 2 μg: 10.6 ± 1.8; G + myrcene, 2 μg: 3:1 ± 1.9; G + limonene, 2 μg: −6.6 ± 2.8; G + α-phellandrene, 2 μg: 0.8 ± 0.5.

The frass essential oil hydrocarbon fraction accounted for GLC peaks 9–12, of which 9 and 11 were present in highest concentrations. The retention volumes (I_k) of these two peaks on the OV-17 column were 1520 and 1555, those of sesquiterpene hydrocarbons. TLC on silica gel impregnated with 15% $AgNO_3$ did not separate peak 9 from 11 completely, but did remove other extraneous compounds. Subsequent GLC on SP-2401 of the mixture of peaks 9 and 11 afforded a separation. Each was rechromatographed to give components essentially free of the other. By GLC-MS investigation, the properties of peak 9 were found to be identical to those of β-caryophyllene (Hedin et al., 1974), and the PMR spectrum agreed closely with that reported by Minyard et al. (1966). By similar GLC-MS investigation, the properties of peak 11 were found to be identical to those of α-humulene (Stenhagen et. al., 1969), but insufficient sample was available for confirmatory PMR analysis. In laboratory bioassays, neither isolated β-caryophyllene or α-humulene was particularly attractive to males (Table 1). The combination of isolates of I + II + β-caryophyllene + α-humulene elicited an index of attraction as great as TLC-2 + TLC-6 (Table 1). By GLC of the total oil as cochromatographed with a known quantity of an internal standard, the daily production of these components (ng/day per female) was determined to be as follows: (±)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol, 0.6 ng; (*Z*)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol, 2.4 ng; β-caryophyllene, 2.4 ng; and α-humulene, 5.1 ng.

Laboratory Bioassay Studies with Synthetic and Other Test Compounds. In laboratory tests (Table 1), I or II plus the cotton bud hydrocarbon fraction attracted about 60% as many males as grandlure attracted females; I or II plus hydrocarbons did not attract females nor did grandlure attract males. Thus, there was a clear difference in the requirements for the attraction of males and females. The addition of I to the mixture of II and the cotton hydrocarbons produced an index of attraction for males that equaled that of grandlure for females.

Next, four commercially available terpene hydrocarbons (β-caryophyllene, myrcene, α-pinene, and limonene) were evaluated as replacements for the cotton bud essential oil hydrocarbon fraction (Minyard et al., 1965, 1966). No individual hydrocarbon, when tested with the two pheromone alcohols, elicited a response above 10 except for β-caryophyllene (Table 1). It was eventually determined that equal weight mixtures of β-caryophyllene, α-pinene, and myrcene (CPM), or of these three + *l*-limonene gave the best responses of several hydrocarbon mixtures when bioassayed with I and II.

It was then determined that grandlure + commercial hydrocarbons attracted each sex (Table 1). This demonstrated that the male pheromone aldehydes III and IV, although unneeded for attraction of males in the laboratory, are not repellents to males. This also provided a rationale for the potential utilization of one formulation, grandlure + hydrocarbons, to attract both sexes in the field.

TABLE 2. FIELD EVALUATIONS OF PHEROMONE CANDIDATES AT HOMESTEAD, FLORIDA, AND WINSTON COUNTY, MISSISSIPPI, 1977

Treatment[a]	Homestead, Fla.[b]		Winston Co., Miss.[c]	
	$\bar{X}$/rep.	% males	$\bar{X}$/rep.	% males
Control	3.3 a	22	1.0 c	
I + II, 2.25:10, 3 mg	3.0 a	17	1.0 c	
Cotton hydrocarbons, 100 mg	2.0 a	25	1.0 c	
CPM (1:1:1), 100 mg	2.0 a	50	1.0 c	
I + II, 3 mg + cotton hydrocarbons, 100 mg	3.7 a	45	1.5 c	
I + II, 3 mg + CPM, 100 mg	18.7 b	46	2.5 bc	50
Grandlure, 3 mg[d]	44.0 c	49	4.7 a	55
Grandlure, 3 mg + cotton hydrocarbons, 10 mg	21.3 b	36	3.5 b	43
Grandlure, 3 mg + CPM 100 mg	47.3 c	45	1.7 c	40

[a]Average catch per replicate, 3–4 replicates. Means not followed by same letter are significantly different at 5% level, Duncan's new multiple-range test.
[b]Laboratory-reared released weevils, mixed sexes.
[c]Native weevils only, none released.
[d]I–II–III–IV (30:40:15:15); 3 mg in 0.5 ml of water–polyethylene glycol–glycerol–methanol (12.5:12.5:25:50) applied to a dental wick and deployed in a Leggett trap (Hardee et al., 1972). Hydrocarbons were dissolved in 0.5 ml heptane and applied to the wick.

Field Evaluations. In the tests conducted at the Florida Experiment Station, Homestead, Florida from February 1 to 4, 1977, and in the tests at Winston County, Mississippi, from June 6 to 14, 1977, various formulations of the components of grandlure plus cotton hydrocarbons were dispensed into wicks saturated with Carbowax 1000 (extender or keeper), and the wicks were

TABLE 3. PHEROMONE TESTS IN LOW- AND HIGH-DENSITY FIELDS IN FLORENCE COUNTY, SOUTH CAROLINA, JULY 28–SEPT. 6, 1977

Test	Low-density field		High-density field	
	Total no. captured	% males captured	Total no. captured	% males captured
Grandlure, 3 mg	45 (7.50 b)[a,b]	6	625 (156.25 a)	42
Grandlure, 3 mg + CPM, 3 mg	51 (8.50 a)	13	528 (130.00 n)	44
Control	2 (0.33 c)	50	76 (19.00 c)	39

[a]Total catch with average catch per week in parenthesis. Means not followed by same letter are significantly different at the 5% level, Duncan's new multiple-range test.
[b]Formulations were as described in Table 2, footnote *d*.

TABLE 4. PHEROMONE TESTS INSIDE AND ADJACENT TO COTTON FIELDS IN CLEVELAND COUNTY, NORTH CAROLINA, OCT. 4–11, 1977

Test	Total no. captured[a]	
	Adjacent	Inside field
Grandlure, 3 mg	12 (3.0 b)	164 (54.7 b)
Grandlure, 3 mg + LCPM, 3 mg[b,c]	19 (4.8 a)	231 (77.0 a)
LCPM, 3 mg	1 (0.3 b)	120 (40.0 b)
Control	0 (0 b)	66 (22.0 b)

[a]Total catch with average catch per week in parenthesis, 3–4 replicates. Means not followed by same letter are significantly different at 5% level, Duncan's new multiple-range test.
[b]Limonene, β-caryophyellene, α-pinene, myrcene; 1 : 1 : 1 : 1.
[c]Formulations were as described in Table 2, footnote *d*.

placed in pheromone traps as described. The Florida test results, summarized in Table 2, show that I + II alone (3.0 insects per replicate) or hydrocarbons alone (2.0) were not attractive. I + II + CPM showed intermediate effectiveness (18.7), but I + II + cotton hydrocarbons was unattractive (3.7). Grandlure alone (44.0) and grandlure + CPM (47.3) attracted approximately equal numbers, and the sex ratio was not altered. Much lower populations were present during the Mississippi tests, but the capture trends were similar.

In the first test conducted in Florence County, traps baited with grandlure and grandlure + CPM were placed in fields with known low and high populations of boll weevils. The results, which are summarized in Table 3, show a significant increase in the percent males trapped in response to added hydrocarbons (Duncan's new multiple-range test). Also, in the second test in Cleveland County, the total captures by grandlure plus β-caryophyllene, α-pinene, myrcene, and *l*-limonene (Table 4) were significantly improved. Since the release of volatiles from the cotton plant decreases in late season (Hedin 1976), the synthetic hydrocarbons may have had greater effect in these tests.

DISCUSSION

The daily production of I and II by female boll weevils is about 1% of that produced by males. The female boll weevil pheromone complex is similar to, but not identical to that of the male in that β-caryophyllene and α-humulene, but not III and IV are also required. In the laboratory, primarily males are attracted to the female pheromone mixture and vice versa. The utilization of plant hydrocarbons as components of pheromone complexes by Coleoptera has been established for a number of species. The performance of the female

pheromone in two field tests was marginal. Since the male pheromone, grandlure (with hydrocarbons) attracted both sexes equally well in the laboratory, the mixture was tested in the field to determine if it possessed efficacy as a simplified system for the attraction of both sexes. There was a marginal (although nominally statistically significant) improvement in total captures and percent males captured in comparison with grandlure. Movement of the male and response to females in the field is known to be very limited (Cross and Mitchell, 1966). Perhaps the normal behavioral patterns of males cannot be greatly altered, even though they are challenged with much greater amounts of pheromone than naturally biosynthesized by the female. The different responses observed in laboratory and field tests may also be attributed to differences in behavior in laboratory tests, as compared to the behavior of native (or laboratory-reared) weevils under field conditions.

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VOLATILE KETONES FROM THE PREORBITAL GLAND OF REINDEER (*Rangifer t. tarandus* L.)

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Abstract—The identification of 4-heptanone and 2-methyl-4-heptanone from the preorbital gland of reindeer (*Rangifer t. tarandus* L.) is reported. The secretion of the gland exhibit marked seasonal and sexual differences.

Key Words—Gland secretion, ketones, mass spectrometry, pheromones, *Rangifer*, skin gland, volatile compounds.

INTRODUCTION

Reindeer (*Rangifer t. tarandus* L.) as well as other cervids posess interdigital, preorbital, tarsal (see Schaffer, 1940), and caudal glands (Müller-Schwarze, et al., 1977). The preorbital gland in reindeer is a pouch anterior to the eye. The secretory epithelium is mostly situated in the bottom of the gland and consists of both holocrine sebaceous and apocrine sudoriferous glands (Schaffer, 1940). Little is known about the function of the preorbital gland in ungulates. One behavior that is often observed is the rubbing of the gland towards objects and thereby applying secretion which could be of importance in chemical communication. This have been observed in several species (Schaller, 1967; Müller-Schwarze, 1971, 1972; Volkman and Ralls, 1973).

Reindeer also rub different parts of their head towards objects but the movement is not strictly directed to the preorbital gland. (Müller-Schwarze, 1975). In reindeer the widening of the gland is frequently observed in agonistic encounters of both males and females. The gland may, in this context, serve as an olfactory as well as a visual stimulus. This assumption is supported by the observation that the animals turn the side of the head towards the antagonist (Mossing, in preparation).

In previous papers the identification of volatile compounds from the

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tarsal (Andersson et al., 1975), caudal (Müller-Schwarze et al., 1977), and interdigital glands (Brundin et al., 1978; Andersson et al., 1979) of reindeer have been reported. This paper reports the identification of volatile ketones from the preorbital gland of the same species.

METHODS AND MATERIALS

Eight reindeer (*Rangifer t. tarandus* L.) of the forest variety, four males and four females, were kept as one group in a pen. Three of the males and all of the females were mature. The animals were fed with lichen, commercial food, and water. Secretion from the preorbital glands was taken with cotton swabs and stored in sealed glass tubes at −20° C until analyzed. Samples were taken once a week over a period of one year. During the rut in October, samples were taken every day.

The volatile compounds from the preorbital gland secretion were collected in dichloromethane by the method described by Andersson et al. (1975). The concentrated dichloromethane solutions were fractionated by gas chromatography with a Pye Unicam GCD gas chromatograph equipped with a flame ionization detector (FID). It was fitted with a glass column (1.8 m × 4 mm) filled with 10% Reoplex 400 on 100–120 mesh Chromosorb W-AW-DMCS, 40 ml/min N_2 flow rate, held at 60° for 10 min, then temperature programmed at 4°/min to 140° C, and then held at 140° C for 20 min.

Mass spectra were recorded on a LKB 9000 mass spectrometer equipped with a Pye Unicam model 64 gas chromatograph. The carrier gas was helium at a rate of 30 ml/min, the column and operating conditions for the gas chromatograph were as above. Operating conditions for the mass spectrometer were: separator temperature, 250° C, ion source temperature, 270° C, and electron energy, 70 eV.

RESULTS AND DISCUSSION

The amount of secretion from the preorbital glands of the females was found to be low at all times. Analysis of the volatile fraction from the preorbital glands was done by gas chromatography. Except for the rutting season in October, the amount of secretion from the mature males was comparable to that from the females. At the end of September (prerut) the amount of secretion from the mature males increased remarkably and was maintained at a high level during the rut. A typical chromatogram from a mature male obtained in October is shown in Figure 1.

The mass spectrum of compound 1 (Figure 2) showed M^+ 114 and base peak at m/e 43. Interpretation of the mass spectrum suggested that the

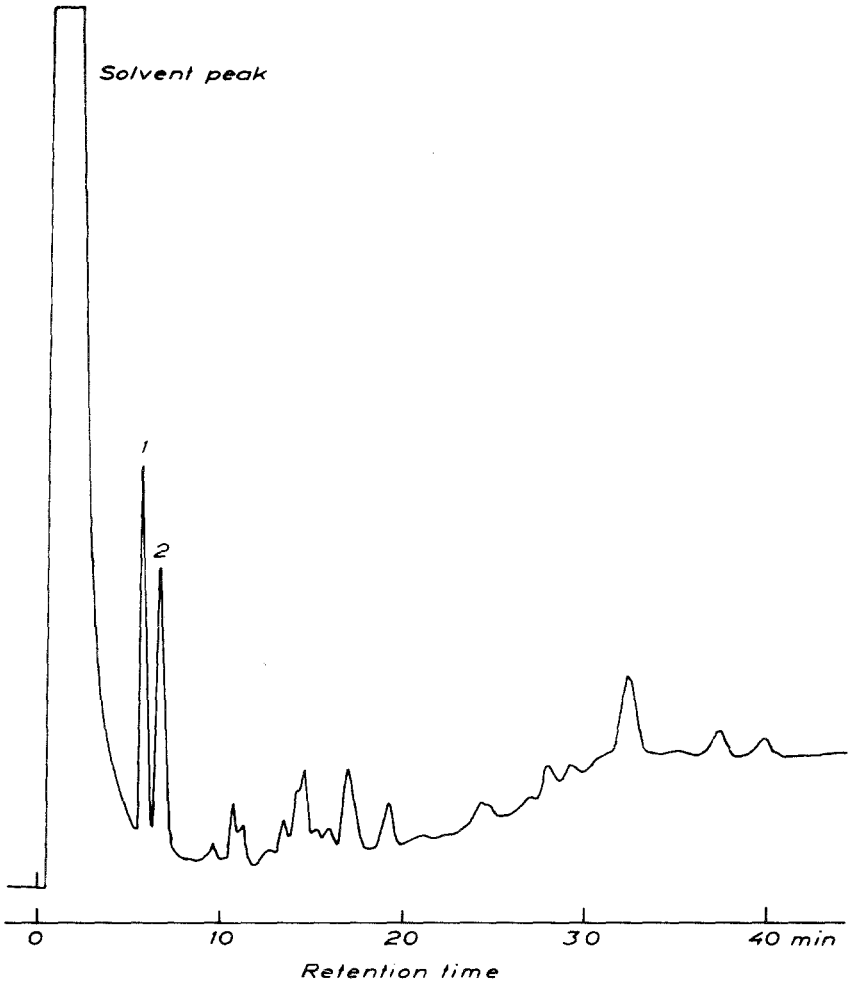
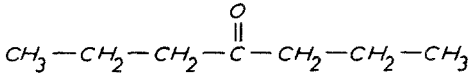


FIG. 1. Gas chromatogram of male preorbital secretion obtained in October 1977.

compound was 4-heptanone (I). Comparison of mass spectra and GC retention time (coinjection with those of an authentic sample) confirmed the structure.



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The mass spectrum of compound 2 (Figure 3) showed M^+ 128 and base peak at *m/e* 57. The prominent peaks at *m/e* 43, 58, 71, and 85 suggested that

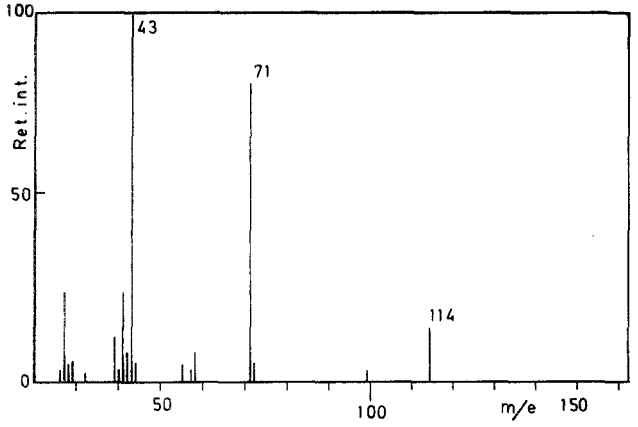


FIG. 2. Mass spectrum of compound 1.

the compound was a C_8-ketone. Two rearrangement peaks at m/e 58 and m/e 86 are evident. Therefore one of the alkyl groups must be C_3H_7. Because the molecular weight of the compound is 128 and the presence of a m/e 58 peak excludes the possibility of α-substitution, the only tenable structures are $CH_3(CH_2)_2COCH_2CH(CH_3)_2$ and $CH_3(CH_2)_2CO(CH_2)_3CH_3$. Synthesis of the two isomers and comparison of their mass spectra and GC retention times (coinjection) with those of compound 2 revealed that the compound was 2-methyl-4-heptanone (II). Because of the small number of reindeer available, none of the other volatile compounds could be collected in sufficient amount for structure elucidation.

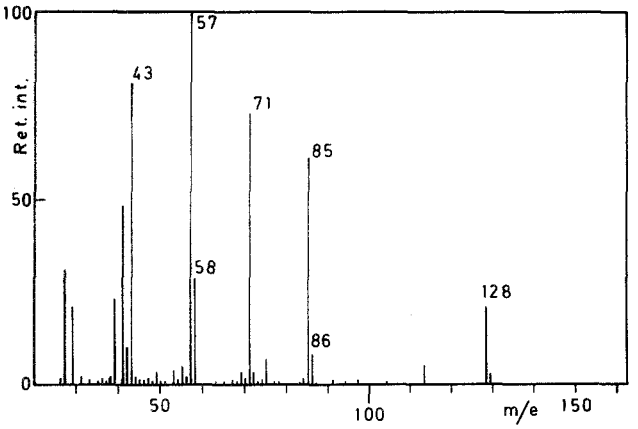
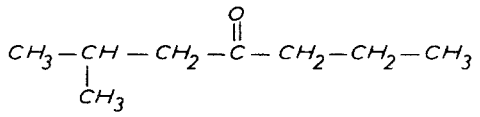


FIG. 3. Mass spectrum of compound 2.



II

According to Källquist and Mossing (1979) the preorbital glands are larger in males than in females, and male preorbital glands are most developed during the rut. These findings were supported by the chemical investigation of the glands. 4-Heptanone (I) and 2-methyl-4-heptanone (II) have also been found in the urine of the mature males during the rut (Brundin, unpublished results). In addition, 4-heptanone has also been found in urine of the red fox (Jorgenson et al., 1978) and in human urine (Liebich and Al-Babbili, 1975). Further investigations of the function of the preorbital gland and its significance in agonostic behavior during the rut are in progress.

Acknowledgments—Mr. Torgny Mossing, University of Umeå, is gratefully acknowledged for valuable biological comments. I am also greatly indebted to Dr. William Dunn, University of Umeå, for help in linguistic matters.

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DEFENSIVE SECRETION OF CHRYSOMELID LARVAE *Gastrophysa atrocyanea* MOTSCHULSKY AND *Phaedon brassicae* BALY[1]

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Abstract—The active substance of the larval defensive secretion of the Japanese chrysomelid beetles, *Gastrophysa atrocyanea* Motschulsky and *Phaedon brassicae* Baly was identified as chrysomelidial.

Key Words—Defensive secretion, *Gastrophysa atrocyanea* Motschulsky, *Phaedon brassicae* Baly, Coleoptera, Chrysomelidae, chrysomelidial, 5-(1′-formylethyl)-2-methyl-1-cyclopentenecarbaldehyde, dehydroiridodial, octadecyl acetate, (*Z*)-11-eicosenyl acetate.

INTRODUCTION

Chemical studies of larval defensive secretion of chrysomelid beetles have been extended to various species (Roth and Eisner, 1962; Eisner, 1970; Weatherston and Percy, 1970). Recently, Meinwald et al. (1977) isolated two new characteristic substances, chrysomelidial (I) and plagiolactone (II), from the defensive secretion of the willow-feeding leaf beetle, *Plagiodera versicolora.* Chrysomelidial was also identified by Blum et al. (1978) as one of the two components in the secretory substances from polygonaceous-feeding beetle, *Gastrophysa cyanea* Melsheimer.

We have investigated the larval defensive secretion of some Japanese chrysomelid beetles (Sugawara et al., 1978, Matsuda et al., 1978). The

[1]This is report No. 2 of the "Defensive Secretion of Chrysomelid Beetles," Report No. 1 is Sugawara et al. (1978).

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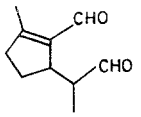
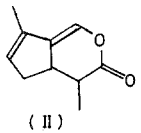


(I)



(II)

Polygonaceae-feeding chrysomelid larva, *Gastrophysa atrocyanea* Motschulsky, secretes the defensive substances from nine pairs of dorsal eversible glands. Our previous work showed that three components (A, B, and C separated on preparative TLC) were involved in the defensive secretion. Component A was found to be a hydrocarbon and B was identified as a mixture of (*Z*)-11-eicosenyl acetate and octadecyl acetate by spectrometric analysis and stereoselective synthesis (Kobayashi et al., 1978). Component C was identified as chrysomelidial, 5-(1′-formylethyl)-2-methyl-1-cyclopentenecarbaldehyde.

While another leaf beetle, *Phaedon brassicae* Baly, has completely different host ranges from *G. atrocyanea*, the defensive secretion involved a single substance which was also identified as chrysomelidial (I).

METHODS AND MATERIALS

G. atrocyanea was selected from our laboratory culture maintained exclusively on *Rumex obtusifolius* L. leaves. *P. brassicae* was reared on *Raphanus sativus* L. or *Brassica campestris* L.

The last instar larvae of *G. atrocyanea* were forced to eject the secretion by stimulating the head with a steel needle. The secretion was collected in glass capillary tubes under a microscope, and stored in a refrigerator. It was dried over Na_2SO_4, extracted with *n*-pentane, and separated on preparative TLC (PF_{254}, developed with benzene). From about 2500 larvae, 220 mg of the secretion was separated into three components: 1.4 mg of A (R_f 0.98), 3.2 mg of B (R_f 0.80), and 10.5 mg of C (R_f 0.23). These R_f values were observed on the 5 × 20-cm silica gel plate developed with benzene–ethyl acetate (25:1).

The collection of the defensive secretion of chrysomelid larvae *P. brassicae* was followed by the method described above. From about 7000 larvae, 353 mg of the secretion was extracted with *n*-pentane which was dried over Na_2SO_4. The extracts (30.9 mg) involved a single substance on TLC ($R_f = 0.38$) which was developed with *n*-hexane–ether (1:1).

The isolated defensive materials were analyzed with the following instruments: A Hitachi gas chromatograph 163 equipped with FID was employed with a 30-m × 0.25-mm glass capillary column (coated with OV-101) at 150°C and a N_2 flow rate of 2 ml/min. PMR spectra were re-

corded on a JEOL JNM PS-100 (100 MHz) spectrometer using CCl_4 or $CDCl_3$ as solvent with 1% TMS as an internal standard. CMR spectra were recorded on a JEOL FX-60 (15 MHz) spectrometer using $CDCl_3$ as solvent. Chemical shifts were calculated from that of TMS. An electron impact mass spectrometer Hitachi M-52G instrument or JEOL JMS-06 connected with JEOL JGC-20K gas chromatograph was used (EV = 70 or 20 eV). CD spectra were recorded on a Union Giken Dichrograph Mark III-J using MeOH as solvent with 0.1 and 0.05% (w/v) concentration of samples.

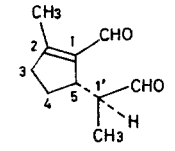
RESULTS AND DISCUSSION

G. atrocyanea. Component A was found to be a hydrocarbon by IR (film on NaCl plates, cm^{-1}: 2920, 2845, 1463, 1380). B consisted of octadecyl acetate and (*Z*)-11-eicosenyl acetate in a 1:4 ratio as described in previous work (Sugawara et al., 1978).

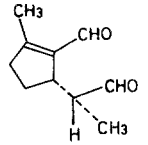
We recorded PMR spectra of the crude extracts in $CDCl_3$ of 22 mg of secretion after drying over Na_2SO_4. Two signals assigned to saturated and unsaturated aldehyde proton at 9.61 and 9.93 ppm made it clear that C was a major component of the unstable extract [B and C were in the ratio of 1:7, calculated from the comparison between the peak intensities of an aldehyde proton (C—CHO, 9.93 ppm) and acetate proton (C—$OCOCH_3$, 2.04 ppm) on PMR spectra]. C could be quickly separated on preparative TLC from the extracts.

Gas chromatographic analysis indicated that C was a single substance with the retention time of 8.6 min. Spectrometric results of IR (2740, 1725, 1665), UV (λ_{max}^{EtOH} = 252 nm; ϵ 5889), GC-MS ($m/e = M^+$ 166, base peak 81), and PMR spectra were identical to chrysomelidial (I) (9.93, 1H, s; 9.61, 1H, d, $J \leqslant 1.0$; 3.70, 1H, br m; 3.15, 1H, q,d,d, J = 7.5, 4.0, $\leqslant 1.0$; 2.48, 2H, m like t, J = 8.0; 2.24, 3H, m like s; 1.98, 1H, complex m; 1.66, 1H, complex m; 0.88 3H, d, J = 7.5). The results of double and triple resonances also supported structure I.

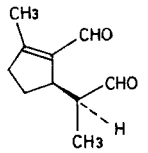
Chrysomelidial possesses two asymmetric centers at C_5 and C_1'. CD spectra ($[\Theta]_{255}$ $+7.7 \times 10^3$, $[\Theta]_{335}$ -2.2×10^3) and optical rotation ($[\alpha]_D^{22}$ = +1.2°) did not conclusively determine the absolute configuration of I. Chrysomelidial was isolated from *P. versicolora* as the mixture of diastereoisomers (Meinwald et al., 1977), and the absolute configuration of the major substance was determined to be III (5*S*, 1′*S*) by comparing the PMR spectra with those of the synthetic products (Meinwald and Jones, 1978).The threo isomer (III, 5*S*, 1′*S*) and erythro isomer (IV, 5*S*, 1′*R*) could be discriminated by the different chemical shifts of protons attached to C-5, C-1′, and C-2′. A similar difference in chemical shifts was also observed in dehydroiridodial isolated from *Actinidia poligama* Miq, and its absolute configuration was



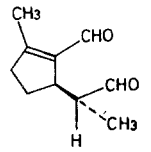
(III) 5S,1'S



(IV) 5S, 1'R



(V) 5R,1'S



(VI) 5R,1'R

established to be the erythro form V (5*R*, 1'*S*) by comparison of chemical shifts of synthesized V with those of the diastereomer VI. The key difference between the insect-derived compounds (III and IV) and the plant-derived compounds (V) is the chirality at C-5. The PMR data summarized in Table 1 show that the chemical shifts of component C were also very similar to those of threo isomer III. Although the diastereomer (IV) was present in *P. versicolora* as a minor component, this isomer was not found in *G. atrocyanea* or *G. cyanea* (Blum et al., 1978).

TABLE 1. CHEMICAL SHIFTS[a] OF COMPONENT C COMPARED TO CHRYSOMELIDIAL OR DEHYDROIRIDODIAL

Substances	Protons at carbon site[b]			
	C_5—H	$C_{1'}$—H	$C_{1'a}$—H	$C_{2'}$—H
Component C. (present authors)	3.70	3.15	9.61	0.88
Synthetic chrysomelidial (Meinwald et al. 1978)				
III (5*S*, 1'*S*)	3.70	3.13	9.72	0.88
IV (5*S*, 1'*R*)	3.43	2.83	9.72	1.01
Synthetic dehydroiridodial (Yoshihara et al. 1978)				
V (5*R*, 1'*S*)	[c]	[c]	9.96	1.01
VI (5*R*, 1'*R*)	[c]	[c]	9.70	0.90

[a]All chemical shifts were calculated from TMS as an internal standard in $CDCl_3$, and each PMR was recorded at 100 MHz.
[b]Numbered according to IUPAC.
[c]Unlisted on their report.

P. brassicae. Gas chromatographic analysis indicated a single peak (retention time = 8.6 min) in the extracts, which was identified as chrysomelidial. CD spectrum ($[\Theta]_{250}$ $+6.8 \times 10^3$, $[\Theta]_{337}$ -3.3×10^3) and optical rotation ($[\alpha]_D^{22}$ $+2.2°$) were similar to those of chrysomelidial from *G. atrocyanea*. CD and PMR data suggested that a single threo chrysomelidial was secreted from *P. brassicae* as was the case for *G. atrocyanea* and *G. cyanea* (Blum et al., 1978).

The CMR spectrum of chrysomelidial had been expected to be a valuable method to elucidate such a substituted cyclopentene carbon skeleton. As chrysomelidial was the sole component in the secretion of *P. brassicae*, we could obtain enough of I (without any contamination) to carry out the CMR analysis. CMR data recorded under complete proton decoupling indicated the presence of ten carbons. Downfield, two carbon signals at 204.7 and 188.3 ppm collapsed to doublets under partial proton decoupling; these were assigned to a saturated aldehyde carbon (C-1 at 204.7 ppm) and an α,β-unsaturated aldehyde carbon (C-1a at 188.3 ppm). Unchanged singlet signals at 165.4 and 137.7 ppm under partial decoupling supported the presence of a tetra-substituted ethylenic structure. The assignments of these two signals followed from the chemical shifts of the ethylenic carbons of 3-methyl-2-cyclopenten-1-one (VII) (Marr and Stothers, 1965). The signal at 165.4 ppm was due to C-2. Chemical shifts of C-2 and C-3 of III were 130.1 and 159.7 ppm, respectively.

Six sp^3 carbon signals were assigned as follows: The two quartet signals under partial decoupling at 7.7 and 14.3 ppm were assigned to two methyl carbons. The more downfield quartet of the two was assigned to C-2a. Two triplet carbon signals at 48.0 and 23.0 ppm were assigned to the two methylene carbons on the cyclopentene ring. As the more downfield triplet of the two, at 48.0 ppm, was assigned to allylic C-3 methylene, the signal at 23.0 ppm was attributable to C-4. The remaining two tertiary carbons at 42.8 and 40.1 ppm were assigned to C-5 and C-1′, but a more specific assignment was not made (Figure 1).

After the larvae of *P. brassicae* were reared on two different species of cruciferous plants, their secretions were extracted and analyzed by TLC and GLC under the same conditions described previously. Since no changes in components were detected, it seems that the quality and quantity of the defensive substance are not affected by the particular host species.

Meinwald et al. (1977) suggested that the origin of a mixture of diastereomers of chrysomelidial was due to a facile enolization of the nonconjugated aldehyde group at the moment of discharge, and the production of plagiolactone was derived from chrysomelidial by autooxidation during storage in the larval, dorsal, eversible glands of *P. versicolora*. As the larvae of *G. atrocyanea* and *P. brassicae* discharge the defensive secretion from the

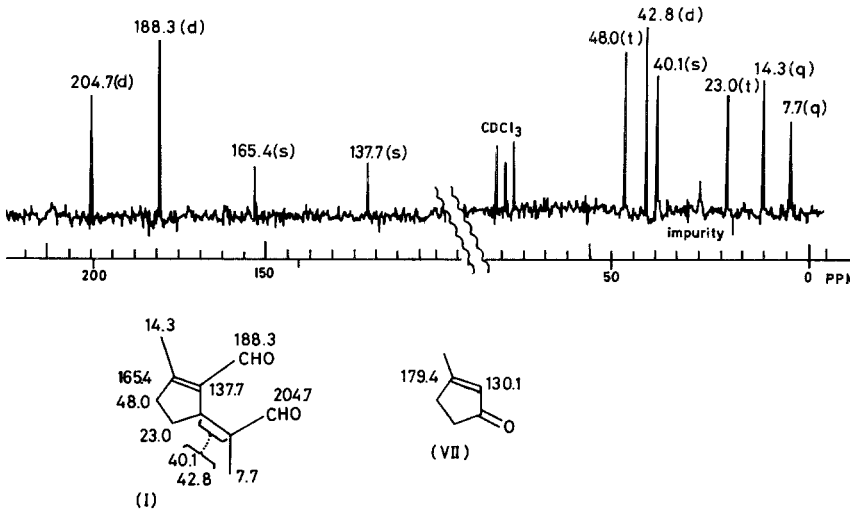


FIG. 1. CMR spectrum of chrysomelidial (I) (*Phaedon brassicae* Baly).

same eversible glands as those of *P. versicolora* (Meinwald et al., 1977) and *G. cyanea* (Blum et al., 1978), the isolation of the erythro form of the isomer and the lactone was expected. However, these two investigated species (*P. brassicae* and *G. atrocyanea*) secreted neither the diastereomer nor the lactone.

The defensive effect of chrysomelidial on arthropod predators has been already described by Meinwald et al. (1977) and Blum et al. (1978). The defensive effect of each components was similarly observed for an ant species (*Lasius niger* L.) by using the techniques of Pasteels et al. (1973). The secretion obtained from one larva of either *G. atrocyanea* or *P. brassicae* showed repellency against ants. In our previous work on the former insect, the mixture of two acetates was assumed to be the most effective component, but the synthetic mixture indicated less effective repellency than the crude extract (Sugawara et al., 1978). Although chrysomelidial was not previously obtained, because of its quite unstable properties, the fast purifications by preparative thin-layer chromatography proved that it was the major compound in the secretion and was the most effective among the three components from *G. atrocyanea*. These results were also supported in the case of *P. brassicae*, which secreted chrysomelidial as a single substance in the larval defensive secretion.

Acknowledgments—Mr. K. Hatakeda is especially thanked for his efforts in obtaining the CMR spectra. Thanks are also due to Dr. T. Sakai for the dehydroiridodial spectra (MS, IR, PMR).

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DEFENSIVE SECRETIONS OF THREE SPECIES OF *Acilius* (COLEOPTERA:DYTISCIDAE) AND THEIR SEASONAL VARIATIONS AS DETERMINED BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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Abstract—A rapid, sensitive, stable, and quantitative high-pressure liquid chromatographic technique was developed for the analysis of defensive secretions obtained from the pygidial and prothoracic glands of dytiscids. Methods were developed for both normal phase (μPorasil) and reverse phase (μBondapak C_{18}) columns. The applicability of this technique was demonstrated when defensive compounds of *Acilius semiculcatus*, *A. sylvanus*, and *A. mediatus* were isolated, identified, and quantitated. No major differences were found in the composition of the defensive secretions between the three species. The seasonal defensive titer of *A. semisulcatus* was determined from June through October 1977. The pygidial defensive agents (benzoic acid, *p*-hydroxybenzaldehyde, and methyl *p*-hydroxybenzoate varied from 9.0 to 67.8 μg and exhibited a maximum in July, and a steroid from the prothoracic gland varied from 7.1 to 33.2 μg and was maximum in October.

Key Words—Coleoptera, Dytiscidae, high-pressure liquid chromatography, *Acilius semisulcatus, Acilius sylvanus, Acilius mediatus,* quantitation, defensive secretions, steroids, benzoic acid, methyl *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde.

INTRODUCTION

The aquatic beetles in the family Dytiscidae have been found to produce two types of defensive secretions (Weatherston and Percy, 1970). One type, produced by the pygidial gland, is composed of low-molecular-weight aromatic compounds such as methyl *p*-hydroxybenzoate and *p*-hydroxybenzaldehyde and appear to be utilized as antimicrobial agents. The second

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type of defensive agent is primarily composed of one or more steroids, produced by the prothoracic gland, although exceptions have been reported (Schildnecht and Tacheci, 1971). These steroids are usually 4-pregnen-3-ones and related derivatives and also include the familiar mammalian sex hormones testosterone and estradiol (see Miller and Mumma, 1976a, for a current listing of the known dytiscid steroids). It is believed that these steroids act as defensive deterrents against vertebrate predation, i.e., fish and amphibians. Bioassays have been developed to study the anesthetic and toxic actions of these steroids on such vertebrates (Schildknecht et al., 1967; Miller and Mumma, 1976a).

Analysis and isolation of these defensive secretions has usually involved thin-layer chromatography (TLC) and gas-liquid chromatography (GLC). Both of these techniques are time consuming and have disadvantages due to the large differences in molecular weight, polarity, and detector response that exist between the pygidial aromatic compounds and the prothoracic steroids. For example, the low-molecular-weight aromatic defensive components are poorly resolved on GLC columns typically used for steroids, and they exhibit poor response with flame ionization detectors.

This manuscript reports an alternative procedure for the analysis of the dytiscid defensive compounds utilizing high-pressure liquid chromatography (HPLC). This procedure has been utilized in the isolation, identification, and quantitation of the defensive compounds of three species of *Acilius: A. semisulcatus, A. sylvanus,* and *A. mediatus.*

METHODS AND MATERIALS

Collection and Extraction. The dytiscids used in this study, *Acilius semisulcatus, A. sylvanus* and *A. mediatus,* were collected with aquatic dip nets from several ponds located around State College, Pennsylvania. The beetles were placed, either individually or in groups according to species, in tap water and electrically shocked with 90 V DC in order to obtain their defensive compounds (Miller and Mumma, 1973). This electrical shocking causes both defensive glands to expel their contents into the water simultaneously. After acidification to pH 1, the defensive compounds were extracted from the water with chloroform and concentrated under nitrogen.

The acidification of the aqueous extract enhanced the chloroform extraction of any ionized species such as benzoic acid. No further clean-up was necessary before chromatographic analysis.

Chromatography. TLC employed a stationary phase of 0.25 mm Supelcosil 12A plus a zinc silicate phosphor (Supelco, Inc., Bellefonte, Pennsylvania) applied to 20 × 20-cm glass plates. The TLC separations of the dytiscid defensive secretions were performed in two steps: (1) separation of

the whole extract into six bands using diethyl ether–petroleum ether (70:30, v/v) as the solvent system and (2) separation of band VI into several bands with cyclohexane–ethyl acetate (30:70, v/v). The addition of acetic acid to these solvent systems greatly improved the resolution of the component bands; however, its use was avoided in preparative separations due to problems with its removal from the purified fraction and the possibility of ester formation with the isolated defensive compounds. Several detection methods were used which included shortwave UV and a chromic acid–H_2SO_4 spray/char for general nonspecific detection and by spraying with a 0.4% solution of 2,4-dinitrophenylhydrazine in 2 N HCl for specific detection of free aldehyde and keto groups.

GLC was performed with a MicroTek 220 instrument using flame ionization detection. The whole defensive extract could be analyzed using a 2% OV-1 phase on a 100/120 Supelcoport support employing a nitrogen flow of 60 ml/min and a temperature program of 110°C held for one min, then 110°–240°C at 5°/min. Analysis of the dytiscid steroids and several steroid standards was also performed using several different phases and temperatures: 2% OV-1 at 230°C, 1% OV-17 at 240°C, and 1% OV-210 at 220°C. All-glass columns (1.86 m × 4 mm ID) were used and contained 160 cm of packing.

HPLC was performed on a model ALC/GPC 244 high-pressure liquid chromatograph with model 6000A pumps, U6K injector, model 660 solvent programmer, and model 440 absorbance detector (Waters Associates, Milford, Massachusetts). The UV detector monitored 254 and 280 nm simultaneously. A 4-mm ID × 30-cm μPorasil column (Waters Associates) using 15% dioxane–85% hexane at a flow rate of 1.0 ml/min was used for normal phase separation. For reverse-phase separations, a 4-mm ID × 30-cm μBondapak C_{18} column (Waters Associates) was employed, using a solvent gradient at 30% acetonitrile–70% water to 60% acetonitrile–40% water following gradient curve 10 on the Waters solvent programmer in 10 mins. The flow rate was 1.5 ml/min.

All solvents for HPLC were filtered (0.45 μMillipore filter) and degassed before use. Organic solvents were of highest purity (Burdick and Jackson Laboratories, Inc., Muskegan, Michigan).

Spectroscopy. A Gilford spectrophotometer (model 250) was used to obtain the UV spectra of the compounds between 200 and 360 nm. Low-resolution mass spectra were taken on a LKB-9000 gas-liquid chromatograph-interfaced mass spectrometer using a 9-ft × 3/16-in. OD glass column packed with either 2% OV-1 or 1% OV-210 on a 100/120 mesh Supelcoport support. A helium flow of 30 ml/min and variable column temperature were used, depending on the compound being analyzed. Mass spectra were taken at an ionizing potential of 70 eV.

Quantification. The UV absorbance monitor of the HPLC was connected with a Spectra-Physics Autolab Minigrator which was used for determining peak areas. Known quantities of pure standard compounds were used to construct standard curves which were used in translating the peak areas into microgram values. Testosterone was used as the standard for constructing the steroid standard curve, utilizing its 4-ene-3-one functional group absorption which was also found in the steroid being quantified.

RESULTS AND DISCUSSION

A mixture of benzoic acid, methyl *p*-hydroxybenzoate, *p*-hydroxybenzaldehyde, testosterone and/or 11-deoxycorticosterone (DOC) was subjected to HPLC analysis using either a normal phase column (μPorasil) or a reverse-phase column (μBondapak C_{18}). Figure 1 shows the separation of these compounds employing a dioxane-hexane solvent system and a μPorasil column, and Figure 2 shows a similar separation using a solvent gradient of acetonitrile-water and a μBondapak C_{18} column. The former method was preferred when doing repetitive analyses because the use of a gradient program in the latter method requires the reequilibration of the column between each sample, lengthening the analysis time.

The lower sensitivity limits of our HPLC UV detector and GLC flame ionization detector were determined. The lower limits of detection by HPLC of the aromatic components was found to be 1-2 ng, and 2 ng for the steroid.

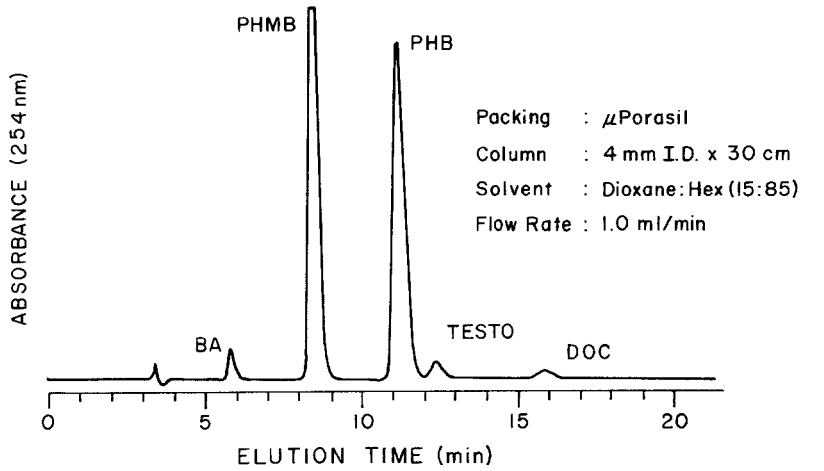

FIG. 1. HPLC separation of standard compounds employing a μPorasil column. BA = benzoic acid, PHMB = methyl *p*-hydroxybenzoate, PHB = *p*-hydroxybenzaldehyde, TESTO = testosterone, DOC = 11-deoxycorticosterone.

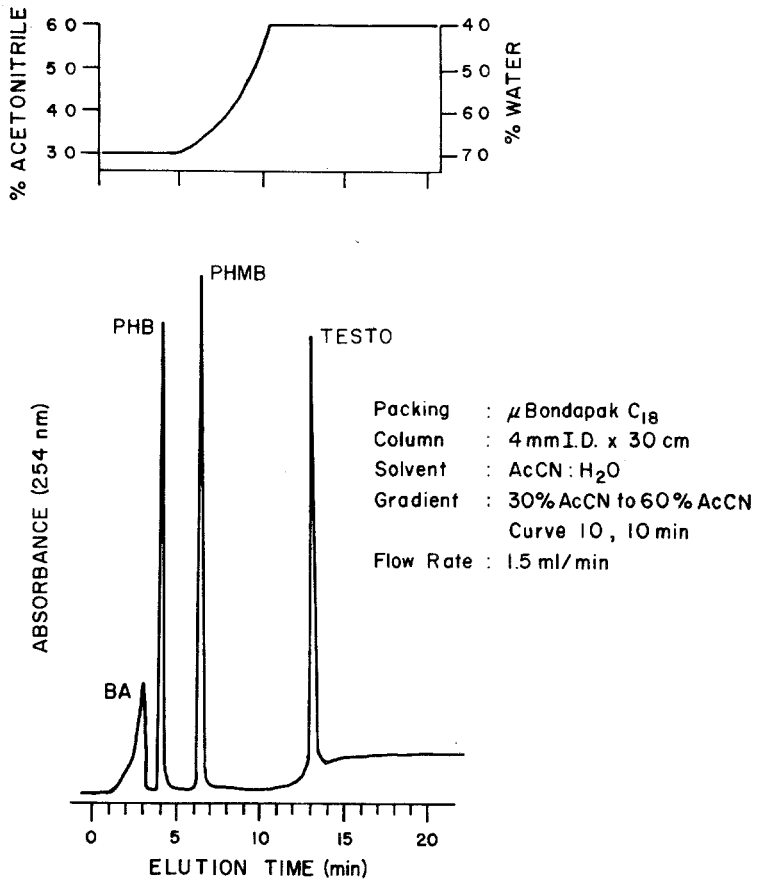

FIG. 2. HPLC separation of standard compounds employing a μBondapak C_{18} column. Abbreviations are the same as in Figure 1.

Lower limits of the GLC flame ionization detector ranged from 100 ng for the aromatic compounds to 10 ng for the steroids (Table 1).

Four species of *Acilius* have been described (Hilsenhoff, 1975) and three of these species, *A. semisulcatus*, *A. sylvanus*, and *A. mediatus*, are found in ponds in the vicinity of State College, Pennsylvania.

A. sylvanus is a newly described species which is similar in size to *A. semisulcatus* but has a distinct yellowish venter as opposed to the dark venter of *A. semisulcatus*. All three species may be found in the same pond, although *A. semisulcatus* is generally the prominent species in ponds near State College. With the HPLC methods described above, the chloroform extracts of all three species were analyzed and compared to standards (Figure 3). Preparative HPLC was used to obtain milligram quantities of four major

TABLE 1. COMPARISON OF LOWER SENSITIVITY LIMITS FOR DYTISCID DEFENSIVE COMPOUNDS USING GLC AND HPLC

Compound	Minimum detectable amount (ng)	
	GLC[a]	HPLC[b]
Benzoic acid	100	2
Methyl *p*-hydroxybenzoate	100	<1
p-Hydroxybenzaldehyde	100	<1
Testosterone	10	2

[a]1% OV-210 column, flame ionization.
[b]μBondapak C_{18} column, 254 nm.

components for further analysis. HPLC, TLC, and GLC methods were used to compare the retention times of the *Acilius* major components with standard compounds. It was found that all three species contain the same major components, the first three corresponding to benzoic acid, methyl *p*-hydroxybenzoate, and *p*-hydroxybenzaldehyde (Figure 3). The identity of these three structures was supported by UV and mass spectroscopy data.

The fourth major component that was isolated from the *Acilius* defensive secretion eluted on HPLC, TLC, and GLC in the region of several steroid standards but did not correspond to any previously identified dytiscid steroids. The UV spectrum of this compound gave a $\lambda_{max}^{EtOH} = 238.0$ nm, suggestive of a 4-ene-3-one conjugated system found in the A ring of many dytiscid steroids. The mass spectrum gave a molecular weight of 288 ($C_{19}H_{28}O_2$) and a fragmentation pattern characteristic of a steroid 4-ene-3-one system. Proof of the structure of this *Acilius* steroid will be presented in a forthcoming paper.

The presence of benzoic acid, methyl *p*-hydroxybenzoate, and *p*-hydroxybenzaldehyde in these three *Acilius* species is not unusual, as some or all of these compounds have been previously reported in other dytiscid species (Schildknecht, 1970). The defensive components of a European *Acilius* species, *Acilius sulcatus*, were analyzed and found to contain the same three pygidial components. Table 2 compares the composition of each defensive component of *A. semisulcatus*, *A. sylvanus*, and *A. mediatus* collected on June 15, 1977. The composition is roughly the same for each beetle, and no statistical differences were found between *A. semisulcatus* and *A. sylvanus*. The smaller beetle, *A. mediatus*, had a slightly higher titer of methyl *p*-hydroxybenzoate and *p*-hydroxybenzaldehyde. Therefore, the composition of the defensive secretions does not appear to be satisfactory as a means of chemical taxonomy between these three *Acilius* species. No difference was observed between the defensive titer of male and female *A. semisulcatus*.

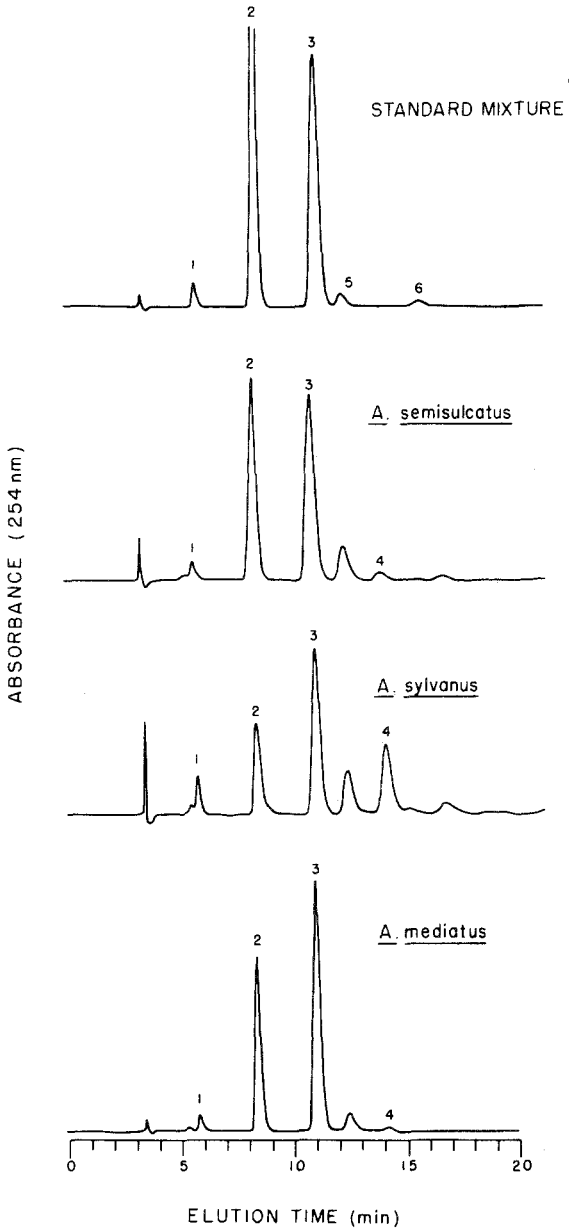

FIG. 3. HPLC separation of the defensive compounds from three *Acilius* species as compared to standard compounds employing a μPorasil column. HPLC conditions are the same as those used in Figure 1. 1 = benzoic acid, 2 = methyl *p*-hydroxybenzoate, 3 = *p*-hydroxybenzaldehyde, 4 = *Acilius* steroid, 5 = testosterone, 6 = 11-deoxycorticosterone.

TABLE 2. QUANTITATION OF DYTISCID DEFENSIVE AGENTS BY HPLC[a]

Species	Date collected	Micrograms/beetle[b] BA	PHMB	PHB	Steroid
Acilius semisulcatus	6/15/77	4.6 ± 1.4a[c]	1.4 ± 0.4a	3.0 ± 0.6a	7.1 ± 1.6a
A. sylvanus	6/15/77	3.2 ± 1.8a	1.5 ± 0.6a	4.9 ± 1.8a	12.6 ± 4.4a
A. mediatus	6/15/77	6.8 ± 2.4a	3.8 ± 0.5b	11.6 ± 1.0a	2.9 ± 0.7a

[a] μPorasil column, 254 nm.
[b] BA = benzoic acid, PHMB = methyl *p*-hydroxybenzoate, and PHB = *p*-hydroxybenzaldehyde.
[c] Numbers followed by different letters are significantly different ($\alpha = 0.05$).

The defensive titer of *A. semisulcatus* was quantitated over a 5-month period from June through October, and these data are presented in Table 3. Large fluctuations were found to occur in the defensive agent titer during this 5-month period. In the pygidial gland, benzoic acid varies from 4.6 to 35.6 μg, methyl *p*-hydroxybenzoate from 1.4 to 11.7 μg, and *p*-hydroxybenzaldehyde from 3.0 to 20.5 μg. All of these three show a maximum titer in July and minimum titers in June and October.

The steroid titer, from the prothoracic gland, remained low during the summer months, averaging 7.1 μg/beetle, then increased to a maximum of 33.2 μg/beetle in October. The total defensive titer of the pygidial gland components and of a prothoracic steroid are plotted versus the month of collection in Figure 4. The two curves show a differing pattern of seasonal variation. The pygidial components exhibit a maximum in July while the prothoracic steroid shows a maximum in October. These data suggest the two defensive glands may be under independent regulation. The large variations

TABLE 3. SEASONAL QUANTITATION OF *Acilius semisulcatus* BY HPLC[a]

Date	Sample size	Micrograms/beetle[b] BA	PHMB	PHB	Steroid
6/15/77	21	4.6 ± 1.4a[c]	1.4 ± 0.4a	3.0 ± 0.6a	7.1 ± 1.6a
7/29/77	22	35.6 ± 8.1b	11.7 ± 1.8b	20.5 ± 3.7b	5.8 ± 1.8a
8/11/77	23	28.5 ± 6.7bc	7.9 ± 0.2c	11.4 ± 0.4c	8.5 ± 2.2a
9/14/77	25	26.2 ± 4.1bc	4.3 ± 0.4d	8.1 ± 1.2ac	19.8 ± 2.2b
10/18/77	29	14.6 ± 4.1ac	6.8 ± 0.4c	9.5 ± 0.8c	33.2 ± 5.3c

[a] μPorasil column, 254 nm.
[b] BA = benzoic acid, PHMB = methyl *p*-hydroxybenzoate, and PHB = *p*-hydroxybenzaldehyde.
[c] Numbers followed by different letters are significantly different ($\alpha = 0.05$).

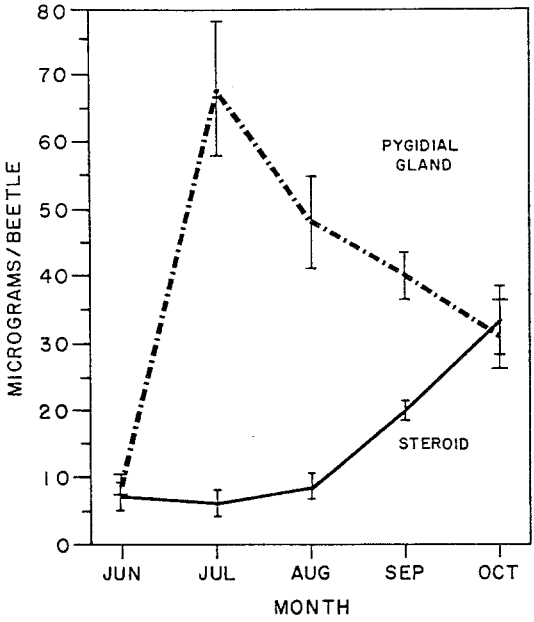

FIG. 4. Defensive titer of pygidial gland secretions and of a steroid from the prothoracic gland of *Acilius semisulcatus*.

in titer may reflect variations in rates of enzymes involved in synthesis and degradation of these compounds, use of these agents for antimicrobial or defensive purposes, or predation pressure, and may be related to the age of the adult beetle. The prothoracic steroids are produced from sterols of dietary origin, and a significant feeding time may be necessary before the titer of newly emerged adults begins to increase to the levels seen in October.

The steroid titer of *Agabus seriatus* (Miller and Mumma, 1973), the only other dytiscid analyzed for seasonal variations, was maximum during midsummer and minimum in the fall, which is in direct contrast to the results obtained with *A. semisulcatus*. These two dytiscids inhabit diverse environments: *A. seriatus* lives in spring-fed streams and is active 12 months of the year, while *A. semisulcatus* lives in shallow stagnant ponds and is active in the warmer months. These differences in habitat probably result in differences in biological development, food availability, and predation pressures, all which may be reflected in defensive titer. Additional studies on the seasonal titer and biology of other dytiscids are needed to clarify the significance of the observed seasonal variation in defensive titer.

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IN VITRO MAINTENANCE OF THE SEX PHEROMONE GLAND OF THE FEMALE INDIAN MEAL MOTH *Plodia interpunctella* (HÜBNER)[1,2]

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Abstract—Sex pheromone glands of *Plodia interpunctella* were isolated from surface-sterilized donors of different ages, freed of most of the attached gut, oviduct, and other tissues; rinsed in sterile medium; and cultured in 1 ml of culture medium. The sex pheromone gland cells that were cultured for 10 days in either chemically defined Grace's medium or modified Grace's medium appeared normal in histological examinations. Bioassays of extracted medium in which pheromone glands from mature females had been incubated showed that a greater percentage of the available pheromone was recovered from modified Grace's medium than from chemically defined Grace's medium.

Key Words—Tissue culture, sex pheromone gland, Lepidoptera, Pyralidae, *Plodia interpunctella,* Indian meal moth.

INTRODUCTION

Although the chemistry of insect pheromones has been studied extensively, there is only limited information available on the control mechanisms involved in the development and activity (production and/or release) of the sex pheromone glands (SPG) in Lepidoptera (Barth and Lester, 1973; Roelofs and Cardé, 1977; Shorey, 1974). Also, the few investigations that have been conducted have been done in vivo. An understanding of the control

[1]Lepidoptera: Pyralidae.
[2]This research was supported in part by NIH Grant 1-F34 GM 06251.
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mechanisms would be greatly facilitated if the glands could be maintained in tissue culture because the isolated tissue could be studied under defined conditions, and the effects of specific hormones and chemicals on the development and activity of the gland could be evaluated. Also, by using appropriately radiolabeled compounds in such an in vitro system, information could be obtained regarding the biosynthesis of sex pheromones. Ultimately, a successful tissue culture system could result in the establishment of in vitro pheromone production systems that would facilitate the isolation and identification of insect pheromones. Consequently, we investigated the requirements for culturing the SPG of *Plodia interpunctella* (Hübner) in vitro. This species was selected because the biology and chemistry of the female sex pheromone in this insect have been investigated in some detail (Brady and Smithwick, 1968; Brady et al., 1971; Kuwahara, et al., 1971; Nordlund and Brady, 1974; Sower and Fish, 1975; Vick and Sower, 1973). The location, histology, and development of the female SPG have also been studied in this species (Smithwick and Brady, 1977a, b). In addition, Oberlander (1976) has established an in vitro system for the culture of various tissues of this species.

METHODS AND MATERIALS

The Indian meal moths were reared by the procedure of Silhacek and Miller (1972). The SPGs, broadly chevron-shaped structures located on the ventrolateral surface of the intersegmental membrane between abdominal scleromata VIII and IX (Smithwick and Brady, 1977b), were isolated from 2-day-old virgin adult females and pharate adult females (18–24 hr prior to emergence). Donors were surface-sterilized by immersing them for 15–20 min in 0.1% $HgCl_2$ solution to which Triton-X had been added. They were then washed in sterile distilled water and allowed to dry for approx. 1 hr. The females were forced to extrude the 8th and 9th abdominal segments (normally retracted in the 7th segment) by gently squeezing the base of the abdomen. The abdominal tip, consisting of the 8th and 9th segments, was severed from the abdomen with a pair of sterile scissors and placed in a drop of the medium. Most of the gut, oviduct, and other adhering tissues were removed from the isolated tips. The tips were then rinsed in several drops of the medium before being placed in culture. Usually 10 tips were cultured in a sterile glass petri dish containing 1 ml of the medium. The cultures were maintained at 25°C in an incubator in which the relative humidity was maintained at approx. 65%.

The SPGs were cultured in chemically defined Grace's medium (Grand Island Biological Co.), a modified Grace's medium containing whole egg ultrafiltrate (10%), fetal calf serum (7%), and bovine serum albumin fraction V (1%) (Yunker et al., 1967) and Ringer's solution. At 2- to 3-day intervals 3–5 glands were removed from these cultures and also from females of similar

ages and fixed in alcoholic Bouin's solution for 4–5 hr, washed several times in deionized distilled water, and stored in 70% ethanol for histological studies.

Histological studies were conducted using the standard techniques for paraffin-embedded tissue. The SPG was embedded in 3% agar before being embedded in paraffin. Sections were cut at 6 μm and stained with Mayer's hemalum and eosin.

The SPG extracts were prepared by immersing the glands in 1 ml of diethyl ether overnight. Culture medium extracts were prepared by washing the medium twice with 1 ml of diethyl ether per extraction. All extracts were dried with Na_2SO_4. The volume of the extracts was adjusted to yield 10^{-2} female equivalents (FE)/2 μl. All experiments were replicated at least three times.

The cultured SPGs and the medium in which the glands were cultured were separately extracted after 1, 72, and 144 hr of incubation to determine the presence of the pheromone. Medium that had received no glands was also extracted at these times as control.

Preliminary analytical studies, using the procedures described by Coffelt et al. (1978), indicated that at 3–4 hr prior to the scotophase 2-day-old virgin females contained approx. 3 ng of the sex pheromone, (*Z*)-9,(*E*)-12-tetradecadien-1-ol acetate. This quantity is close to that estimated by Nordlund and Brady (1974). Dissection and the subsequent preparation of the tissue for tissue culture required approx. 1 hr, and resulted in a >90% loss of pheromone, as determined by analytical studies and bioassay. Thus we estimate that the material placed in culture contained <0.3 ng of pheromone/gland. Subsequent estimates of pheromone titer were made from bioassay data. An eye-fitted regression line based on the percent male response to serial dilutions of the female extract (10^{-2} FE = 81%, 10^{-3} FE = 61%, 10^{-4} FE = 30%, 10^{-5} FE = 16%) indicated that a tenfold change in concentration resulted in an approx. 20% change in male response.

Bioassays were conducted using the apparatus described by Sower et al. (1973). The procedure for the bioassays was similar to the one described by Vick and Sower (1973) and was as follows: Groups of 8–12 male (1- to 3-day old) moths were placed in 42–45 cm long × 2 cm (ID) Plexiglas tubes held in portable Plexiglas 15-tube racks 1 hr after the onset of the scotophase. Test substances were pipetted onto stainless-steel applicators, approx. 2 × 0.5 cm, held in disposable stoppers. Treatments were inserted 2 cm from the upwind end of each tube after the ether had evaporated. Males that moved upwind to within 4 cm of the source were considered to be responding to the pheromone and were counted after 15 and 30 sec, and the counts were averaged to obtain the mean response. The number of males within 4 cm of the application point just prior to insertion of a sample was subtracted from the total number of sampled population. Each assay was replicated at least 10 times.

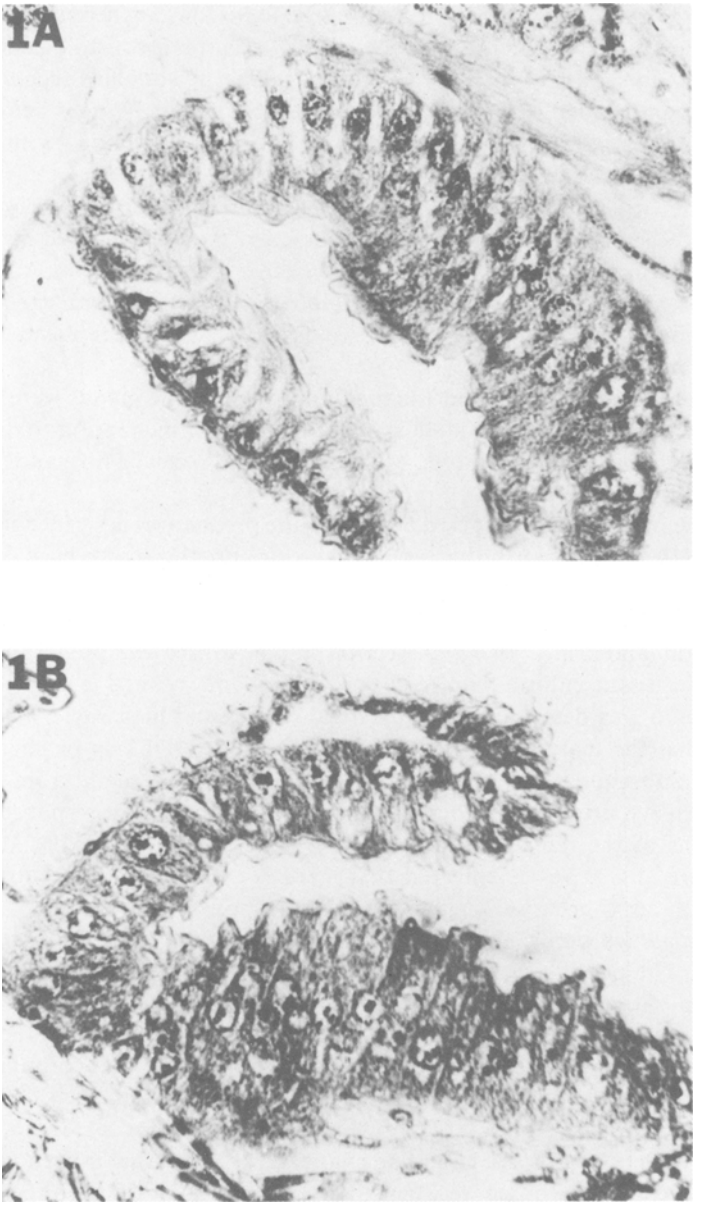
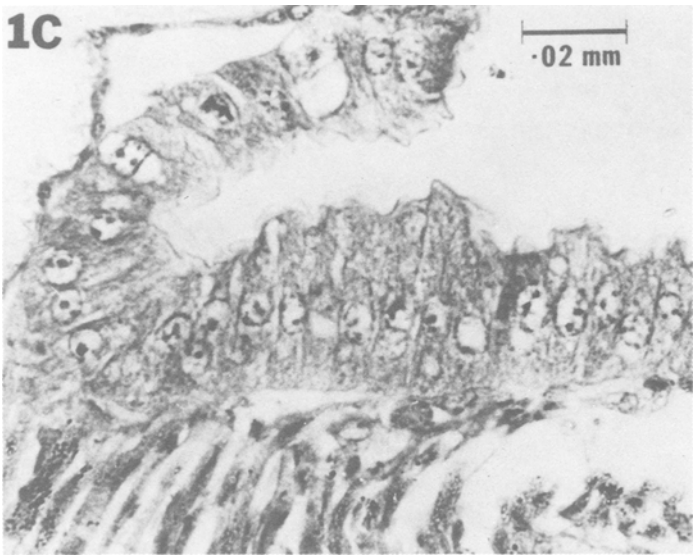


FIG. 1. (above and on facing page) Sections of pharate adult female *P. interpunctella* SPG 18–24 hr prior to emergence: (A) before being placed in culture; (B) after 10 days in chemically defined Grace's medium; (C) after 10 days in modified Grace's medium.



RESULTS

Morphology. The SPG of the female *P. interpunctella* consists of a single layer of specialized columnar epidermal cells. Figures 1A and 2A show sections of the SPG of pharate adult female and 2-day-old virgin adult female, respectively, before the glands were placed in tissue culture. The SPG cells appeared healthy after 10 days in culture in either chemically defined Grace's medium (Figure 1B and 2B) or modified Grace's medium (Figure 1C and 2C).

No gross differences were noted under the dissecting microscope for as long as 12 days after the abdominal tips were cultured in chemically defined or modified Grace's medium or in Ringer's solution. However, histological examination revealed that the SPG cells had disintegrated in the SPGs maintained for 3 days in Ringer's solution, even though the cuticle overlaying the SPG cells appeared intact. The SPG cells in the 5-day-old virgin adult females appeared normal except for the cytoplasm, which was less dense around the nucleus.

Pheromone Content. When 2-day-old virgin female SPGs were cultured for 3 or 6 days, approx. 100× more pheromone was recovered from the modified Grace's medium than from the chemically defined Grace's medium (Figure 3). The quantity of the pheromone in the SPG diminished during incubation. Little pheromone was detected in extracts prepared from glands

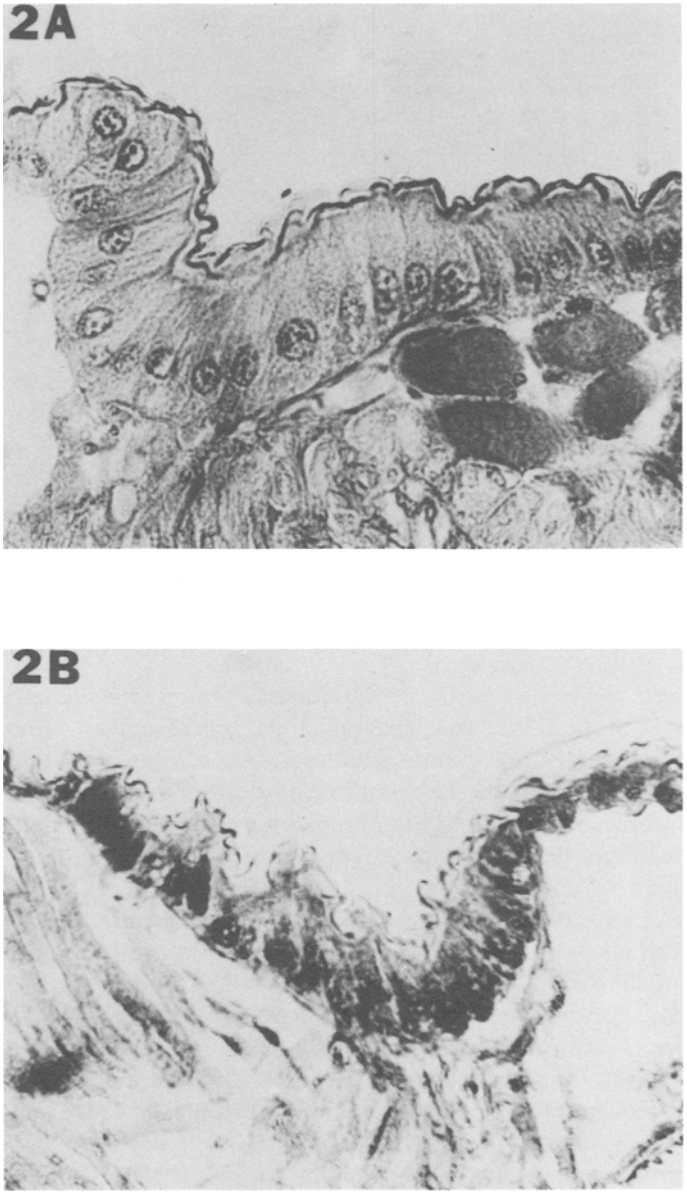
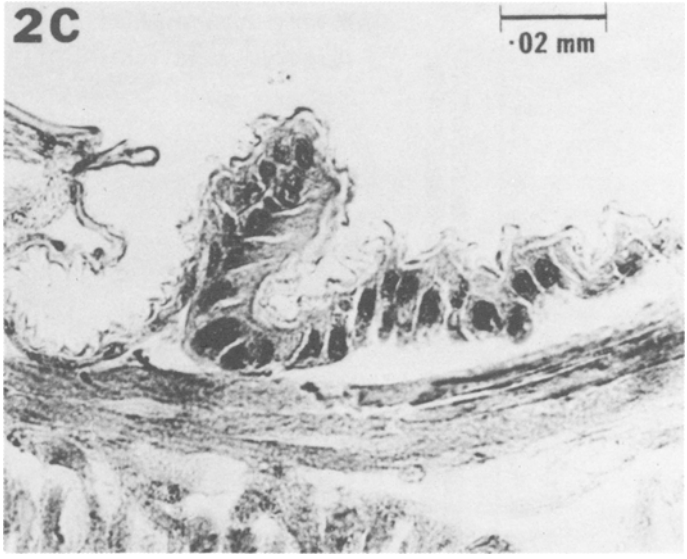


FIG. 2. (above and on facing page) Sections of 2-day-old virgin female *P. interpunctella* SPG: (A) before being placed in culture; (B) after 10 days in chemically defined Grace's medium; (C) after 10 days in modified Grace's medium.



in culture for 6 days (Figure 3). Ninety percent of the pheromone present in the glands of the 2-day-old virgin female was removed when the glands were rinsed with culture medium. Of the "removed pheromone" only about 10% could be recovered from the medium used for rinsing. However, ten times as much pheromone was recovered from the modified Grace's medium used for rinsing than from the chemically defined Grace's medium.

No pheromone was detected in extracts prepared from SPG of pharate adult females either before the glands were placed in culture or after the glands were cultured for 3 or 6 days in either chemically defined Grace's medium or modified Grace's medium, or from the media in which the glands were cultured.

DISCUSSION

In the only other successful attempt at culturing a lepidopteran SPG tissue, White et al. (1972) found that 50 or 100 μg/ml of juvenile hormone (JH) was essential for maintaining the integrity of the SPG cells of *Diatraea saccharalis* (F.) in tissue culture. In the absence of JH, they found massive destruction of the cytoplasmic matrix, and the nuclei appeared highly pycnotic. They indicated that such disintegration of the SPG cells also occurs in vivo approx. 4 days after emergence, and they speculated that the sparing action of JH on the SPG cells might be associated with some nutritional

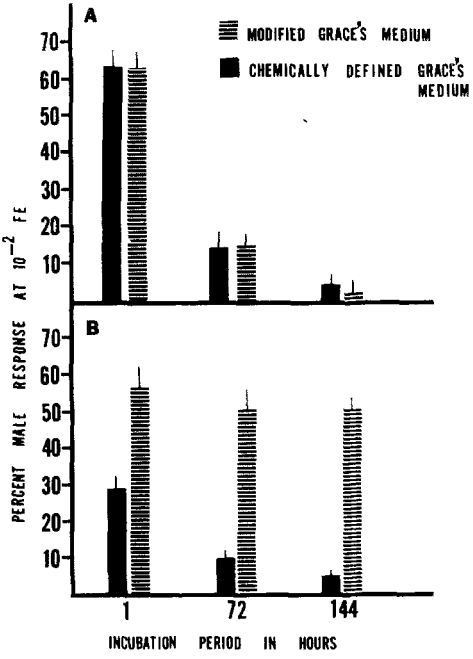


FIG. 3. Comparison of male response to sex pheromone extract from different sources. Vertical line indicates ±1 SE. Response to the controls (solvent only), solvent extracts of the modified Grace's medium, and chemically defined Grace's medium were 3.9 ± 1.9, 3.6 ± 0.9, and 4.1 ± 1.3, respectively. (A) Sex pheromone extract from SPG in culture. (B) Sex pheromone extract from culture medium after incubation with SPG.

requirement of the cells or with the activity of inhibitors originating in the serum. The SPG cells of *P. interpunctella* not only did not require hormonal supplements in the medium but also did not need any serum or albumin in the medium for survival. Thus, even the chemically defined Grace's medium supplied the nutritional requirements of *P. interpunctella* SPG cell for survival. Also, we did not observe any massive destruction of the cytoplasmic matrix of the SPG cells in the 5-day-old virgin adult females. The materials present only in the modified Grace's medium were therefore not essential for survival, but they were necessary if pheromone was to be recovered from the culture medium. The pheromone was readily lost from the chemically defined Grace's medium, presumably because of evaporation and/or decomposition, whereas such a loss is prevented in the modified Grace's medium by the presence of these additional components. In fact, we were able to recover an amount of pheromone almost equal to that present in rinsed 2-day-old virgin female SPG.

Ninety percent of the pheromone was removed from the 2-day-old virgin female glands when they were rinsed in the medium, but only about 10% could be recovered from the medium used for rinsing. The rest was either degraded or evaporated. Thus, the rather high recovery from the modified Grace's medium in which the glands were cultured for 3 or 6 days suggests that either the pheromone becomes stable when it is released into this medium or the glands in culture were synthesizing the pheromone and we were recovering only a portion of the pheromone that was synthesized. Because of the very low level of pheromone in the SPG after 144 hr of incubation (approx. 1% of the 1-hr level, Figure 3A), we cannot conclude that the glands have produced pheromone after isolation. However, we recognize that further studies using radiolabeled precursors will have to be conducted to determine whether or not the cultured glands synthesize pheromone in vitro.

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A QUANTITATIVE FEEDING BIOASSAY FOR *Pissodes strobi* PECK (COLEOPTERA:CURCULIONIDAE)[1]

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Abstract—A feeding bioassay for *Pissodes strobi* Peck was developed that consisted of plastic petri dishes containing paired agar disks immersed in paraffin wax. Lens paper covering the top surface of the disks provided 1, 2, or 3 weevils in each replicate with a surface through which they made regular feeding punctures which could be easily counted. The number of feeding punctures was correlated with the amount of agar ingested. Candidate feeding stimulants or deterrents were applied to the paper covering one of the disks in the dishes, while the other served as a solvent control. Feeding stimulants were tested using disks of pure agar, and deterrents were assayed on agar disks that contained 2% ground, dried Sitka spruce bark, *Picea sitchensis* Carr. The weevils exhibited a concentration-dependent response to the amount of spruce bark in the agar disk. Feeding stimulation by Sitka spruce bark extracts, and deterrency by leaf oil of western red cedar, *Thuja plicata* Donn, was demonstrated. The bioassay would be useful in chemical isolation programs aimed at identification of feeding stimulants and deterrents.

Key Words—*Pissodes strobi* Coleoptera, Curculionidae, *Picea sitchensis* Carr., *Thuja plicata* Donn, feeding bioassay, feeding deterrents, feeding stimulants.

INTRODUCTION

The trend toward the development of environmentally acceptable methods of insect control has led to considerable research on plant-produced chemicals that modify insect behavior (e.g., Hedin et al., 1974; Munakata, 1977).

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Within this scope, attractants, feeding stimulants, and antifeedants show potential in insect pest management.

The white pine weevil, *Pissodes strobi* Peck, relies on visual (VanderSar and Borden, 1977a) as well as chemical (Anderson and Fisher, 1956, 1960; VanderSar and Borden, 1977b) cues to find its hosts. VanderSar and Borden (1977b) showed that feeding stimulants present in an ethanolic extract of Sitka spruce bark, *Picea sitchensis* Carr., caused the weevils to feed on extract-impregnated elderberry pith disks. The weevils fed little on Sitka spruce twigs soaked in pressure-extracted fluids of western red cedar, *Thuja plicata* Donn. They hypothesized that the ultimate step in host selection was mediated by olfaction and contact chemoreception, involving chemical feeding stimulants as well as deterrents.

As a prerequisite to identifying the chemicals involved, it was essential to develop a reliable, quantitative bioassay for the response of *P. strobi* to feeding stimulants and deterrents. The spruce twig bioassay (VanderSar and Borden, 1977b) required extensive preparations and was subject to genetic variations in individual trees. An alternative inert substrate to elderberry pith disks (VanderSar and Borden, 1977b) was also needed, as the weevils made irregular feeding cavities on them, instead of the round neat punctures that they make in host bark. Agar is a common inert substrate used in feeding bioassays for other coleoptera, including the smaller European elm bark beetle, *Scolytus multistriatus* (Marsham) (Peacock et al., 1967), and the boll weevil, *Anthonomus grandis* Boheman (Keller et al., 1962). This paper describes the development and evaluation of an agar-based feeding bioassay for *P. strobi.*

METHODS AND MATERIALS

The weevils used in these experiments were obtained as mature larvae infesting Sitka spruce leaders collected in the fall of 1977 and immediately placed in screened cages at room temperature. Emergent weevils were stored at 3° ± 2°C in petri dishes containing the rearing diet described by Zerillo and Odell (1973), modified by the incorporation of Sitka spruce bark instead of white pine bark. Prior to their use in experiments, the weevils were held for 24 hr at room temperature, on moist filter paper.

Description and Standardization of the Feeding Bioassay. After experimentation with several substrates and designs, a feeding bioassay was developed which exploited the fact that *P. strobi* will readily feed on agar containing Sitka spruce bark, whereas they will feed very little on agar alone.

Each experimental unit (a replicate) (Figure 1) consisted of a 5.5-cm-diam plastic petri dish that contained two agar disks (1.5 cm diam. × 0.4 cm

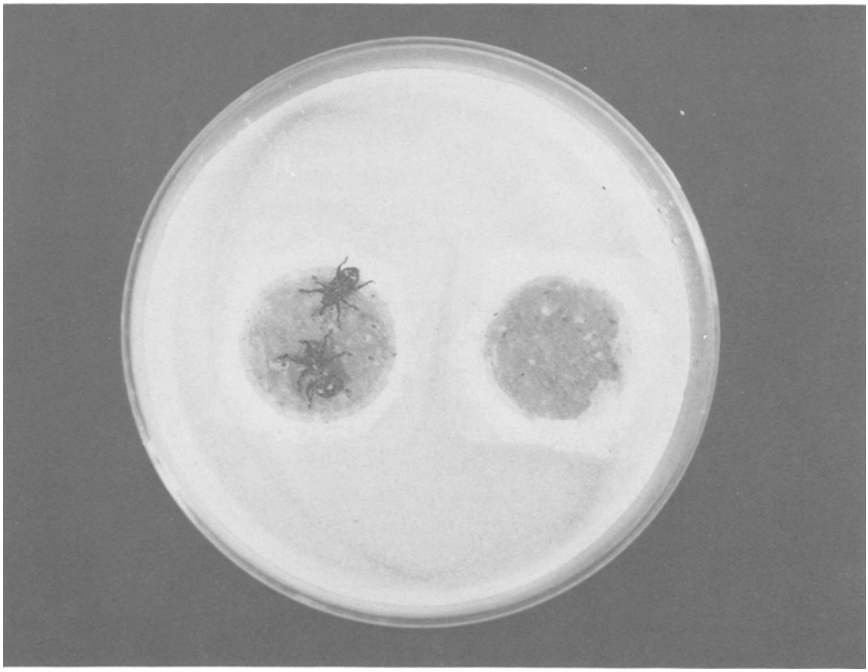

FIG. 1. Bioassay apparatus showing *P. strobi* feeding through lens paper into agar disk containing 2% Sitka spruce bark.

high). The agar disks were covered on their upper surface with a piece of Fisher standard lens paper, the most acceptable of a number of coverings tested. After placing the agar disks 2 cm apart in the petri dishes, hot paraffin wax was added to a height equal to that of the disks. Thus, only the upper, paper-covered surface of the agar disks was exposed, retarding dehydratation and preventing shrinkage of the agar away from the paper. The paper was kept in contact with the agar by embedding its edge in the hot wax. Insects placed in the dish had ready access to the surface-level test materials as they walked over the disks.

Stimulants were applied to the paper surface of the disks or were incorporated into the agar as finely powdered, oven dried (105° ± 2°C for 2 hr) Sitka spruce bark from lateral branches. Feeding deterrents were applied to the paper surface on agar disks that contained powdered Sitka spruce bark. Feeding activity was evaluated by comparing the number of feeding punctures made by the test insects in the paper over treated and control disks.

The relationship between the number of feeding punctures and the area and volume of agar ingested was investigated in ten 20-replicate experi-

ments. Two weevils per replicate were allowed to feed for 24 hr in bioassay preparations with only one agar disk, containing 2% Sitka spruce bark. An estimate of the volume of each feeding puncture was obtained by multiplying its area at surface level, calculated by planimeter from a camera lucida drawing, by its depth, measured by carefully introducing an insect-pin depth gauge into each cavity. By addition, a "total volume" and "total area eaten" were obtained for each disk. Correlation coefficients were calculated between the number of feeding punctures produced and (1) surface area, and (2) volume of agar ingested.

The number of weevils to use per replicate was evaluated in a 4-treatment, 15-replicate experiment, in which the number of punctures made in 24 hr by 1, 2, 3, or 4 weevils on single 2% spruce bark–agar disks were counted.

Test of the Bioassay for Evaluating Sitka Spruce Bark and Bark Extracts as Feeding Stimulants. An experiment was designed to test whether the weevils would exhibit a concentration-dependent feeding response to different amounts of Sitka spruce bark incorporated into the agar. Each replicate consisted of one agar–bark disk plus a plain agar control disk. Eight different concentrations of bark, ranging from 0.01% to 3% were tested; 15 replicates, each with two insects, were run for 24 hr for each treatment and sex of *P. strobi.*

Two extracts of Sitka spruce bark were tested for feeding stimulatory activity. Dried Sitka spruce bark (30 g) obtained from lateral branches, was sequentially extracted for 24 hr in a Soxhlet extractor with pentane and ether. The two solvent extracts were concentrated to 25 ml by distillation, and then 10 μl of extract (the equivalent of 0.012 g of spruce bark) was applied with a micropipette to the paper surface of one of two pure agar disks in each dish. The control disk in each dish was treated with 10 μl of either ether or pentane. The number of replicates and insects and the experimental duration were the same as in the previous experiment.

Test of the Bioassay with Feeding Deterrents. Branchlets of western red cedar (200 g) were macerated in a Waring blender with water (1750 ml). The mixture was transferred to a 3-liter distilling flask fitted with a modified Nielsen-Kryger condenser (Veith and Kiwus, 1977), and steam distilled for 3.5 hr. The continuous extraction section of the condenser was charged with approximately 5 ml of doubly distilled pentane. The pentane solution of cedar leaf oil was washed with 2% $NaHCO_3$ solution and water, dried (Na_2SO_4), and concentrated by distillation. Residual solvent was removed by brief vacuum pumping.

The cedar leaf oil was tested for feeding deterrent activity in an experiment in which both agar disks in a dish contained 2% Sitka spruce bark. A pentane solution of the cedar leaf oil was applied to the paper surface covering one of the disks in each replicate. The control disk received an

equal volume of distilled pentane. The amounts of extract applied were: 1, 10, 50, and 100 μg. Fifteen replicates, each with two weevils, were run for 24 hr for treatment and sex of *P. strobi.*

RESULTS AND DISCUSSION

The weevils readily fed on agar containing spruce bark (Tables 1–3, 5) or treated with either of the spruce bark extracts (Table 4). The feeding punctures in the lens paper were very distinct and easy to count.

High correlation coefficients were obtained between the number of feeding punctures produced and the area and volume of agar ingested by the insects (Table 1). Therefore, the number of punctures is a good indicator of the amount of feeding by the test insects, justifying their use in evaluating subsequent experiments. If poor correlations were found, the number of feeding punctures would be indicative of weevil biting response rather than feeding activity.

The number of feeding punctures in the agar disks increased linearly with the number of female weevils per replicate. Males, however, apparently fed less when four instead of three insects were used (Table 2). This observation may indicate a behavioral difference between sexes in response to crowding. The differences in feeding between males and females were not significant for any number of weevils. The data in Table 2 suggest that our decision to use two weevils per replicate in further experiments resulted

TABLE 1. LINEAR CORRELATION COEFFICIENTS BETWEEN NUMBER OF FEEDING PUNCTURES PRODUCED AND VOLUME AND AREA OF AGAR INGESTED BY 2 *P. strobi* ON SITKA SPRUCE BARK (2%)-AGAR DISKS[a]

Experiment	Punctures/area	Punctures/volume
1	0.89	0.90
2	0.84	0.72
3	0.72	0.54
4	0.93	0.94
5	0.85	0.90
6	0.91	0.89
7	0.91	0.84
8	0.82	0.92
9	0.86	0.85
10	0.88	0.91

[a] $N = 20$ replicates for each experiment.

TABLE 2. MEAN NUMBER OF FEEDING PUNCTURES PRODUCED ON SITKA SPRUCE BARK (2%)-AGAR DISKS BY DIFFERENT NUMBERS OF *P. strobi*[a]

Number of weevils	Mean number of feeding punctures[b]	
	Males	Females
1	30.4 a	26.7 a
2	45.8 a	43.4 ab
3	68.9 b	49.1 b
4	60.0 b	70.1 c

[a] $N = 15$ replicates for each mean.
[b] Means within each column followed by the same letter are not significantly different (Newman-Keuls test, $P < 0.05$).

TABLE 3. MEAN NUMBER OF FEEDING PUNCTURES PRODUCED PER PAIR OF *P. strobi* ON AGAR DISKS CONTAINING DIFFERENT CONCENTRATIONS OF SITKA SPRUCE BARK[a]

Bark concentration (%)	Mean number of feeding punctures[b]			
	Males		Females	
	Agar-spruce disks	Untreated disks	Agar-spruce disks	Untreated disks
3.0	20.3	0.0**	14.5	0.5**
1.5	12.1	0.1**	8.8	0.2**
0.5	11.5	0.1**	20.5	0.1**
0.25	12.5	0.5**	15.3	0.3**
0.10	3.1	0.1**	3.7	0.1**
0.05	1.6	0.4*	9.3	0.4**
0.02	0.3	0.1	1.8	0.3*
0.01	1.1	0.3	1.5	0.0*

[a] $N = 15$ replicates for each mean.
[b] Student's t test on difference between paired means for each sex indicated by: $^{**}P < 0.01$; $^{*}P < 0.05$.

in reliable data. Use of two weevils in each dish eliminated variability in the data.

There was a concentration-dependent feeding response of *P. strobi* to the various concentrations of Sitka spruce bark in the agar disks (Table 3). Linear regression equations for the data transformed to natural logarithms were: NFP = 13.8454 + 3.3537 × ln concentration, for males, and NFP =

TABLE 4. MEAN NUMBER OF FEEDING PUNCTURES PRODUCED PER PAIR OF *P. strobi* IN RESPONSE TO PENTANE AND ETHER EXTRACTS OF SITKA SPRUCE BARK APPLIED TO LENS PAPER COVERING PLAIN AGAR DISKS[a]

	Mean number of feeding punctures[b]			
	Males		Females	
Extract	Treated disks	Untreated disks	Treated disks	Untreated disks
Pentane (10 μl)	36.3	2.3**	19.7	1.1**
Ether (10 μl)	19.3	0.1**	46.5	0.4**

[a] $N = 15$ replicates for each mean.
[b] Student's t test on difference between means for each sex indicated by: $^{**}P < 0.01$.

TABLE 5. MEAN NUMBER OF FEEDING PUNCTURES PRODUCED PER PAIR OF *P. strobi* ON SITKA SPRUCE BARK (2%)-AGAR DISKS TREATED WITH WESTERN RED CEDAR LEAF OIL AT DIFFERENT CONCENTRATIONS[a]

	Mean number of feeding punctures[b]			
	Males		Females	
Amount of cedar leaf oil applied (μg)	Treated disks	Untreated disks	Treated disks	Untreated disks
100	3.3	47.7**	7.6	42.5**
50	6.0	37.7**	10.0	28.8*
10	22.2	35.4	13.8	24.6
1	19.7	33.4*	19.7	29.7

[a] $N = 15$ replicates for each mean.
[b] Student's t test on difference between paired means for each sex indicated by: $^{**}P < 0.01$; $^{*}P < 0.05$.

$13.7777 + 2.4197 \times \ln$ concentration, for females, where NFP = number of feeding punctures.

Correlation analysis yielded correlation coefficients of $r = 0.93$ ($P < 0.01$) and $r = 0.70$ ($P < 0.01$) for males and females, respectively, indicating that the feeding response of males was more closely dependent on concentration than the response of females. By extrapolation of the fitted curves, the threshold of responses to bark in the agar were found to be 0.016 and 0.0034% for males and females, respectively. This difference is possibly due to a superior sensory capability in females, a phenomenon also noted by VanderSar and Borden (1977b).

The weevils readily responded to the pentane and ether extracts of

spruce bark (Table 4). The high level of feeding obtained on the treated disks is in agreement with earlier findings that extractable feeding stimulants are present in Sitka spruce bark (VanderSar and Borden, 1977b).

Western red cedar leaf oil proved to be highly deterrent to feeding by *P. strobi*. The bioassay effectively quantified the increasing deterrent effect of higher stimulus concentrations (Table 5). As the amount of cedar oil increased on the treated side, the weevils fed preferentially on the untreated agar disks.

CONCLUSION

The experiments reported herein show that an agar disk bioassay can be effectively utilized to evaluate quantitatively the feeding stimulatory and deterrent properties of natural plant products on *P. strobi*. Stimuli can either be incorporated into the agar or applied to an absorbent paper surface covering the disk.

Evaluation of feeding in this bioassay can be done by counting the numbers of feeding punctures, which are highly correlated with the amount of material actually ingested by the insects. The bioassay can be carried out using 1–3 insects per replicate without affecting the behavior of the weevils.

We have used the bioassay to confirm that feeding stimulants for *P. strobi* occur in the bark of Sitka spruce and that chemicals from western red cedar leaf oil inhibit feeding on an otherwise acceptable feeding substrate. The large differences between feeding on treated and untreated disks (Tables 3 and 4) make this bioassay particularly suitable for detecting compounds that are even mildly stimulatory to feeding by *P. strobi*.

Acknowledgments—We thank L. Chong for assistance in experimentation, and R.G. Long for photography.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS

IX. Investigations of Various Kairomone-Treatment Patterns for *Trichogramma spp.*[1,2,3]

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Abstract—A kairomone from adult *Heliothis zea* (Boddie) scales is an important factor in the host selection process of *Trichogramma pretiosum* Riley. If the host density is sufficiently high (i.e., 1 egg/500 cm^2 and higher), a complete coverage or solid treatment of kairomone spray may be the optimum for increasing parasitization rates, but this is not the case at lower host densities (e.g., 1 egg/2000 cm^2). At the lower densities, the kairomone must be distributed in such a way as to retain the parasitoids in the target area without inhibiting their movement from one oviposition site to the next. Simulated moth scale particles appear to fill this need since their density can be regulated to provide the optimum frequency of parasitoid stimulation and thus maximum rates of parasitization at prevailing host densities.

Key Words—*Trichogramma*, Hymenoptera, Trichogrammatidae, kairomone, *Heliothis zea*, biological control, pest management, parasitoids.

[1]Hymenoptera: Trichogrammatidae.
[2]In cooperation with the University of Georgia College of Agriculture Experiment Stations, Coastal Plain Station, Tifton, Georgia 31794. Accepted for publication November 21, 1978.
[3]Mention of a commercial or proprietary product in this paper does not constitute endorsement by the USDA.

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INTRODUCTION

Scales left by ovipositing *Heliothis zea* (Boddie) moths are a source of kairomone that stimulates host-seeking behavior by *Trichogramma* spp. (Lewis et al., 1971, 1972; Jones et al., 1973). The initial paper of this series reported that field-plot application of the appropriate kairomone resulted in increased parasitization rates by certain *Trichogramma* spp. (Lewis et al., 1975a). It was demonstrated that the kairomone did not have to be limited to the immediate vicinity of the host egg but increased parasitization if applied as a complete coverage or solid spray over the entire plot. Data from a subsequent study (Lewis et al., 1975b) indicated that the primary process by which the kairomone functions is by releasing and maintaining an intensified search behavior rather than as an attractant or steering mechanism. In these studies, solid treatment of the target area resulted in the highest parasitization rates because it elicited host-seeking responses more quickly from more females and continuously reinforced their searching behavior throughout the target area.

However, as studies with the kairomone advanced, it became evident that the solid-treatment pattern did not necessarily produce the highest rates of parasitization. Therefore, the present study was conducted to appraise the effectiveness of various patterns of kairomone application with different experimental designs and host-egg distributions.

METHODS AND MATERIALS

The 1/1000 dilution of a hexane extract of *H. zea* moth scales (MSE) used in this study was formulated according to the procedure of Jones et al. (1973). In some of the experiments, simulated moth scales (SMS) which consisted of moth scale extract-impregnated particles, were used. Two particle types were used: Anakrom U (Analabs, North Haven, Connecticut) (diatomaceous earth), 40/50 mesh, which were white, and Anakrom C-22 (diatomaceous earth), 40/50 mesh, which were red. These particles were impregnated with moth-scale extract by mixing 5 g of either particle type with 1 ml of crude moth-scale extract and 50 ml of hexane in a boiling flask and vacuum-evaporating the liquid. Unimpregnated particles were used as controls.

The treated *H. zea* eggs used in this study (and used for rearing the *Trichogramma*) were obtained from laboratory cultures processed with a sodium hypochlorite wash as described by Burton (1969), irradiated with 25 krad (^{60}Co source) when 8–36 hr old, and stored at approx. 10° C.

The *Trichogramma* stock used in the study originated from Hermosillo, Mexico. It crossed successfully with a stock from Los Mochis, Mexico

(Gonzalez and Allen, 1975, Division of Biological Control, University of California, Riverside, CA 92501, unpublished results) that was identified as *Trichogramma pretiosum* Riley (Oatman et al., 1970). These parasitoids were reared from treated *H. zea* eggs at approx. 26°C and 70% relative humidity, according to the method of Lewis and Redlinger (1969).

Dissection of host eggs to determine parasitization was done according to the procedure described by Lewis and Redlinger (1969).

Statistical analysis was performed by the paired *t* test. Arcsin transformations were conducted on percentages before analysis. Means given in the text are followed by the standard error in parentheses.

RESULTS

Experiment I. Previous evaluations of solid-coverage treatment of field plots were based on parasitization rates of artificially applied host eggs. However, because naturally oviposited eggs would have a liberal but yet undetermined amount of kairomone immediately around them, we decided that a more adequate appraisal of the influence of the kairomone treatment would be derived by simulating this natural distribution with a 7.6-cm spot of kairomone around the eggs. Therefore, this experiment was conducted to compare parasitization rates of artificially applied eggs in plots receiving both spot treatment around the eggs and solid treatment vs. plots with only spot treatment immediately around the egg.

The experiment was conducted in soybean plots 6 rows wide (rows were planted 91.44 cm apart) and 4.6 m long. Populations of naturally occurring *H. zea* and *Trichogramma* spp. were insignificant relative to the number applied. A chromatographic sprayer (Lewis et al., 1972) was used to apply kairomone at 1 ml/0.3 m of row on the solid-treated plots and a 7.6-cm-diameter spot around the eggs to simulate a natural oviposition site. The 7.6-cm spot was applied by spraying through a template with a 7.6-cm-diameter hole held against the foliage with the egg at about the center of the hole. *T. pretiosum* were released from 16-dr plastic cups at 6 cups of approx. 50 parasitoids/plot. Host eggs were placed on the plants (1 egg/0.9 m) using Plantgard® (Polymetrics International, New York, New York) (Nordlund et al., 1974). This experiment was replicated 8 times.

Mean parasitization rate was 10.8% (±2.6) in the solid-treated plots as compared with 24.1% (±1.8) in the spot-treated plots (significantly different at the $P < 0.01$ level).

These results were contrary to those found in laboratory and greenhouse studies of spot-treatment vs. solid-treatment patterns reported earlier (Lewis et al., 1975b).

Thus, the 150 × 15-mm petri dishes and pea cotyledons growing in

22.8-cm pie pans used in previous studies proved too small an arena with too dense host egg populations to allow full expression of the host-finding behavior by the ovipositing *T. pretiosum* females. To reconcile these apparently conflicting results, we conducted further evaluations of different treatment patterns under controlled laboratory conditions.

Experiment II. An expanded laboratory universe was designed and tested to determine whether the effectiveness of kairomone-treatment patterns could be accurately compared in the laboratory. The testing arenas consisted of sheets of white butcher paper (Schrier Bros., Mamaroneck, New York) (117.5 × 30.4 cm) placed on a laboratory table. The butcher paper sheets were separated at each end by 30 cm and on the sides by 60 cm. Three eggs were attached to the sheet with a camel's hair brush moistened with Plantgard. Eggs were spaced in a line along the middle of the sheet with 1 egg in the center, i.e., 15.2 cm from the side and 58.75 cm from the ends of the arena. The other 2 eggs were placed 45.72 cm to either side of the center egg, 15.2 cm from the sides and 13.03 cm from the respective ends of the sheet. Two vials of 5 *T. pretiosum* females were released, one each midway between the center egg and the egg on each end. The parasitoids were allowed to function for 30 min, and the 3 eggs were then dissected to determine parasitization. Three paired comparisons of two treatment patterns were conducted at a time. Means of three readings of each treatment constituted one replication. Each comparison was replicated 15 times.

To evaluate this universe as a bioassay tool, we compared the standard solid-coverage treatment vs. an untreated control. The treated sheets were sprayed evenly with approx. 25 ml of MSE with a chromatographic sprayer.

Mean percentage of parasitization was 23.6% (±19.0) for the solid-treated universe compared with 14.0% (±10.3) for the controls (differences significant at the $P < 0.05$ level). These results were similar to those obtained with other laboratory and greenhouse techniques and demonstrated that there was an effective response by *T. pretiosum* females to kairomone(s) in this system.

To determine whether this expanded laboratory universe allowed a more complete expression of the host-finding behavior and more adequately reflected the field results, we made a second comparison of solid treatment vs. spot treatment around the egg. The spot treatment consisted of a circular zone 7.6 cm in diameter treated with 0.25 ml of MSE with a pipette.

Mean percentage of parasitization in the spot-treated arenas (33.8% ± 20.0) was significantly higher ($P < 0.05$) than in the solid-treated arenas (18.0% ± 16.1). These results were different from those obtained with the petri dish and greenhouse studies reported earlier but agreed with the results of field studies presented above. These findings supported the belief that a more expanded universe than previously used in laboratory and

greenhouse studies, with adequate spacing of the eggs, is required to allow a sufficient expression of the host-finding behavior of *T. pretiosum* and to assess effectively the various kairomone-treatment patterns. The results further demonstrate that, contrary to previous indications, the solid-treatment pattern is not the optimal treatment pattern except perhaps when high host egg densities are present. Although the kairomone does not attract or guide the parasite directly to the host, it does concentrate search in egg-site locations. The solid treatment, although it results in high parasite retention in the overall target area, falsely signals equal probability of eggs throughout the site.

In the case of moderate to low host densities, the kairomone around the egg sites is insufficient to provide reinforcement of the searching behavior at the frequency needed for high parasite retention in the area. Therefore, the optimal pattern would achieve high parasitoid retention in the target area and parasitoid concentration in the high probability areas. One possibility was to apply a more dilute kairomone solution, thus providing frequent stimulation, while permitting parasitoids to seek out high concentration areas around eggs. However, it became apparent in other aspects of the studies that there were few differences in the response by *Trichogramma* to different dosages of the kairomone and that application of dilute dosages among the oviposition sites would not provide a sufficiently detectable gradient to allow concentration around the high-probability sites. Furthermore, because the natural system provides for contact with scales and other residue particles from the moth, it was supposed that tightness of the search pattern was dependent more on contact frequency with these sources than on molecular concentration at a given contact point. Therefore, we decided to use SMS and to manage the search pattern by regulating the density of SMS. It was supposed that by providing the appropriate density of SMS among the oviposition sites, adequate retention could be obtained without significantly impeding movement from one site to the next and that parasitization could be increased above that of spot treatment.

This possibility was investigated by a third comparison of the spot-treatment pattern with a pattern of spot treatment plus red SMS in the expanded laboratory universe. The SMS were applied at 144/sheet evenly distributed (approx. 6-cm grid) over each sheet. A camel's hair brush moistened with Plantgard was used for application. Control particles were applied to the substrate in this comparison to compensate for any possible influence of the particles themselves. Mean percentage parasitization was 27.2% (±17.0) for the spots with control particles treatment and 43.9% (±18.8) for the spots plus SMS (differences significant at the $P < 0.05$ level). These results demonstrate that the application of SMS could be used to increase parasitization in this expanded universe system.

Experiment III. Greenhouse tests were conducted to further evaluate

these treatment patterns. Each arena consisted of twelve 22.8-cm pie pans of cotyledonous-stage crowder peas arranged in a 3 × 4-pan design. Thus, each arena consisted of approx. 0.7 m^2 of foliage. Two *H. zea* eggs, one on each of a pair of cotyledons, were placed in the center of 4 pans, one on alternating ends of each of the 4 rows of pans. Six vials of one *T. pretiosum* female each were released from 6 locations in the arena. Six paired comparisons of treatment patterns were conducted at a time. Mean percentage parasitization from two readings (1-hr exposure each) from each of six arenas of two treatments constituted one replication.

The first test involved arenas in which the pans were sprayed solid (1 ml/pan) with MSE vs. arenas that received no kairomone treatment (control). Eight replications of this test were conducted. Mean percentage parasitization in the solid-treated arenas was 12.6% (±2.0) and was significantly higher ($P < 0.05$) than that in control arenas, which was 8.3% (±2.5). These data demonstrate that a solid treatment of kairomone increases parasitization when compared with an untreated control, as demonstrated in earlier studies (Lewis et al., 1975b). This type of situation would not occur in nature, however, because each naturally oviposited egg would have some kairomone associated with it.

Thus, a second test was conducted in which arenas with the four egg sites treated with a 5.1-cm-diameter spot of MSE were compared with arenas that were sprayed solid, as in the previous test. This smaller spot was used because the universe was comparatively small. This test was replicated six times. Mean percentage parasitization in the spot-treated arenas was 11.5% (±3.2), which was not significantly different ($P < 0.05$) from the 12.6% (±5.3) in the solid-treated arenas. At this egg density, there was no difference between the parasitization rates produced by a solid-spray treatment or a spot-spray treatment. These results were not surprising, since solid spray resulted in a relatively higher percentage parasitization in previous studies (Lewis et al., 1975b). However, at a lower host density, the solid treatment would be expected to produce a relatively lower percentage parasitization as shown in experiments I and II.

A third comparison involved arenas in which the four egg sites were treated with a 5.1-cm-diameter spot of MSE and five white SMS were applied to each of the 12 pans vs. arenas that were only spot treated, as previously described. Mean percentage parasitization was 36.5% (±3.0) in the spot and SMS arenas, which was significantly higher ($P < 0.05$) than the 23.2% (±8.9) for the spot-treated arena. These data demonstrate the effectiveness of the SMS, even when the egg density is as high as 8 eggs/0.7m^2.

Experiment IV. The final objective of this investigation was to appraise the use of SMS to increase parasitization by *T. pretiosum* in the field. The experiment was conducted in cotton approx. 0.9 m high. Naturally occurring populations of *H. zea* and *Trichogramma* spp. were insignificant

in this field. Seven pairs of plots, approx. 7.6 × 7.6 m, were selected with a minimum of 30.5 m separation between plots. Host eggs were placed on the plot at 1 egg/0.9 m, and kairomone was spot sprayed on a 7.6-cm-diameter spot around the egg. This simulated a natural density of approx. 5000 *H. zea* eggs/0.4 hectare. One plot of each pair was designated as treated and sprayed with white SMS at approx. 10 particles/0.3 m of row. These particles were applied as a suspension in a solution of Plantgard and water (1:4) with a pneumatic sprayer powered with compressed CO_2. *T. pretiosum* were released from 16-dr cups in the plots at approx. 50,000/0.4 hectare. Host eggs were collected after approx. 24 hr and dissected to determine parasitization. This experiment was replicated seven times.

Mean percentage of parasitization was significantly higher ($P < 0.01$) in the plots receiving a spot treatment around the egg, plus kairomone-impregnated particles (64.1% ± 4.9) than in the plots receiving only a spot treatment around the egg (43.4% ± 7.0). These results demonstrated that the SMS, unlike the solid treatment in experiment I, increase parasitization above that obtained by spot treatment around the egg under field conditions. Therefore, it is apparent that SMS provides adequate reinforcement of the host-seeking behavior between egg sites without impeding movement and are superior to solid-pattern application of kairomones. By regulation of the density of these particles based on the host density, this procedure should provide optimum treatment patterns for managing *Trichogramma* in varying natural host densities.

DISCUSSION

Earlier work with a kairomone from *H. zea* moth scales indicated that a solid treatment was optimum because the kairomone stimulated intensive search behavior but did not attract. Data from experiment I indicated that this was not the case at low host densities. The simulated oviposition sites stimulated sufficient numbers of parasitoids in areas where eggs were present to cause higher parasitization rates than in the plots which were solid treated.

In the expanded laboratory universe of experiment II, use of SMS between simulated oviposition sites improved the parasitization rates significantly over spot treatment. This indicates that the SMS provided the parasitoids sufficient contact with kairomone sources to stimulate searching and retention without impeding movement from one simulated oviposition site to the other. Similar results were obtained in the greenhouse tests of experiment III.

Experiment IV was an appraisal of the effectiveness of SMS in the field. Data indicate that the use of SMS at the appropriate density can significantly increase parasitization rates in the field.

These data demonstrate that the optimum distribution of the kairomone is dependent on host density, that solid treatment is not effective at low host densities, and that SMS can be used to increase parasitization rates at intermediate to low host densities.

Important factors that were not addressed in this study include the size, shape, and texture of the SMS. We have preliminary data that indicate that there are more returns to, and thus greater retention by, larger kairomone sources. Particles used in these studies caused significantly increased parasitization rates by *T. pretiosum*; however, with a greater understanding of the influence of size, shape, and texture of particles on the behavior of these parasitoids, we feel certain that even greater efficiency can be achieved.

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SYNTHESIS AND FIELD TESTING OF 4,6,6-LINEATIN, THE AGGREGATION PHEROMONE OF *Trypodendron lineatum* (COLEOPTERA:SCOLYTIDAE)[1]

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Abstract—Authentic 4,6,6-lineatin (3,3,7-trimethyl-2,9-dioxatricyclo-[3.3.1.$0^{4,7}$]nonane) (I) was produced in low yield via three synthetic pathways. In field tests, microgram amounts of the product from all three syntheses attracted large numbers of *Trypodendron lineatum* of both sexes. These results confirm that 4,6,6-lineatin (I) is a population aggregation pheromone for *T. lineatum*.

Key Words—*Trypodendron lineatum*, 4,6,6-lineatin, 3,3,7-trimethyl-2,9-dioxatricyclo[3.3.1.$0^{4,7}$]nonane, pheromone, attractant, tricyclic acetal, Scolytidae, aggregation pheromone, ambrosia beetle.

INTRODUCTION

Secondary attraction involving pheromones produced by female *Trypodendron lineatum* (Olivier) was originally demonstrated in the field (Rudinsky and Daterman, 1964a,b; Chapman, 1966) and verified in the laboratory

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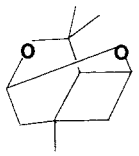
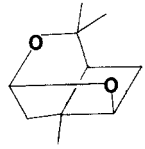
(Borden et al., 1968; Borden and Slater, 1969). An attractant compound isolated from the frass produced by female *T. lineatum* was tentatively identified as one of two isomeric tricyclic acetals (I or II), and given the trivial name lineatin on the basis of congruent chemical and biological properties of the isolated compound and a compound produced by a synthesis that was not unambiguous; biological activity was compared in a laboratory bioassay (MacConnell et al., 1977). Structures I and II were named 4,6,6- and 4,5,6-lineatin, respectively, based on the size of the fused rings in each system.



I



II

Structure I (3,3,7-trimethyl-2,9-dioxatricyclo[3.3.1.$0^{4,7}$]nonane) was chosen as the initial target compound for synthesis because of coupling in the NMR spectrum between the methine proton and that on the oxygen-bearing carbon in the four-membered ring. This, however, does not unequivocally rule out II since long-range, cross-ring couplings of this magnitude are known. We now present confirmation of structure, by further synthesis, and results of field tests of the synthesized compound.

CHEMISTRY

The spectral, chromatographic (including cochromatography), and biological properties of compound I, synthesized by three independent sequences (schemes 1–3), were congruent with those of the natural lineatin (MacConnell et al., 1977). Scheme 1, although cumbersome and with poor overall yield, provided an unambiguous synthesis of compound I.

The "unit resolution" mass spectrum of isolated lineatin (MacConnell et al., 1977) was actually based on a high-resolution spectrum. It differed somewhat in detail from subsequent spectra on unit resolution instruments, particularly in the intensities of the m/e 135 and 153 peaks. A spectrum of isolated 4,6,6-lineatin obtained on a Hitachi RMU-6 instrument is shown in Figure 1.

We conclude that the pheromone is compound I, and we present below the synthesis schemes (1–3) that confirmed this identification. None of these schemes gave a satisfactory yield. Scheme 4, although still in process, is presented here, since exploratory work shows promise. Details will be published elsewhere.

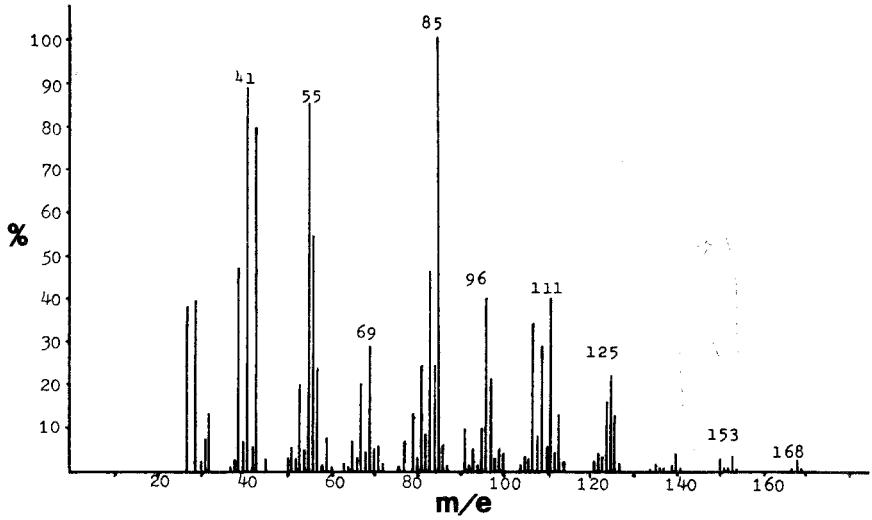


FIG. 1. Unit resolution mass spectrum of lineatin.

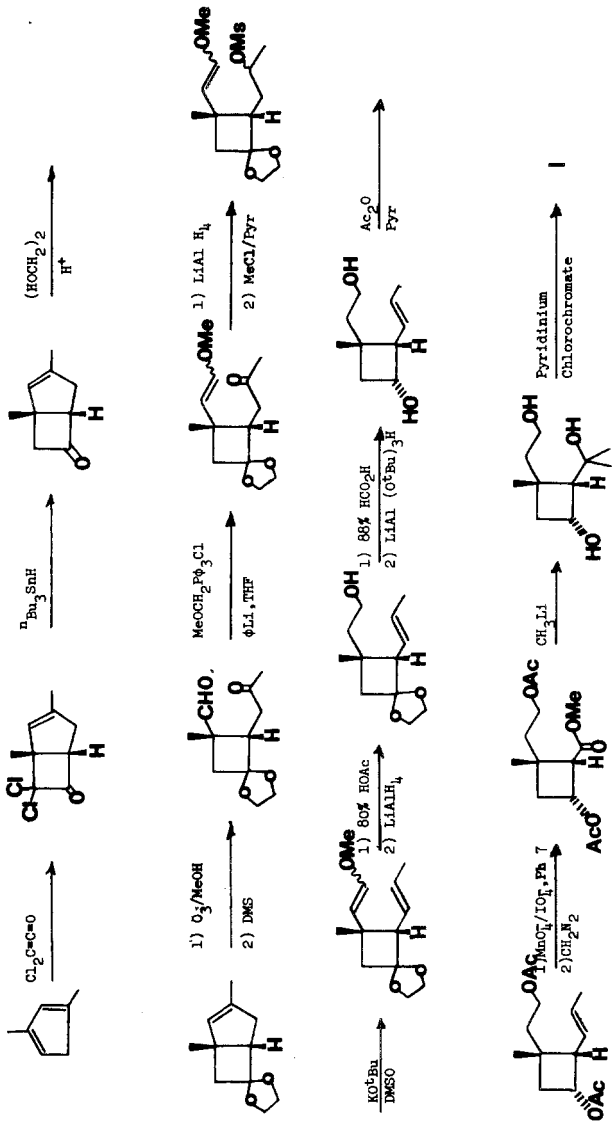
Cycloaddition in scheme 2 appears to take place only in the presence of zinc salts produced in the formation of dichloroketene by dehalogenation. No significant amount of adduct could be identified when dichloroketene was generated by dehydrohalogenation with trimethylamine.

A small amount of pure 4,6,6-lineatin was obtained with scheme 3, a variation of MacConnell's method (MacConnell et al., 1977). Since separation of the individual components of the photoadduct product mixture was difficult, the sequence was carried through on the crude mixture. 4,6,6-Lineatin (200 μg) was isolated from the complex product mixture on a 6.1-m × 4.0-mm 5% C20M column (on Chrom GHP 60/80 mesh, 60 ml/min He flow; retention time = 31 min at 127°) followed by a 6.1-m × 4.0-mm 5% FFAP column (on Chrom GHP 60/80 mesh, 60 ml/min He flow; retention time = 31 min at 131°).

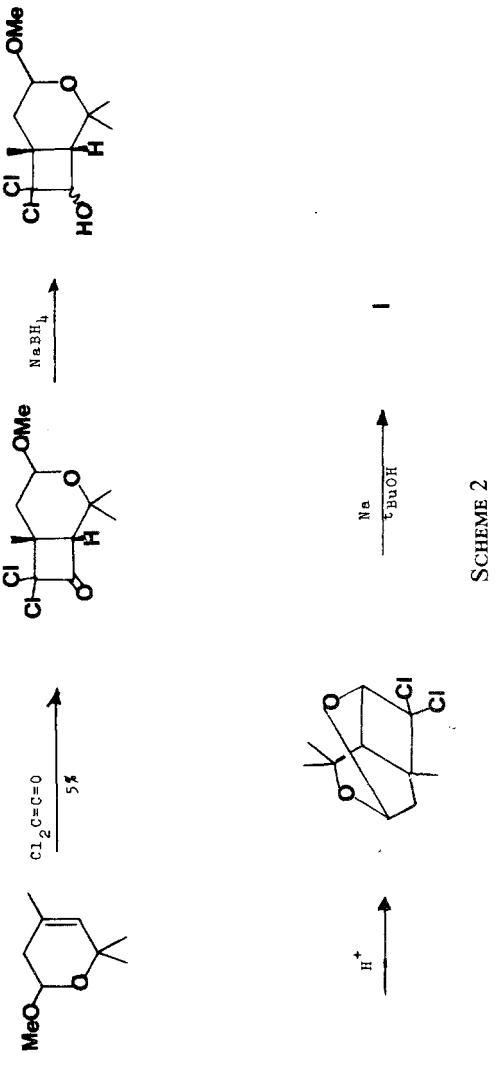
Since the isomeric abundances of the dehydromevalonolactone photoadduct in scheme 3 precluded an economical synthesis via this method, we are now investigating an alternative synthesis (scheme 4).

BIOLOGY

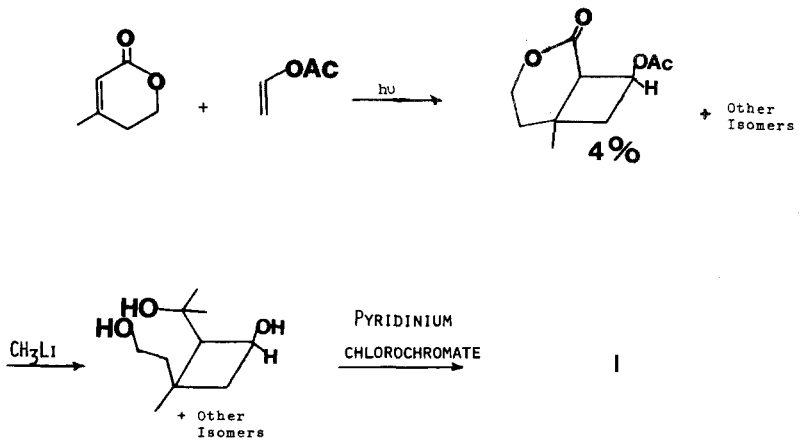
Synthetic 4,6,6-lineatin from each of the three synthesis pathways was tested in early spring field experiments in a 70-year-old stand of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, at the B.C. Forest Service Experiment Station at Cowichan Lake. The 4,6,6-lineatin was formulated in benzene and placed in a vial-within-a-vial release system inside 0.3-m^2, cylin-



SCHEME 1



SCHEME 2



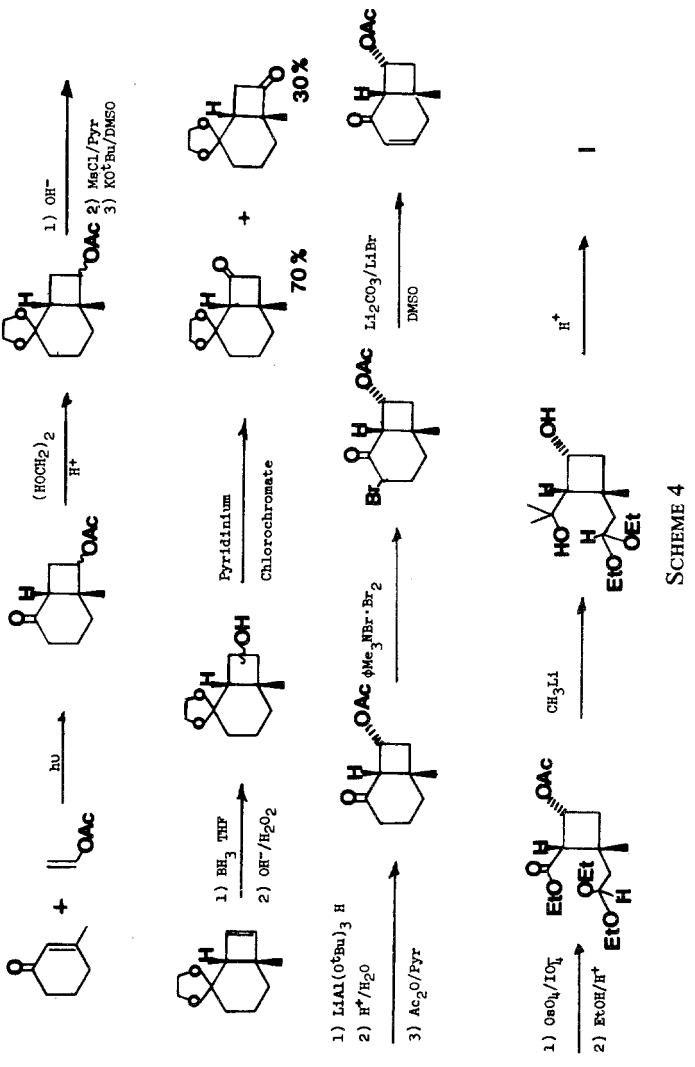
SCHEME 3

drical, wire-mesh traps coated with Stikem Special (Michel and Pelton, Manufacturing Chemists, Emeryville, California) (Byrne et al., 1974). Amounts of 4,6,6-lineatin varied between 20 and 30 μg within the three experiments. Control traps were baited with benzene alone. The traps were placed at intervals of 40–50 m along service roads through the forest.

Experiments 1–3 (Table 1) tested 4,6,6-lineatin produced by syntheses 1–3, respectively. There were two experimental and two control replicates in experiment 1, and 5 replicates of each stimulus in experiments 2 and 3. The traps in experiment 1 were ordered in alternate treatment and control order. In experiments 2 and 3, the traps were randomly placed.

In experiment 1, the traps baited with synthetic 4,6,6-lineatin caught several *T. lineatum* in the first hour on April 28, 1976, indicating that a flight was in progress. Therefore, they were left in continuous operation. In experiments 2 and 3, the bait vials were opened during sunny weather and closed during cool periods and at night. However, one baited trap in experiment 2, left open inadvertently, caught 364 beetles during a brief warm period from May 6–8, 1978. The bait vials in the remaining traps were left open during the next warm period from May 18–20. All beetles removed from the traps were held in ethanol until they were sexed in the laboratory.

Lineatin from all three syntheses proved to be highly attractive to both sexes of *T. lineatum* (Table 1), thus biologically verifying the authenticity of the synthetic product. Moreover, these results constitute positive proof that the pheromone is the 4,6,6-isomer of lineatin (I), and strongly support the conclusion of MacConnell et al. (1977) that secondary attraction in *T. lineatum* results from a single-component pheromone. However, the enantiomeric composition is not known (compare Byrne et al., 1974).


hυ
1) BH3 THF
2) OH⁻/H2O2
Pyridinium
Chlorochromate
30 %
70 %
(HOCH2)2
H+
1) OH⁻
2) MsCl/Pyr
3) KO tBu/DMSO
1) LiAl(O tBu)3 H
2) H+/H2O
3) Ac2O/Pyr
ϕMe3NBr·Br2
Li2CO3/LiBr
DMSO
1) OsO4/IO4⁻
2) EtOH/H+
CH3Li
H+

SCHEME 4

TABLE 1. RESPONSE OF *Trypodendron lineatum* TO TRAPS BAITED WITH SYNTHETIC LINEATIN FROM 3 SYNTHESIS PATHWAYS, B.C. FOREST SERVICE, COWICHAN LAKE EXPERIMENT STATION

Experiment no. and dates	Stimulus description	No. replicates	Beetles captured			Sex ratio (♂:♀)
			Males	Females	Total	
Expt. 1, Apr. 28–May 6, 1976	Est. 23 μg synthetic lineatin in impure reaction mixture (scheme 1)	2	205	145	350	1.41
	Benzene control, 1.4 ml	2	0	1	1	
Expt. 2, May 2–20, 1978	Est. 20 μg synthetic lineatin in impure reaction mixture (scheme 2)	5	413	318	731	1.30
	Benzene control, 1 ml	5	0	0	0	
Expt. 3, May 2–20, 1978	Approx. 30 μg synthetic lineatin (scheme 3)	5	601	239	840	2.51
	Benzene control, 1 ml	5	0	0	0	

Because of the extremely small amounts of pheromone available, no estimates of the release rates were made. However, weather records indicated approximately three "good" flight days for each experiment in which temperatures rose well above the 16°C flight threshold (Chapman and Kinghorn, 1958). If all of a 30-μg bait evaporated over a 3-day period, the mean release rate would be 0.4 μg/hr. A high response, such as in Table 1, to a pheromone released at such a low rate is indicative of an extremely high potency, particularly when the experimental forest site is considered. There were no large populations of overwintering beetles that might have been encountered in the proximity of active logging sites or timber processing areas. Rather, there appeared to be a relatively small endemic population, maintained in periodically windthrown trees. The result in experiment 1 is even more striking when one considers that, in the same forest, no response occurred during the experimental period to glass barrier traps baited with ethanol and/or α-pinene—baits that attracted numerous beetles at Port Renfrew, 32 km distant (Nijholt and Schonherr, 1976).

An early attempt to utilize 4,6,6-lineatin in a pest management pro-

gram is definitely warranted because of the continued economic importance of *T. lineatum* (Dobie, 1978; Nijholt, 1978), the high aggregation response of both sexes to synthetic lineatin (Table 1), and the success of a pheromone-based pest management program on another British Columbia ambrosia beetle (McLean and Borden, 1977, 1978).

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DEFENSIVE REACTION TIME OF BOMBARDIER BEETLES
An Investigation of the Speed of a Chemical Defense

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Abstract—Legs of bombardier beetles (*Brachinus* spp.) were pinched with forceps which were closed electromagnetically. The timing of resulting defensive discharges was recorded. The shortest latency between stimulus onset and chemical response was 52 msec; when responses were grouped in 10-msec intervals, the mode occurred at 70–80 msec. These values are similar to latencies for evasive antipredator responses reported for a variety of species. This result indicates: (1) response latencies for chemical defenses are not intrinsically longer than latencies for evasive responses, and (2) they are not necessarily lengthened by selective pressures unique to chemical defense.

Key Words—Chemical defense, reaction time, Coleoptera, Carabidae, bombardier beetles.

INTRODUCTION

Antipredator evasive responses are of special interest to biologists because of their speed and simplicity. Much of the work on these responses has been that of neurophysiologists hoping to find a simple neural network responsible for behavior (e.g., Horridge, 1959; M.B.V. Roberts, 1962; A. Roberts, 1968). Less effort has been directed towards interpreting the importance of speed in fulfilling the biological function of the behavior—escaping from predators. A

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notable exception is the work of Roeder and his associates (Roeder, 1959, 1967b). Roeder's comparison of cockroach evasive response latencies and mantid strike speed, although the two species are not a natural predator and prey combination, led him to the conclusion that coevolution of predators and prey has pushed the speed of both attack and defense behaviors to limits set by constraints of neural organisation and optimal allocation of resources.

A second antipredator strategy particularly prevalent among arthropods is that of chemical defense. Although much work has been devoted to elucidating the bewildering variety of chemicals and glands used for chemical defense (for a review see Eisner, 1970), the speed of such defenses has not been previously investigated. As Eisner points out, chemical defense as a category subsumes two types of defensive strategies differing not only in the type of substance employed but also in temporal characteristics and in consequences for an individual attacked by a predator. Defensive substances such as emetics and toxins often require minutes to hours to become effective; typically they are dispersed throughout the body of the insect so that application to the predator occurs as the prey is injured. These defenses generally do not save the individual under attack but act indirectly to confer advantage on the responsible genes. Repellents, on the other hand, act to protect the individual and therefore must be effective before the predator can inflict serious injury. The defensive substance is stored in specialized glands, often with elaborate provisions for deployment against predators. Repellent defenses are analogous to evasive responses in that speed is critical.

The bombardier beetle defense is a well-studied example of a repellent discharge (Schildknecht, 1957; Eisner, 1958; Schildknecht and Holoubek, 1961; Aneshansley et al., 1969). The repellent is a quinonoid spray ejected from gland openings at the tip of the abdomen. The discharge is unusual in that it is simultaneously produced and ejected by a tiny, explosive chemical reaction resulting in a hot, high-velocity discharge. Beetles typically discharge in response to mechanical disturbance. The resultant spray is effective in repelling most predators that have been tested. In the experiment reported here, the latency of the beetle's chemical response to short pinches delivered to a leg was measured in order to estimate the speed with which a beetle can respond to attack.

METHODS AND MATERIALS

Bombardier beetles (*Brachinus* spp.) collected locally around Ithaca, New York, were maintained in the laboratory in small plastic boxes provided with a layer of sand, a folded piece of paper towel as a refuge, and a small moist sponge cube. Freshly chopped pieces of mealworm or cockroach were provided three times a week. A small aluminum hook was attached with wax to the elytra to allow the beetles to be handled without eliciting discharges.

For testing, beetles were restrained by firmly inserting their aluminum hooks into a short piece of plastic tubing fixed to a metal rod. The tips of electromagnetically closable forceps were manually positioned around a leg. When the beetle was standing quietly the magnet was turned on, closing the forceps and delivering a pinch. Pinches were directed both at the tibia and at the femur of meta- and mesothoracic legs. During an experimental series, each beetle was stimulated repeatedly with frequent alternation between legs until the beetle either failed to respond or produced only small, faintly audible discharges; this testing of a beetle to exhaustion defined a single beetle-test. The intervals between successive stimuli varied greatly due to the time needed to position the forceps and allow the beetle to become still but the shortest interval was more than several seconds.

The stimulus forceps were constructed from 1-mm-thick sheet metal filed to 0.5 mm at the tips to eliminate injuries to the beetles. The breadth of the forceps tips was 1 mm, and the maximum spread between them was restricted to 1 mm by a mechanical stop. Two electromagnets were made by winding metal sewing machine bobbins with magnet wire (Lafayette Radio Electronics; total resistance of one magnet equaled 26 ohms). The magnets were mounted on bolts in one arm of the forceps. One magnet alone proved sufficient to stimulate beetles without injuring their legs. It was powered by a 7 V DC power supply [Kepco PAR-7(c) regulated DC supply, output voltage 7 V $\pm$ 0.01% over 0–10 amps]. Digibit logic circuits (BRS/LVE, a Division of Tech Serv, Inc.) controlling current to the electromagnet limited pinch duration to less than 50 msec. During tests, the forceps were held by the arm containing the magnets, and the beetle's leg was allowed to rest against the tip of this arm. Activating the magnet attracted the free arm of the forceps, closing it onto the beetle's leg. The onset of current to the magnet was recorded and used to calculate the beginning of stimulation.

Actual closing time and stimulus duration were calibrated by using a wire of appropriate diameter to mimic a beetle's leg (0.2–0.4 mm) and arranging a simple circuit so that closure of the forceps was signaled electrically. Measured by this calibration procedure, the delay between activation of the magnet and effective stimulus onset ranged in different experiments between 6 and 14 msec. Over the course of a single experiment, the delay varied less than 2 msec; within a group of successive calibration measurements, the standard deviation of the delay was typically less than 1 msec. Closing time was decreased by 1–2 msec by increasing the diameter of the wire used for calibration from 0.25 mm to 0.5 mm, corresponding to the difference in diameter between bombardier beetle tibias and the thickest part of their femurs.

The beetle's defensive discharges elicited by the pinches were timed using either a bead thermistor (VECO 31A7 2D1) to record the heat or a microphone (Jerrold Solid State Commander Professional Cap 25T) to record the

sound. The ability of the thermistor to clearly register response onset depended on its receiving sufficient heat from the spray, which in turn required that the thermistor be close to the gland opening and in the path of the spray. However, the spray direction of bombardier beetles is not fixed; the end of the abdomen is quite maneuverable, allowing the beetles to aim their spray at a predator attacking almost any part of their bodies (Eisner, 1958). In order to maximize the probability of spray reaching the thermistor during the experiments, the thermistor was placed inside the end of a slightly flattened plastic tube (Intramedic PE 320; vertical axis 1.9 mm, horizontal axis 2.7 mm) fitted loosely against the tip of the beetles abdomen. In addition, the thermistor was offset from the middle of the tube towards the side of the legs to be stimulated. However, as the tip of the abdomen was free to move, some audible discharges failed to hit the thermistor.

In later experimental series, a microphone was positioned within 5 cm of the beetle to enable the recording of discharges missing the thermistor and to corroborate the thermistor's accuracy. Although the microphone's response was not dependent on the direction of the spray, the sound of the forceps opening did mask any discharges occurring within 15-20 msec after the end of a pinch. For beetle discharges which caused a sharp onset signal in the thermistor, the microphone and thermistor measurements of response beginning agreed to within 1 msec.

Thermistor, microphone, and electromagnet signals were recorded on tape (Sangamo Data Cartridge) at 1 7/8 ips [frequency response: thermistor and electromagnet FM channels, DC to 624 Hz $\pm$ 0.5 db; microphone direct record channel, 300-7500 Hz $\pm$ 3db)] and displayed on a storage oscilloscope (Tektronix Series 5000). The interval between activation of the magnet and response by the beetle was measured either during the experiment or during playback of taped records. The actual response latency was determined by subtracting from this interval the time required for the forceps to close, as determined in a calibration series.

RESULTS

In 84 beetle-tests from 34 beetles, 437 discharges were recorded over 3 preliminary and 4 main experiments. The shortest latency was 52 msec; the longest was more than a second. Of all discharges 76% occurred within 200 msec of stimulation. Tabulated in 10-msec intervals, these responses show a single peak between 50 and 220 msec, with a single mode in the interval between 70 and 80 msec (Figure 1D).

Of the two pinch durations used in the main tests, long pinches (40-50 msec, Figure 1C) were found to be slightly more effective stimuli than short ones (22 msec, Figure 1B), in that the resultant distribution of response

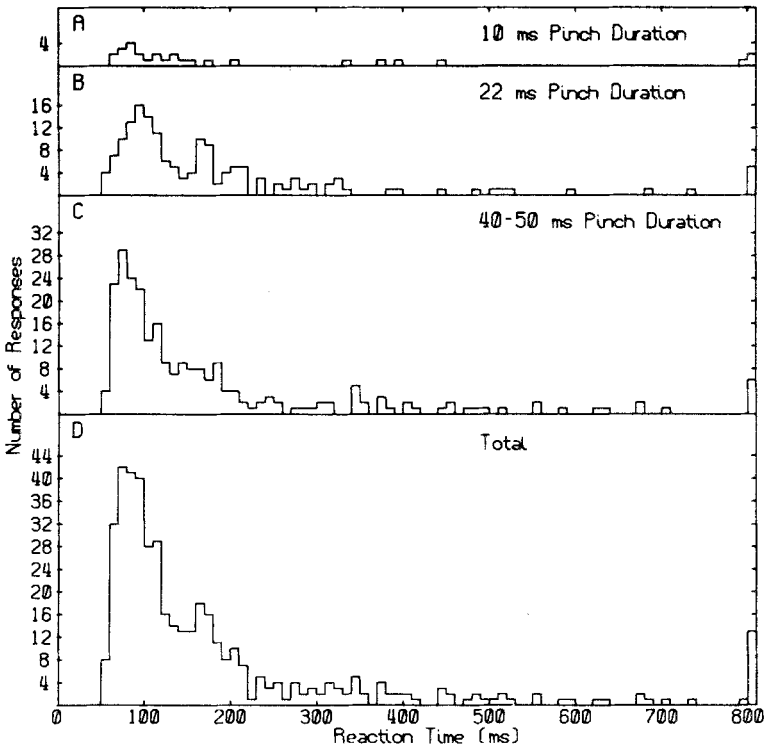

FIG. 1. Bombardier beetle reaction times: latencies from stimulus to response. Histograms plot number of responses in 10-msec intervals. Parts A, B, and C show response latency according to pinch duration. (A) 10-msec pinch duration: 29 responses from two preliminary experiments in which the pinches unintentionally injured the beetles' legs. (B) 22-msec pinch duration: 161 responses of which 15 were from a preliminary experiment in which the pinches unintentionally injured the beetles' legs. (C) 40- to 50-msec pinch duration: 247 responses. (D) All responses together.

latencies for long pinches was skewed more towards faster responses and the median response latency was 15 msec faster (115 vs. 130 msec, $P = 0.035$ one-tailed Mann Whitney U Test with z conversion).

Using the method of OD curves (Darlington, 1974), comparison of the response distributions for the two stimulus durations indicated that the significant difference covered response latencies between 76 and 108 msec: response latencies within this range were significantly more frequent for long than for short pinches (P less than 0.05, one-tailed). This period is slightly longer than the difference in stimulus duration 32 msec vs. 18-28 msec). It begins 54 msec after the divergence of the two stimulus conditions (76 msec minus the 22-msec duration of the short pinches): the beetle's shortest

reaction latency following stimulus cessation was of the same order as its latency following stimulus onset. The shortest latencies recorded under the two conditions were essentially identical—54 msec for long pinches vs. 52 msec for short ones.

The number of discharges produced by individual beetles in single tests ranged from 0 to over 40. Within any test, response latencies were not temporally ordered; that is, the first responses were not necessarily the fastest nor were the last responses necessarily the slowest. Nor was there any clear differentiation of the beetles into fast and slow responders: 75% of the beetle-tests elicited at least one response latency shorter than 100 msec (Figure 2D), while response times for an individual beetle in a single test typically varied by several hundred milliseconds. When pinched for 40-50 msec, 50% of the beetles responded in less than 75 msec to at least one pinch (Figure 2C).

In 32 cases, beetles discharged more than once to a single stimulus. The shortest interval between the onsets of individual discharges in such a multiple response was 22 msec. Eleven such intervals (29%) were less than 50 msec—the shortest latency between stimulus and first response, while 26 (68%) were less than 100 msec.

The stimuli used were strong ones capable of evoking maximally fast responses from the beetles. This is indicated by the fact that in preliminary experiments in which beetles' legs were unintentionally injured by the pinch-

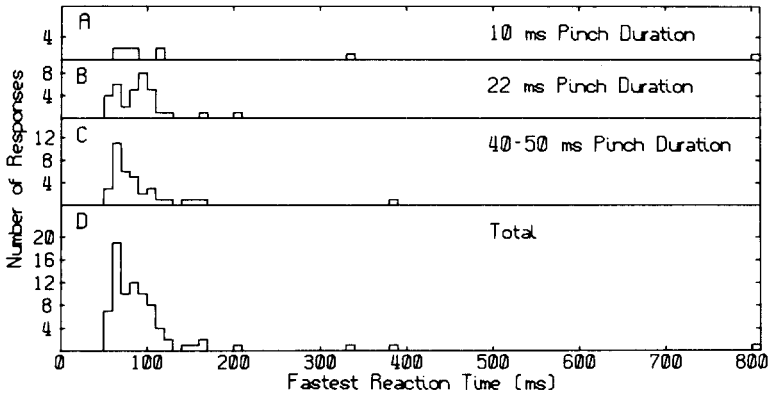

FIG. 2. Bombardier beetle reaction times: shortest latencies for each beetle-test. Histograms plot number of beetle-tests vs. shortest latency elicited. Parts A, B, and C show results according to pinch duration. (A) 10-msec pinch duration: 10 beetle-tests from two preliminary experiments in which pinches unintentionally injured beetles' legs. (B) 22-msec pinch duration: 34 beetle-tests of which 6 were from a preliminary experiment in which pinches unintentionally injured beetles' legs. (C) 40- to 50-msec pinch duration: 36 beetle-tests. (D) All beetle-tests grouped together. Not shown are 4 beetle-tests in which no discharges were elicited.

ing, the responses were no faster than those elicited during the main group of experiments in which no obvious injuries resulted. (These preliminary experiments included pinch durations of 10 msec, Figure 1A, as well as 22- and 40- to 50-msec pinches included in Figure 1B and C.)

DISCUSSION

The shortest response latency (52 msec) and the concentration of latencies in the interval between 52 and 125 msec are similar to latencies found for such other fast reactions as withdrawal, startle, and evasive responses (Figure 3A). The majority of these reactions show shortest latencies between 40 and 70 msec. These reaction times are similar to the time required for fast prey-catching behaviors (Figure 3B). This similarity among limiting values for fast behaviors of different species suggests that common neuromuscular limits have been approached. The use of a chemical repellent does not appear to introduce new speed contraints over and above the general limits characteristic of neuromuscular systems.

Chemical defenses have a unique limitation in that they are expendable and may require days to regenerate. As a result, the tactics evolved for repellent defenses differ from those for evasive responses. Chemical defenses typically are not employed before the prey is physically contacted by a predator and serious threat is imminent. The function of the discharge is to cause the predator to release the prey after capture and not, as in evasive responses, to avoid capture altogether. The crucial temporal factor for a prey possessing an effective repellent is the speed with which it is likely to receive crippling or fatal injuries. Although the speed with which insect predators injure their prey has not been studied directly, observation suggests that insect prey may quickly lose legs or receive fatal injections of venom. For example, a bombardier beetle introduced into an antlion trap repelled the larva but only after it had received a fatal amount of venom (Eisner, personal communication). Clearly, exposure to such predators can select for ever faster defensive responses.

Viewed from the perspective of a predator, the predator's injection of venom or its establishment of firm contact with the prey does not instantaneously accomplish its goal of subduing the prey. In general, one would not expect this second phase of predation—the subduing of captured prey—to approach the speed of the fast prey-capture behaviors (Figure 3B and C), if only because the configuration of predator and prey at the completion of capture cannot be completely controlled by the predator. This expectation is born out by studies of several rapid prey-catching behaviors (Figure 3D). Therefore, a prey with a rapid chemical defense should have sufficient time to employ it, and the predator may be repelled and lose the benefit of its

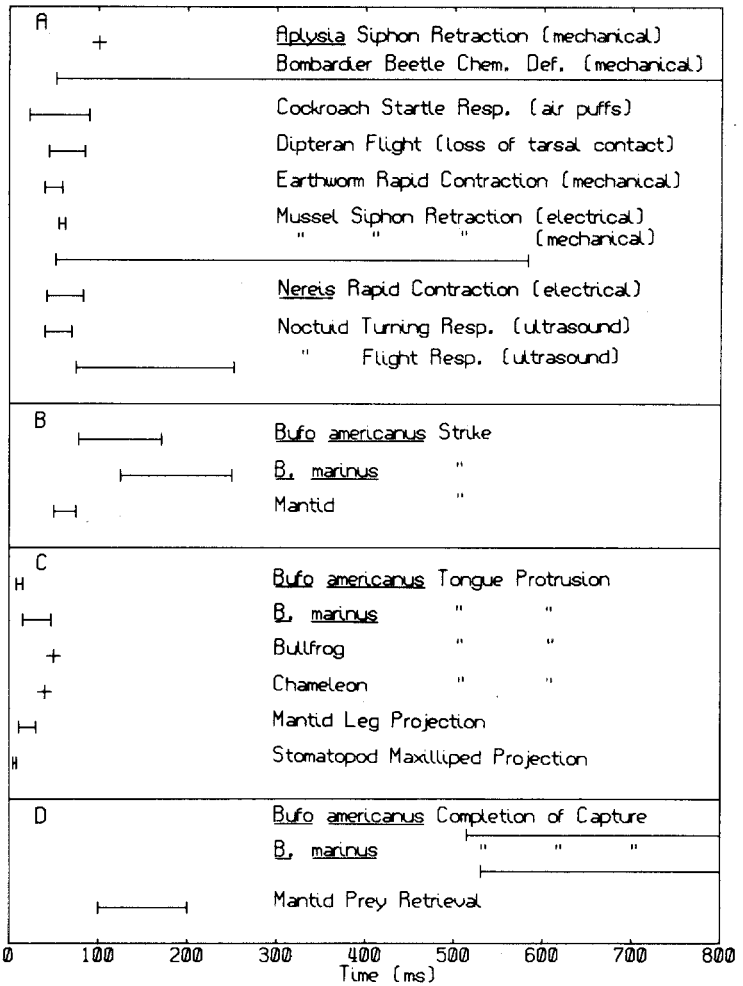

FIG. 3. Timing of various fast behaviors. Bars represent ranges; crosses indicate single reported values. (A) Latencies of defensive, evasive, and other fast responses. In parentheses is the stimulus used to elicit the reaction. References are, in order: Lubowiak and Jacklet, 1972; Dean, this paper; Roeder, 1959 (2×); Pieron, 1941 (2×); Horridge, 1959; Roeder, 1967a; Treat, 1956. (B) Complete predator attacks from initial motion to contact with prey. References: Dean, in press (2×); Rilling et al., 1959. (C) Duration of ballistic components of fast predator attacks. References: Dean, in press (2×); Gans, 1961; Gans, 1967; Rilling et al., 1959; Burrows, 1969. (D) Time to secure captured prey after initial contact. References: Dean, in press (2×); Rilling et al., 1959.

capture. A successful predator counterploy, illustrated by certain orb-weaving spiders (Eisner and Dean, 1976) and by toads (Dean, in press), is to avoid the direct contest of speed by attacking with a minimum of disturbance to the beetle so as not to elicit discharges until after the beetle has been immobilized or has been positioned so that the effects of the discharge will be mitigated.

The results discussed above concern only one aspect of the bombardier beetle chemical defense—the maximum speed with which it can be brought to bear on an attacker. The question of how quickly the defense can be reused was not directly tested, as the individual stimuli were delivered at intervals of at least several seconds. However, the fact that the intervals between discharges in a multiple response to a single stimulus can be as short as 22 msec shows that new discharges can be generated virtually instantaneously. Apart from the possible depletion of precursors, the mechanism of the bombardier beetle discharge introduces only a miniscule delay, if any, before the defense can be repeated. In natural encounters, this speed of repetition allows beetles to meet each new predator with a discharge. Moreover, the capability for multiple discharges serves as a means of adjusting the defensive effort in individual encounters to the severity of the threat.

The response speed itself may also reflect an adjustment to the level of threat, although the failure of responses to obviously damaging pinches to be uniformly fast shows that speed is not solely dependent on stimulus intensity. A more exact investigation of adjustment of response to the stimulus intensity requires a stimulus that is precisely defined both physically and temporally. In my experiments the geometry of contact between forceps and beetle leg could not be controlled with sufficient precision to accurately relate response speed and discharge duration to stimulus intensity.

Comparison of the results from long and short pinch durations indicates that the beetle's evaluation of the threat reflects stimulus duration in addition to stimulus intensity. The continued presence of the external stimulus is not required; in fact, all discharges in these experiments occurred after the end of the pinch. However, the stimuli used here potentially cause effects, such as cuticle deformation or tissue damage, which outlast the pinch itself. These effects may cause or contribute to excitation leading to the production of a discharge. Nevertheless, longer stimuli tend to produce faster responses.

In contrast to the absence of pronounced change in latency with repeated stimulation, the presence of changes in the threshold for responding was clear. Beetles discharged less and less often when pinched repeatedly in the same location and yet responded readily when pinched on another part of the same leg or on another leg.

Given the expendable character of chemical repellents, the accurate discrimination between threat and nonthreat must be as important to survival as

response speed. An animal must balance the risk that it may quickly suffer critical injury if the chemical response is slow against the risk that premature or inappropriate discharges may exhaust the defensive supply and leave the animal unprotected. For chemically protected arthropods, the first risk is decreased through the possession of additional defenses, such as thickly armored cuticle; thus the application of the defensive chemical may be more judicious. The bombardier beetle, in contrast, is unusual in the extent to which the second risk has been reduced through the storage of sufficient material to produce many discharges. This stockpile allows the beetle to risk faster responses: the bombardier beetle is one of only two cases in which chemically defended insects occasionally discharge before physical contact with a predator. [The other is a stick insect which presumably is rapidly injured by its avian predators (Eisner, 1965).] This heavy investment in the defense of the adult may in part compensate for the precariousness of the bombardier's existence in its parasitic larval stages. Part of this commitment to chemical defense is the speed with which it can be employed.

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TERMITE PREDATION BY *Megaponera foetens* (FAB.) (HYMENOPTERA: FORMICIDAE)

Coordination of Raids by Glandular Secretions

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Abstract—Termite predation by the ponerine ant, *Megaponera foetens*, is coordinated by chemicals from at least two glands. Columns of ants are guided to termite foraging areas by pheromones originating from the poison apparatus. On finding groups of termites, ants release alkyl sulfides (dimethyl disulfide and trisulfide) from their mandibular glands which attract sister workers who dig, into the termite galleries, in response to other unidentified mandibular gland pheromones.

Key Words—*Megaponera foetens*, Hymenoptera, Formicidae, chemical coordination, termite predation, dimethyl disulfide, mandibular gland.

INTRODUCTION

Megaponera foetens is a termitophagous ponerine ant with a specialized recruitment and foraging regime (Collart, 1927; Wheeler, 1936; Levieux, 1966; Fletcher, 1973; Longhurst and Howse, 1979). At Mokwa, Nigeria, the main prey items are the fungus-growing termites *Macrotermes bellicosus* and *Odontotermes* spp (Longhurst et al., 1978) which forage for food over an area of several square meters at some distance from their nests. The termites are sought by single major workers referred to as 'scout ants' (Fletcher, 1973). The scout ant, having located its prey from cues in the covering of soil sheeting constructed by foraging termites (Longhurst and Howse, 1978), returns to the nest where it recruits a column of sister workers to the foraging area of the termites. During the raid, the ants spread out and break open any soil sheeting constructed by the termites and then small groups of ants dig into

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the underlying termite galleries. After the raid has been completed, worker ants pick up termites in their mandibles and the column forms again and returns to the nest by the same route taken on the outward journey (Longhurst and Howse, 1979). In this paper the use of glandular secretions in coordinating this predation by *M. foetens* on termites is described. The *M. foetens* studies relate to observations made in the field which were followed by field and laboratory experiments.

Scout ants of *M. foetens* lay a scent trail which guides the ant column to the foraging termites, and the reinforcement of this trail then appears to guide ants back to the nest (Longhurst, 1978, Longhurst and Howse, 1979). The existence of such trails was demonstrated by Levieux (1966), who found that physical removal of the trails disorientated the ants and that the trail could be obliterated with kerosene but not with water. Levieux also indicated that, in the Ivory Coast where scout ants were not found in this species, the trails were very long-lasting, and he suggested that they were colony-specific. When the trails of two colonies crossed, a column followed only its own scent trail.

The source of recruitment trail pheromones in ponerine ants appears to differ in different species. The hind gut of *Termitopone laevigata* (Blum, 1966) and the poison gland of *Leptogenys* spp. (Fletcher, 1971) have been implicated as the source of trail pheromones in these two genera. In *Leptogenys ocellifera,* an Asiatic species which feeds mainly on earthworms and termites, the poison gland secretion also has orientating and recruiting effects (Maschwitz and Mühlenberg, 1975). In the related *Leptogenys chinensis,* Maschwitz and Schönegge (1977) have found an additional gland between the last and penultimate gastral segments. This dorsal gland has a secretion that is synergistic with that of the poison gland secretion in releasing orientation and recruitment and in coordinating nest moving. In this study the use of scent trails, their source in the ant, and longevity in both field and laboratory conditions were investigated in *M. foetens.*

A second possible use of chemical signals is during the attack of the recruited ants on the termites. After arriving at the termite foraging area, ants spread out over several square meters and break into the termites' foraging galleries which are protected by soil sheeting. Sister workers are attracted to points where single ants are digging and assist them; termites are usually retrieved from these excavations. In some cases the single ants are not in direct line-of-sight with the attracted ants, suggesting that nonvisual cues may be used. Pheromones which release attraction and digging behavior have been found in other ant species. In *Acanthomyops claviger n*-undecane and other *n*-alkanes from the Dufour's gland release attraction and "excitement" (Regnier and Wilson, 1968) and in *Oecophylla longinoda n*-hexanol, part of a multicomponent pheromone system, releases attraction (Bradshaw et al.,

1975). High concentrations of 4-methyl-3-heptanone from the mandibular glands of *Pogonomyrmex* spp. release alarm and digging (Wilson, 1958) and alkyl sulfides from the mandibular glands of *Paltothyreus tarsatus* release digging behavior when the pheromone source is buried (Crewe and Fletcher, 1974). In view of these findings studies were carried out on the role of pheromones in attraction and digging in *M. foetens*. The source and identity of pheromones was also investigated. To distinguish attraction and digging from alarm behavior seen in disturbed ant columns, the alarm-defense system of *M. foetens*, and some of the chemicals controlling this system, were investigated.

METHODS AND MATERIALS

Field Observations

Field observations were carried out in primary savanna woodland, located approximately 17 km north of Mokwa, Nigeria (9°18′ N, 5°5′ E; Wood et al., 1977; Collins, 1977).

Culture Conditions

Colonies of *M. foetens*, obtained from primary savanna woodland, were kept in large Plexiglas nest boxes with plaster of Paris bases (Longhurst, 1978). At Southampton University the relative humidity was 65-85%, temperature 24-29°C and a 12-hr light-12 hr dark cycle was maintained. At Mokwa experiments were carried out at ambient temperature and humidity under a natural light cycle of approximately 13 hr light-11 hr dark. The ants had access from their nest boxes to foraging arenas, 1×0.35 m at Southampton and 1.5 m^2 at Mokwa. The foraging arenas were covered with clean sand (Southampton) or sterilized topsoil (Mokwa). Bioassays were carried out at the beginning and end of the light regime when ants were foraging for termites (*Macrotermes bellicosus* or *Reticulitermes* spp.) supplied in petri dishes.

Coordination of Attacks on Termites

Exploring scout ants recruited sister workers to the termites. Because the distance between the petri dishes of termites and the nest entrance was less than 1 meter, a single discrete column of ants (Wheeler, 1936; Fletcher, 1973, Longhurst and Howse, 1979) was not observed, but a continuous column of ants formed, moving in both directions. Some ants would leave this two-way column and forage in the arena. It was these ants which encountered the experimental stimuli. Major workers were observed as they approached a

stimulus and their behavior noted. Distances were judged in relation to 1-mm-diameter metal stakes pushed into the soil (Mokwa) or marks in the sand (Southampton).

In order to study the effects of the ants' own secretions on sister workers, body components and excised glands of ants were crushed onto filter papers (13 mm diameter). Ants for assay were killed by plunging them into a bed of dry-ice and breaking off the head and gaster from the thorax. The body sections were then crushed with a clean spatula onto filter papers, and presented by placing the paper approximately 200 mm from the edge of the foraging ant column. Further tests were carried out on extracts of active glands which had been fractionated by micropreparative gas chromatography (Baker et al., 1976). Pure chemicals were also presented to the ants in a similar manner after allowing the dichloromethane solvent to evaporate. Both fractions and pure chemicals were presented at a level of 1 gland-equivalent (GE) of the component on the filter paper.

Pure chemicals were also presented to ants foraging naturally in primary savanna at Mokwa. Columns of ants were followed on their outward journey to sites where they attacked termites, and tubes containing the chemicals, or control tubes, were sunk into the soil about 200 mm to the side of the foraging trails. The reactions of the ants were then observed on their return journey. The chemicals for assay were injected into melting-point tubes (1 mm OD × 30 mm long, sealed at one end). When dimethyl disulfide and dimethyl trisulfide were tested, 5 μl was injected into each tube. For benzyl-methyl sulfide a solvent-free preparative technique was used (Longhurst 1978). The compound was eluted from the gas chromatograph (GC) into an open-ended tube (1 mm OD) surrounded by dry ice. The tube was sealed at both ends until needed for use, when one end was removed to leave a tube 30 mm long containing about 200 μg of the synthetic compound.

Alarm Behavior

Field and laboratory observations were made in order to distinguish alarm behavior from other chemically mediated behavior. Two states of alarm behavior were defined, preceded by an alerting stage. An alerted ant was in a state of arrest, with its antennae porrect, usually oriented towards the stimulus. Low-intensity (directional) alarm behavior was defined as that in which the ant stood with its antennae porrect and mandibles open. The ant sometimes approached the stimulus with its antennae and mandibles held in these positions. High-intensity (undirectional) alarm was characterized by the ants moving rapidly in all directions, often colliding with sister workers. Bursts of stridulation (Markl 1973) were often heard during this type of alarm. To investigate intraspecific alarm communication, ants were presented

with crushed gasters, Dufour's glands and poison glands, crushed on 13-mm-diameter filter papers.

The Dufour's gland was examined by GC (Figure 2) and some of the components were identified. *n*-Undecane and *n*-tridecane, two of the major components, were presented both to ants in culture and to foraging columns of ants using the methods described previously.

Trail-Following Behavior

Field Studies on Duration of Trails. In order to establish the duration of recruitment trails in the field, columns of ants foraging in the morning (Longhurst and Howse, 1979) were observed and the trails marked with wooden stakes (1 mm diameter) at 0.2-m intervals. Major workers were captured from the returning column of ants by trapping them separately in specimen tubes. At recorded times the ants were released onto the trail at least 15 m from the nest. The tube was inverted over the trail and left for at least 60 sec before the ant was released. It was then followed for at least 10 meters if it followed the trail or 300 sec if it lost the trail and entered the surrounding vegetation. A number of ants were also released 2 meters to the side of the trail and observed.

Laboratory Studies on Source of Trail Substances. Twenty major and twenty minor workers from cultures at Southampton were placed in traps made from 100 × 50-mm polythene containers, with a sliding door at one end. Extracts of various parts of major workers, killed by plunging in dry ice, were made in purified hexane at the following concentrations: 1 head + thorax in 500 μl hexane; 1 gaster in 500 μl; 1 Dufour's + poison gland on sting apparatus in 2 ml; 1 hind gut, removed by dissection in 1 ml; 1 poison gland in 2 ml; and 1 Dufour's gland in 1 ml.

The Dufour's and poison glands were separated by dissection under distilled water; any ruptured glands were rejected. The hind gut was taken as a piece of gut approximately 5 mm long, starting from a point as close to the anus as possible.

The extract (100 μl) to be tested was taken up in a Drummond microcapillary tube and streaked along a convoluted pencil line, about 250 mm long, on cartridge paper (300 × 200 mm). Presentations to sister workers were made so that any ant had to make a choice between two potential trails. Presentations were paired as follows: head + thorax with gaster; Dufour's + poison gland with solvent blank; hind gut with pencil blank; poison gland with hind gut and Dufour's gland with poison gland.

The start of the trail was made flush with the sliding door of the trap. Ants were released from the trap and scored as responding positively if they followed a trail for 200 mm from the door. A fresh piece of cartridge paper was

used for each ant, as observations suggested that ants could be laying trails of their own on the paper.

To compare trails laid by foraging ants with poison gland extract from sister workers a different experimental situation was used. Petri dishes of termites were placed under the traps used above and ants were allowed to forage to them and establish trails approximately 500 mm long. When the ants had finished foraging, 20 major and 20 minor workers from the same colony were placed in the trap which was replaced in its original position in the foraging arena. The natural trail was fairly wide (about 60 mm), but narrowed where it entered the trap, and was in a straight line between the trap and the nest entrance. The artificial trail was laid from the trap to the nest at one side of the natural trail, and was slightly longer than the latter. The ants were released from the trap and scored according to which trail they followed. No attempt was made to provide a new trail for each ant, but ten trials were made with one natural trail against 0.1 or 1.0 GE of poison gland extract. Statistical analysis, using the binomial test (Siegel, 1956), was carried out to compare the two trail stimuli presented.

Identification of Mandibular and Dufour's Gland Components

Identification was carried out by gas chromatography on 5% Carbowax 20M (A), 5% OV101 (B), 5% PPGA (C) and 10% E30 (D) 2-mm ID × 3-m all-glass columns with nitrogen carrier gas. Excised glands were examined using solid-sample gas chromatography (SSGC) (Morgan and Wadhams, 1973). Whole ant heads were examined using a modified Morgan-Wadhams solid sampler which allowed the use of biological material with a maximum diameter greater than 1 mm (Longhurst, 1978). Extracts of heads and excised Dufour's glands were made in purified dichloromethane which was reduced in volume under oxygen-free nitrogen for analytical work. GC-mass spectra were obtained on a MS 12 with a VG Digispec 16 data system calibrated with perfluorokerosene.

Synthesis

Benzylmethyl Sulfide. Sodium hydrogen sulfide reagent was made by the method of Khormandaryan and Brodovich (1930). Benzyl chloride was refluxed with a 50% ethanolic solution of the reagent (50 ml ethanol, 50 ml of aqueous NaSH solution) for 4 hr. The reaction mixture (75 ml) was added to saturated sodium hydroxide solution (100 ml). Dimethyl disulfide (50 ml, Koch Light) was added and the solution refluxed for three hours. The products were taken up in ether, and this extract was dried with magnesium sulfate. The benzylmethyl sulfide was purified by GC (column A) and the product trapped in cooled capillary tubes (Longhurst, 1978).

Dimethyl Trisulfide. Dimethyl disulfide (10g) was refluxed with sodium hydrogen sulfide solution (30 ml) in ethanol (30 ml) with the addition of sulfur (5 g) and ethylamine (5 ml). The reaction mixture was refluxed overnight, the products taken up in ether, and the extract dried with magnesium sulfide; the product was purified as above.

RESULTS

Identification of Exocrine Secretions

Mandibular Glands. Modified SSGC studies showed the presence of at least 13 compounds (Figure 1) and that the castes did not differ in the proportion or patterns of compounds. In the major workers, the most abundant component (B, Figure 1) was present at the level of only 20 ng/ant. SSGC-MS and GC-MS studies with solvent extracts produced the following information about the major mandibular gland components.

Component B was identified as dimethyl disulfide by comparison of its

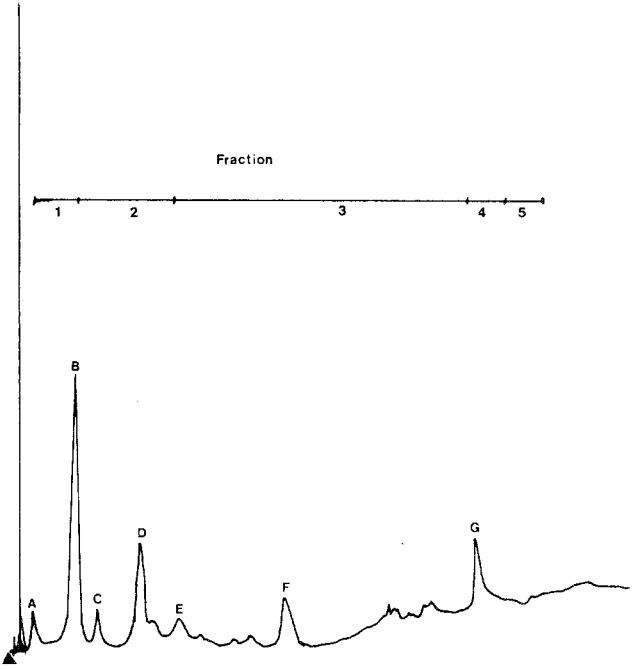

FIG. 1. Chromatogram of the mandibular gland secretion of a major worker of *Megaponera foetens*. 5% Carbowax 20M; 80–180°C at 6°/min.

mass spectrum with published spectra (Crewe and Fletcher, 1974) and co-elution with an authentic sample on GC columns A, C, and D.

The mass spectrum of component C revealed a base peak at m/e 108 (100%) which was also the molecular ion and large fragments at 80 (89), 45 (49), 47 (33), 76 (31), 94 (28), 79 (26). Fragments at m/e 109 (4) and 110 (11) suggested a sulfur-containing compound and the large fragment at 80 (89%) is probably from the loss of C_2H_4 from the molecule. No published mass spectra corresponding to the natural product could be found. The evidence points to a compound with the empirical formula $C_3H_8S_2$; the loss of the ethyl radical suggests $C_2H_5S_2CH_2$, ethylmethyl disulfide, as a possible structure.

The mass spectrum of component D corresponded to the published spectrum of dimethyl trisulfide (Crewe and Fletcher, 1974). A synthetic sample gave a mass spectrum congruent with, and that coeluted with, the natural product on GC columns A, C, and D.

Component F exhibited a molecular ion at m/e 138 (89%) with additional fragments at 139 (2%) and 140 (8%), characteristic of the isotopic ratio of sulfur. The base peak at m/e 91 ($C_7H_7^+$) is characteristic of an aromatic ring with a substituted methyl side chain ($C_6H_5CH_2X$). The loss of 47 from the molecular ion to form the base peak (91, 100%) suggests the loss of CH_3O_2 or CH_3S, the latter being most likely because the presence of sulfur was indicated by the isotopic ratios. A synthetic sample of benzyl-methyl sulfide gave a mass spectrum congruent with the natural product and coeluted on GC columns A, B, and D.

Dufour's gland. The identity of the compounds found in the Dufour's gland (Figure 2) are presented in Table 1. Chromatograms from *M. foetens* workers of all sizes exhibited similar proportions of components.

Poison Gland. Although crushed poison apparatus had a strong ester-like odor, only 10 minor components (1–2 ng, major workers) could be resolved by GC. Attempts at identification were not successful.

Alarm Behavior

Alarm behavior was defined in the Methods and Materials section. High-intensity (undirectional) alarm behavior was released by crushed gasters, crushed Dufour's glands, as well as two of the major components of this gland: *n*-undecane and *n*-tridecane.

Coordination of Attacks on Termites

Worker ants, when presented with crushed heads, turned towards them and then moved rapidly towards the stimulus. The ants surrounded the odor source, making biting movements with their mandibles into the sand and the filter papers, and digging into the sand with their forelegs. Two major be-

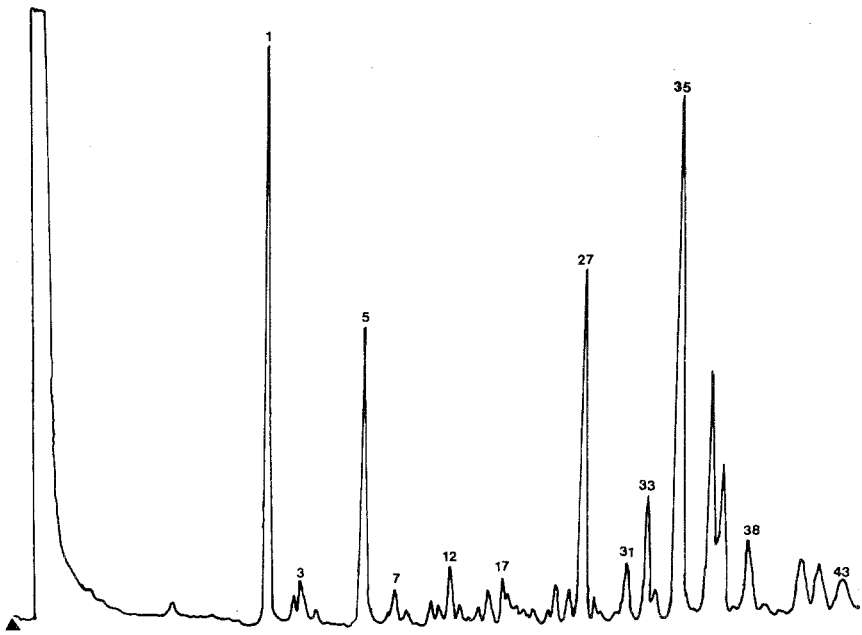

FIG. 2. Chromatogram of the Dufour's gland secretion of a major worker of *Megaponera foetens*. 5% OV101; 70-250°C at 8°/min.

havioral components were thus distinguishable—attraction and digging. Excised, crushed mandibular glands elicited the same reaction as crushed whole heads (Table 2).

Presentation of crushed thoraces elicited no reaction. Ants presented with crushed gasters were initially alerted, and then showed low-intensity alarm. A period of high-intensity alarm then followed. As this high-intensity alarm subsided (30–50 sec after presentation), a number of ants approached the odor source slowly and then stood around the crushed gaster, biting at it. Some of the ants remained with their mandibles fixed in the remains of the gaster or the filter paper for up to 60 sec. The rapid biting and stinging seen in reaction to some species of crushed termite soldiers (Longhurst, 1978) was not observed. The overall reaction to crushed gasters was a complex one, probably because the contents of more than one gland were released on crushing.

Of the reactions to crushed body sections, only the behavior elicited by crushed heads was similar to that seen in columns of ants after they spread out after locating the termite foraging area and commenced their attack on groups of termites. Ants were attracted to a sister worker digging into the soil at the entrance to the termite galleries, and then also began to dig. In view

TABLE 1. IDENTIFICATION OF SOME COMPONENTS IN DUFOUR'S GLAND OF *Megaponera foetens*[a]

Component No.	Identity	Method of identification[a]
A	*n*-Decane	LP,RT
1	*n*-Undecane	MS,LP,RT
4	*n*-Dodecane	LP,RT
5	*n*-Tridecane	MS,LP,RT
8	*n*-Tetradecene	LP
9	*n*-Tetradecane	MS,LP
12	*n*-Pentadecene	MS,LP
13	*n*-Pentadecane	MS,LP,RT
17	*n*-Hexadecene	MS,LP
18	*n*-Hexadecane	LP,RT
24	*n*-Heptadecene	LP
27	*n*-Heptadecane	MS,LP,RT
31	*n*-Nonadecene	MS,LP
33	*n*-Nonadecane	MS,LP
38	*n*-Heneicosene	LP
39	*n*-Heneicosane	MS,LP
41	*n*-Docosane	MS,LP
43	*n*-Tricosane	LP

[a]MS, mass spectrometry; LP, log plot; RT, relative retention time with standard.

of these observations the reactions of ants to crushed excised mandibular glands, and fractions prepared by GC (Figure 1) for these glands, were observed.

Crushed mandibular glands released the same behavior as did crushed heads. When fractions 1 and 2 (Figure 1) were presented worker ants orientated towards the odor source and then rapidly moved towards it. They did not stop at the odor source, but usually overshot by 30-50 mm, and then turned about and reorientated towards it. The ants eventually explored the odor source, moved away, but were usually attracted back towards it until 60-120 sec after their initial orientation to it. No reaction was observed to either fractions 3 or 5 (Figure 1). Worker ants encountering the treated filter papers showed no difference to ants encountering solvent-treated controls.

Ants encountering a filter paper, treated with fraction 4 (Figure 1), in the course of their explorations usually bit at the odor source and dug into the surrounding sand with their forelegs, in a similar manner to that observed in response to crushed heads. When fractions 1 and 4 were presented together, both attraction to and digging at the odor source were observed; this was the same as behavior observed to crushed heads.

Three of the known constituents of the mandibular glands were then bioassayed. Dimethyl disulfide and dimethyl trisulfide were present as the main components in active fractions 1 and 2, respectively, and benzylmethyl sulfide was present in inactive fraction 3. The reactions of ants to dimethyl disulfide and trisulfide, at a level of 100 ng/presentation, was the same as the reactions of ants to fractions 1 and 2, namely attraction to the odor source. Ants did not respond to benzylmethyl sulfide at a level of 50 ng/presentation. Dimethyl disulfide (20 ng), presented with 1 GE of fraction 4, released the same behavioral repertoire as crushed heads—attraction to the odor source followed by digging at and around the source. If dimethyl disulfide (on filter paper) was buried in the sand of the foraging arena, ants

TABLE 2. REACTIONS OF *Megaponera foetens* TO CRUSHED BODY SEGMENTS AND MANDIBULAR GLAND FRACTIONS AND COMPOUNDS FROM SISTER WORKERS.

Presentation	Concentration	No. of presentations	Behavior observed	No. of times behavior observed[a]
Crushed heads	1	10	Approach, digging	10
Crushed thoraces	1	10	No reaction	
Crushed gasters	1	10	Undirected alarm, followed by approach, arrested at source	10
Mandibular glands	1 pair	10	Approach, digging	10
Fraction[b]				
1	1 GE[f]	10	Approach	10
2	1 GE	10	Approach	8
3	1 GE	5	No reaction	
5	1 GE	5	No reaction	
4	1 GE	15	Digging	10
DMDS[c]	100 ng	10	Approach	9
DMTS[d]	100 ng	5	Approach	5
BMS[e]	50 ng	5	No reaction	
DMDS + fraction 4	20 ng + 1/2 GE	10	Approach, digging	7
DMDS buried in sand	100 ng	5	Approach	5

[a]At least one ant responding.
[b]Mandibular gland fractions (Figure 1).
[c]Dimethyl disulfide.
[d]Dimethyl trisulfide.
[e]Benzylmethyl sulfide.
[f]Gland equivalent.

responded in the same manner as they did to the compound presented on the surface. These results show that burying this alkyl sulfide does not release digging behavior in *M. foetens*, as it does in *Paltothyreus tarsatus* (Crewe and Fletcher, 1974). The reactions of *M. foetens* workers to mandibular gland contents are summarized in Table 2.

After the presentation of dimethyl disulfide to foraging ants, workers left the foraging column, oriented towards the stimulus, and then moved rapidly towards it. If the ants overshot the stimulus, they usually reoriented towards it. This behavior continued for up to 150 sec, after which the ants either located the trail again and rejoined the remainder of the column, or moved into the surrounding vegetation. On some occasions, especially when large numbers of ants were moving towards the odor source, high-intensity alarm occurred, with bursts of stridulation. The overall reaction to the disulfide was attraction, but the large numbers of ants involved in the interaction appears to have released alarm. In similar conditions, neither benzyl-methyl sulfide nor control tubes elicited any reaction.

TABLE 3. ABILITY OF MAJOR WORKERS OF *Megaponera foetens* TO FOLLOW NATURAL RECRUITMENT TRAILS LAID BY COLUMNS OF SISTER WORKERS DURING MORNING RAIDS ON TERMITES[a]

Hours elapsed since trail was laid	Number of major workers (out of 10) following the trail for at least 10 meters	Number of major workers (out of 5) which, after being released 2 meters to the side of the trail, moved in the direction of the trail for 10 m
0.25	9	0
0.5	8	
0.75	6	
1.0	7	1[b]
1.5	6	
2.0	5	
2.5	5	
3.0	1	0
3.5	0	
4.0	0	
4.0	0	
7.0	0	0

[a]Trails followed for at least 10 meters.
[b]Trail relocated and followed to the nest.

TABLE 4. TRAIL-FOLLOWING RESPONSES OF *Megaponera foetens* MAJOR AND MINOR WORKERS TO EXTRACTS OF PARTS OF THE BODY OF SISTER WORKERS

Extract[a]	No. of major workers (of 20)	No. of minor workers (of 20)
Head/thorax	4	2
Gaster	14	6
No trail followed	2	12
Dufour's/poison gland	13	6
Solvent blank	1	0
No trail followed	6	14
Hind gut	4	3
Pencil blank	0	0
No trail followed	16	7
Poison gland	17	10
Hind gut	2	4
No trail followed	1	6
Dufour's gland	3	6
Poison gland	15	11
No trail followed	2	3
Natural trail	12	12
0.1 GE poison gland	7	5
No trail followed	1	3
Natural trail	3	
1 GE poison gland	16	
No trail followed	1	

[a]For extract concentrations see Methods and Materials.

Trail-Following Behavior

Duration of Trail in Primary Savanna. The results of releasing major workers along existing trails of their own colony, at different time intervals after its origin, are presented in Table 3. Trails showed some activity for up to 3 hr, but their efficiency was greatly reduced after $1\frac{1}{2}$ hr.

Source of Trail Substance. Fourteen major workers followed the gaster extracts and four the head + thorax extracts (Table 4). Although these differences were not statistically significant ($P = 0.09$), further studies were carried out on gaster extracts because it was believed that the presence of alkyl sulfides in the head extracts, which released attraction, may have caused the ants to follow the head + thorax extract trails.

The Dufour's + poison gland complex proved to be a more powerful releaser of trail-following behavior in major workers than the hind gut ($P =$

0.01), and separate assays on these two glands showed that the poison gland was more active than the Dufour's gland ($P = 0.04$), suggesting that the former gland is the major source of trail pheromones in *M. foetens*. Comparison of the poison gland extract with the natural trail showed that at a level of 0.1 GE, 12 of 20 major workers followed the natural trail and 7 of 20 the poison gland extract ($P = 0.08$). At the higher presentation level of 1 GE, 16 ants followed the extract and 3 the natural trail ($P = 0.01$).

Minor workers were less competent at following artificial trails than major workers (Table 4). When the head + thorax extract was compared with the gaster, only 6 ants followed the latter, 2 ants followed the head + thorax extract, and 12 ants did not follow any trail at all. Comparison of different glands in the gaster did show that major worker poison gland extract was more potent than that of the Dufour's gland or hind gut. The results were not always statistically significant because of the large number of ants not following any trail at all.

DISCUSSION

The obligate termite predator, *M. foetens*, shows at least two refinements of its chemical communication system to allow it to act efficiently as a predator of foraging termites. Prevous authors (Levieux, 1966; Fletcher, 1973) have demonstrated the use of trails by *Megaponera*, and in this study the trail substance has been shown to originate in the poison gland. No attempt was made to identify the trail pheromone(s), although SSGC of excised poison glands did reveal the presence of more than 10 compounds, all at a level of a few nanograms per major worker.

In the related ponerines, *Leptogenys attenuata* and *L. nitida*, Fletcher (1971) has demonstrated that the source of the trail pheromone is also in the poison gland. In both *Leptogenys* species the trail pheromone is used in the coordination of emigration to new nests and in *L. nitida* is also used in the recruitment of ants to their isopod prey. In the termitophagous ponerine, *Termitopone laevigata*, the glandular source of the trail pheromone is the hind gut (Blum, 1966).

To allow efficient utilization of the foraging termite party, the column of ants must arrive as a group. An odor trail achieves the efficient guiding of the ants to the foraging termites where other chemical cues can complete the coordination of predation. The scent trails at Mokwa did not last more than 3 hr; which in an area where foraging occurs only in the morning and evening (Longhurst and Howse, 1979) may be of use in preventing confusion with the colonies' trails from previous raids. In the Ivory Coast, where the details of recruitment differ from Mokwa and a scout ant is not used, Levieux (1966) claims that the trails last for up to 24 hrs. It is possible that the different foraging pattern observed by Levieux has led to a lower threshold of detection

of the trail odors. The low level of response of minor workers to artificial trails may reflect the fact that this caste never needs to follow trails in the absence of major workers (Longhurst and Howse, 1979) and may stay with the foraging column by visual or tactile means.

Mandibular gland pheromones from ponerine ants have been found to be very diverse. In *Gnamtogenys pleurodon* methyl-6-methylsalicylate acts as an alarm pheromone (Duffield and Blum, 1975), as do 4-methyl-3-heptanone in *Neoponera villosa* (Duffield and Blum, 1973) and alkyl pyrazines in *Odontomachus*, *Hypoponera*, and *Ponera* species (Wheeler and Blum, 1973; Duffield et al., 1975; Longhurst et al., 1979). Dimethyl disulfide and trisulfide have been found previously in the ponerine *Paltothyreus tarsatus* (Crewe and Fletcher, 1974) where, like 4-methyl-3-heptanone in *Pogonomyrmex* spp. (Wilson, 1958) they act as releasers of digging behavior. By contrast, in *M. foetens* these two components are attractants, acting in conjunction with other unidentified mandibular gland components which release digging, as part of a multicomponent pheromone system. Field observations and tests support the interpretation of these alkyl sulfides as part of the predation system, and not just alarm attractants. After an ant has broken open the entrance to a termite foraging gallery, other ants move towards it from all directions. They then distribute themselves around the gallery entrance and commence to dig before any minor workers enter the galleries and predate the termites. This regular spacing does suggest that other pheromones—perhaps the digging pheromone in fraction 4—may be repellent at high concentrations or that other unknown stimuli may be involved.

Although the trail pheromone leads the ants to the termite foraging area, it does not locate ants at all the points were termites are foraging in the area. A second chemical system (attraction and digging) coordinates the final attack within the overall foraging area and allows a more efficient utilization of the prey. If each ant had to search out its prey, or if groups of ants had to be led to foraging termites by new recruitment trails, many termites might escape. If a number of ants can be concentrated at the entrance to the termite galleries, more prey can be secured if the retreating termites can be cut off before they reach the gallery entrance.

Some comment must be made on the different roles of dimethyl disulfide in *M. foetens* and *Paltothyreus tarsatus*. In the latter species Crewe and Fletcher (1974) found that the workers were ". . . indifferent to crushed worker heads and filter papers impregnated with [1 μl of] dimethyl disulphide," but that buried whole ants and buried filter papers treated with 0.1 μl of the disulfide elicited digging and rescue of ants or filter paper. The lack of response to unburied compound is difficult to explain; Crewe and Fletcher suggest that as *P. tarsatus*, unlike *M. foetens*, lacks stridulatory organs (Markl, 1973), the response is needed only to release sister workers trapped in nest falls. In *M. foetens* dimethyl disulfide has a completely dif-

ferent function, as an attractant in a multicomponent system with another less volatile component (fraction 4) that releases digging. The amounts of dimethyl disulfide and dimethyl trisulfide also differ markedly in each species. *P. tarsatus* contains 4.6 μg of the disulfide and 23.3 μg of the trisulfide, whereas *M. foetens* major workers contain 28 and 10 ng, respectively, of the two compounds.

Acknowledgments—This work was carried out in collaboration with the Termite Research Project sponsored by the Ministry of Overseas Development (U.K.) and Ahmadu-Bello University (Nigeria). C.L. was supported by a joint Centre for Overseas Pest Research/Science Research Council CASE award and is grateful for research facilities provided at Mokwa Agricultural Research Station (A.B.U.). We should also like to thank Drs. W.A. Sands, T.G. Wood and R.A. Johnson for helpful advice.

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(*Z*)-9-TETRADECEN-1-OL ACETATE

A Secondary Sex Pheromone of the Fall Armyworm, *Spodoptera frugiperda* (J.E. Smith)[1,2,3]

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Abstract—(*Z*)-9-Tetradecen-1-ol acetate [(*Z*)-9-TDA], identified originally as the sex pheromone of the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), acted as a secondary sex pheromone when it was tested in sticky traps in field tests. Low-level synergism was obtained when 2 and 10% quantities of (*Z*)-9-TDA were added to 100 μg of (*Z*)-9-dodecen-1-ol acetate, which is now considered the primary sex pheromone.

Key Words—Fall armyworm, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, pheromone, sex attractant, secondary sex pheromone, (*Z*)-9-tetradecen-1-ol acetate, (*Z*)-9-dodecen-1-ol acetate.

INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), an important pest of corn and other crops in the United States, has had two chemicals identified as its sex pheromone. Sekul and Sparks (1967) identified an isolate from abdominal tips of the female as (*Z*)-9-tetradecen-1-ol acetate [(*Z*)-9-TDA], and later they (Sekul and Sparks, 1976) identified (*Z*)-9-dodecen-1-ol acetate

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[(*Z*)-9-DDA] from the same source. The former chemical elicited wing fanning and copulatory behavior in the laboratory but subsequently proved to be inactive in the field. (*Z*)-9-DDA was later found to be an effective attractant (Sekul and Sparks, 1976; Tingle and Mitchell, 1975; Mitchell and Doolittle, 1976). As the role of (*Z*)-9-TDA in the reproductive behavior of this insect had not been determined, this study was designed to evaluate the synergestic or inhibitory effects of (*Z*)-9-TDA on the attractiveness of (*Z*)-9-DDA in the field.

METHODS AND MATERIALS

The chemicals used in this study were purchased from Chemical Samples Co. (Columbus, Ohio), purified by high-pressure liquid chromatography on a 6-mm × 15-cm silica column (5μ) impregnated with 20% silver nitrate, kept frozen, and discarded if not used within 1 week.

The material to be tested was placed on a dental wick in 2 ml of hexane. All wicks were prepared daily and were placed in Stikem®-coated pie-plate traps (Snow and Copeland, 1969) within 1 hr before sunset. The traps were arranged around corn fields in a randomized block design. Counts of trapped insects were made the following morning, and all insects and the test wicks were removed. When virgin females were used as bait, the traps contained 4 insects. Data were subjected to analysis of variance and Duncan's new multiple-range test.

RESULTS AND DISCUSSION

The first test was conducted in 1974 near Belle Glade, Florida, at a time of high populations of fall armyworm. Results are shown in Table 1. The mixture of 25 μg (*Z*)-9-TDA and 500 μg (*Z*)-9-DDA caught significantly more males than did the 500 μg (*Z*)-9-DDA alone. The increases in catch produced by the other mixtures were not significant. Addition of (*E*)-9-DDA to (*Z*)-9-DDA did not significantly increase catch.

Additional and more extensive testing of mixtures of (*Z*)-9-TDA and (*Z*)-9-DDA was conducted in two trials in corn fields in Tift County, Georgia, during July 1976. Results of these tests are shown in Table 2. Again, the addition of (*E*)-9-DDA to (*Z*)-9-DDA did not enhance the catch significantly. However, the addition of 2-10% (*Z*)-9-TDA to (*Z*)-9-DDA increased the attractiveness to males from two- to threefold.

In tests conducted in October 1976, a comparison was made of 100 μg (*Z*)-9-DDA and 100 μg (*Z*)-9-DDA + 10 μg (*Z*)-9-TDA. The results (16 replicates) showed that the synergized (*Z*)-9-DDA caught five times as many

TABLE 1. CAPTURE OF FALL ARMYWORM MALES IN TRAPS CONTAINING MIXTURES OF TWO PHEROMONES (BELLE GLADE, FLORIDA, NOVEMBER 1974)

Chemical (μg)		
(*Z*)-9-DDA	(*Z*)-9-TDA	No. males/trap/night[a]
500	0	62.50 bc
500	5	99.00 cd
500	25	103.25 d
500	50	87.25 cd
500	125	88.25 cd
0	125	5.50 a
Virgin females		36.50 ab

[a]Means not followed by the same letter are significantly different at the 0.05 level of probability.

TABLE 2. CAPTURE OF FALL ARMYWORM MALES IN TRAPS CONTAINING MIXTURES OF TWO PHEROMONES (TIFT COUNTY, GEORGIA, JULY 1976)[a]

	Chemical (μg)			
Trial	(*Z*)-9-DDA	(*Z*)-9-TDA	(*E*)-9-DDA	No. males/trap/night[b]
I	100	—	—	10.11 b
	100	2	—	14.06 bc
	100	10	—	18.94 c
	—	10	—	2.11 a
	Virgin female			12.50 bc
II	—	—	25	1.44 a
	—	25	—	2.56 ab
	50	—	—	3.99 ab
	100	—	—	6.22 abc
	200	—	—	4.33 abc
	500	—	—	9.22 abc
	1000	—	—	9.56 abc
	100	—	2	10.67 bcd
	100	—	10	6.44 abc
	100	—	25	9.44 abc
	100	2	—	17.89 de
	100	10	—	18.78 e
	100	25	—	13.89 cde
	Virgin female			17.94 de

[a]Trial I consisted of 18 replicates, and trial II consisted of 9 replicates.
[b]Means within trial not followed by the same letters are significantly different at the 0.05 level of probability.

males as the (*Z*)-9-DDA alone (28.6/trap per night to 5.3/trap per night). A *t* test showed this difference to be significant at 0.001 level of probability. Virgin female traps caught 15.2 males/trap per night. In this test new traps were put out each night, since earlier tests indicated that the effect of synergism declined rapidly with the age of trap. Apparently the Stikem absorbed the pheromone, and there was a gradual buildup of interfering chemicals.

The various tests therefore showed that (*Z*)-9-TDA synergizes (*Z*)-9-DDA but to a lesser degree (3–5×) than has been reported in the literature for the pheromone blends of some other insects. In addition, the ratio of (*Z*)-9-TDA to (*Z*)-9-DDA was not critical, whereas loss of activity has often been reported when a proper ratio of components was not used.

The significance of these findings becomes clear when we consider the nature of pheromone blends. Roelofs and Cardé (1977) divided sex pheromones into primary and secondary components: The primary component is responsible for the long-range upwind attraction of the male, and it may consist of more than one chemical. When that is the case, ratios are critical, and degrees of synergism are often infinite. The secondary component(s) is effective over a short range and is responsible for such activities as landing, fanning, and copulatory attempts. Thus, the addition of the secondary component to a pheromone preparation usually results in a low level of synergism, although this obviously depends heavily on trap design. If a trap entangles males in flight, then a very low level or perhaps no synergism will be observed. If a trap requires the male to land, walk, or crawl to become entangled, medium to high levels of synergism will be observed.

The roles of primary and secondary chemicals have been delineated for the redbanded leafroller *Argyrotaenia velutinana* (Walker) (Baker et al., 1976), the oriental fruit moth *Grapholitha molesta* (Busck), (Cardé et al., 1975), and *Spodoptera litura* (F.) (Tamaki and Yushima, 1974a; Nakamura, 1976). Also, Yushima (1976) determined that (*Z*)-9,(E)-11-tetradecadien-1-ol acetate elicited an upwind flying response (primary component) in *S. litura* and that (*Z*)-9,(*E*)-12-tetradecadien-1-ol acetate elicited a downwind, walking, crawling response (secondary component). The addition of this secondary component in amounts of 5–20% to the primary component increased trap catch fourfold for *Spodoptera littoralis* (Boisd.) (Tamaki and Yushima, 1974b).

Since (*Z*)-9-TDA is produced by the female, elicits wing fanning and copulatory movements in the male, and synergizes trap catches when it is added in uncritical amounts to (*Z*)-9-DDA, we conclude that (*Z*)-9-TDA is a secondary sex pheromone for the fall armyworm, *S. frugiperda.*

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IRIDODIALS AND NEPETALACTONE IN THE DEFENSIVE SECRETION OF THE COCONUT STICK INSECTS, *Graeffea crouani*

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Abstract—The defensive secretion of the coconut stick insect, *Graeffea crouani* Le Guillou (Phasmatodea: Phasmatidae) from the Pacific Islands has, as major constituents: *trans,trans*- and *trans,cis*-iridodials and nepetalactone. *Cis,trans*-iridodial is a minor constituent. A minor iridoid has yet to be identified. Male and female insects yield the same constituents.

Key Words—Coconut stick insect, defensive secretion, *Graeffea crouani*, iridodial, nepetalactone, gas chromatography-mass spectrometry, Phasmatodea, Phasmatidae.

INTRODUCTION

Cyclopentanoid monoterpene derivatives have been reported from a wide range of insect and plant sources (Cavill, 1969, and references therein). Of these, the dialdehydes iridodial (I) and dolichodial (II) are well-known constituents of insect defensive secretions. Iridodial has been reported as a major component of the defensive secretion of various species of ants (cf. Cavill, 1969), and beetles (Vidari et al., 1973; Bellas et al., 1974; Fish and Pattenden, 1975). Dolichodial has also been isolated from several species of ants, while anisomorphal (dolichodial) was obtained from a phasmid or stick insect (Meinwald et al., 1962). More recently dolichodial was isolated

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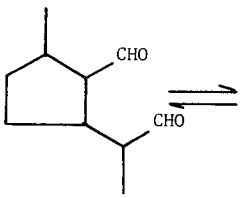
from a plant source, *Teucrium marum* (Pagnoni et al., 1976). The iridoid dialdehydes are structurally related to nepetalactone (III) (McElvain and Eisenbraun, 1955; Meinwald, 1954), the physiologically active principle of the catmint plant *Nepeta cataria* (Eisner, 1964), through the nepetalinic acids (IV, R = H), (Bates et al., 1958, and references therein).

The present paper reports on the chemistry of the defensive secretion of the coconut stick insect, *Graeffea crouani* Le Guillou (Phasmatodea: Phasmatidae), a widespread economic pest of coconut palms in the South Pacific region (Dumbleton, 1954; Swaine, 1971). The major constituents have been characterized as *trans,trans*-iridodial (VI),[4] *trans,cis*-iridodial (VII), and nepetalactone (III).



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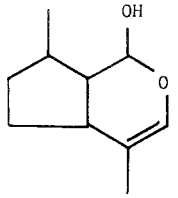

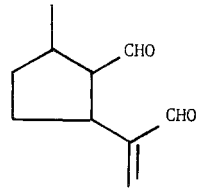

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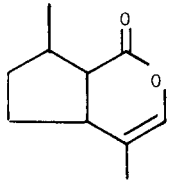

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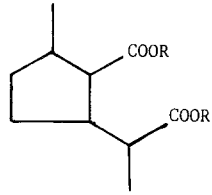

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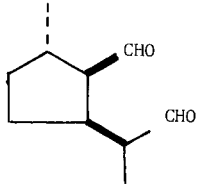

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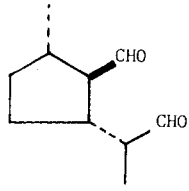

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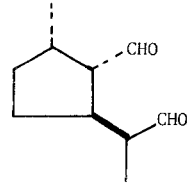
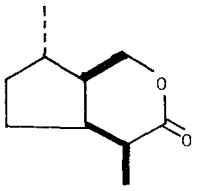


(VII)

[4]In the designation of configuration, the relationship of the propional moiety to the formyl group is given first, and that of the formyl to the methyl group second, see V, VI, and VII.



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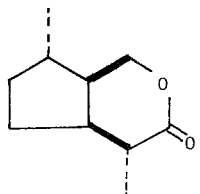

(IX)

METHODS AND MATERIALS

Isolation of Defensive Secretion of G. crouani. Samples of the secretion were collected by milking a colony of male and female stick insects, maintained on coconut fronds. The neck glands were squeezed to yield a milky secretion which was stored in sealed ampoules at −5°C; large females yielded up to 11 mg of secretion. When required, this neutral aqueous secretion was extracted with carbon tetrachloride.

Gas Chromatography and Mass Spectrometry. Analytical gas chromatography was carried out using a Perkin-Elmer F33 with a FID detector and fitted with glass columns (2 m × 3 mm) packed with: (1) 3% OV-101 on Gas Chrom Q, 80-100 mesh at 100° and 150°; and (2) 2.5% XE-60 on Chromosorb G, 80-100 mesh at 160°. Nitrogen was used as carrier gas (30 ml/min.).

Gas chromatography-mass spectrometry (GC-MS) (electron impact) was carried out using either a Shimadzu GC 6A, or a Varian 1740 gas chromatograph, with a flame ionization detector and helium as carrier gas. The GC was directly coupled to an AEI MS12 spectrometer using a straight-split separator. The MS was operated at 70 eV, ion source at 225°C. The following columns were used for GC-MS and for analytical purposes: (3) glass, 2 m × 3 mm, packed with 3% OV-1 on Gas Chrom Q, programed from 120° to 190° at 3° min^{-1}; (4) stainless steel, 2 m × 3 mm, 10% XE-60 on Chromosorb W at 145°; (5) glass 44 m × 0.5 mm Carbowax 20M, SCOT column at 160°; and (6) stainless steel, 2 m × 3 mm, 3% SE 30 on Chromosorb W at 140°.

Spectroscopy. Infrared and ultraviolet data were recorded on Perkin-Elmer 177 and Perkin-Elmer 402 instruments, respectively.

Reference Compounds. A mixture of *cis,trans-*, *trans, trans-*, and *trans,cis*-iridodials was synthesized by the method of Clark et al. (1959), starting from citronellal.

Derivatives. Oxidation of the total secretion was carried out using Jones' reagent, chromic acid in acetone (Bowden et al., 1946). The mixture of acids, when separated, was methylated with diazomethane.

RESULTS

The total secretion showed an absorption in the ultraviolet region, $\lambda_{max}(H_2O)$ 230 nm. The infrared spectrum showed $\nu_{max}(CCl_4)$ 2810, 2710, 1760, 1725, 1695, 1130 cm^{-1}, suggesting the presence of aldehyde and ester/lactone carbonyl groups.

On gas chromatography the secretion, and its extract in carbon tetrachloride, showed the presence of four volatile components (see Figure 1). Peaks 1, 2, and 3 represent major components, peak 4 was not observed in all samples. The retention times of the four peaks, on two columns, are consistent with these substances being monoterpenoids. There is no significant variation in the gas chromatographic data for the first three peaks between collections, or between samples of the secretion isolated from male and female phasmids.

After treatment with 2,4-dinitrophenylhydrazine, gas chromatography of the extract showed only peak 3 to be present. The carbonyl reagent has presumably formed derivatives with the components represented by peaks 1, 2, and 4. Purification by thin-layer chromatography and recrystallization yielded a yellow, crystalline 2,4-dinitrophenylhydrazone, mp 221–224°. The melting point was not depressed on admixture with a specimen of iridodial bis-2,4-dinitrophenylhydrazone, from *Iridomyrmex detectus* (Cavill et al., 1956).

Combined gas chromatography–mass spectrometry established that peaks 1 and 2 (Figure 1) are iridodials. Peak 1 shows M^+, m/e 168 (3%), and

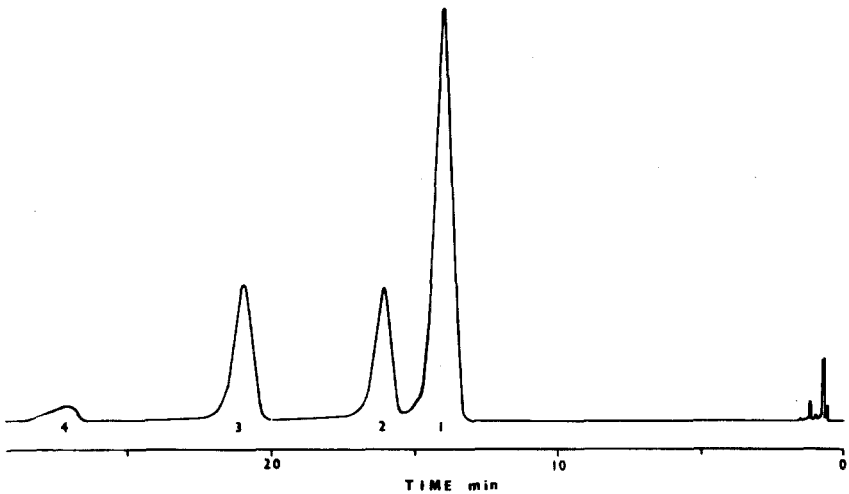

FIG. 1. Representative gas-liquid chromatogram of the fresh defensive secretion of *G. crouani*. Conditions column (1), isothermal 100°.

TABLE 1. LINEAR RETENTION INDICES OF IRIDODIALS AND DERIVED DIMETHYL NEPETALINATES

GC Column[a]	Iridodials of *G. crouani*	Reference iridodials: ex *I. nitidiceps*		Reference iridodials: Synthetic mixture		
		cis,trans-V	*trans,cis*-VII	*cis,trans*-V	*trans,trans*-VI[b]	*trans,cis*-VII
(3)	1246, 1271[c]	1235, 1247	1272	1238	1248	1272
(4) (Fig. 2)	1730, 1765, 1797[c]	1700, 1729	1757, 1804	1702, 1730	1766	1804

	Methylated oxidation Products of *G. crouani*	Reference dimethyl nepetalinates (IV, R = CH_3)		
		cis,trans-	*trans,trans*-[b]	*trans,cis*-[b]
(5)	1764, 1800, 1824	1759, 1781	1800	1818
(6)	1419, 1427, 1446	1412, 1430	1425	1442

[a]See Methods and Materials for description of column.
[b]Epimers in side chain not resolved.
[c]Nepetalactone (III) is also present LRI:1412 (3) and 1800 (4).

m/e 153 (3%), 150 (6), 135 (35), 111 (50), 109 (49), 95 (25), 93 (45), 81 (100), 71 (60), 67 (70), 58 (75), 55 (70), 43 (70), 41 (90). Peak 2 shows M^+, *m/e* 168 (2%), and *m/e* 153 (2%), 150 (6), 135 (25), 111 (60), 109 (45), 95 (25), 93 (35), 81 (100), 71 (70), 67 (70), 58 (60), 55 (50), 43 (70), 41 (90). These spectra closely correspond with that reported for iridodial, isolated from *Iridomyrmex nitidiceps* (Cavill et al., 1976).

Gas chromotographic comparisons and peak enhancement studies (see Table 1 and Figure 2) with authentic specimens of the *cis,trans*- and *trans,cis*-iridodials from *I. nitidiceps*, and with the synthetic *cis,trans*-V, *trans,trans*-VI and *trans,cis*-VII iridodials[5] show the presence of all three isomers in the defensive secretion of the coconut stick insect. Of these, the trans,trans isomer is the major constituent. The minor cis,trans isomer cochromatographs with *trans,trans*-iridodial on OV-101 (see Figure 1, peak 1).

Oxidation of the total secretion, using Jones' reagent, then methylation of the derived acids, gave a mixture of at least three methyl esters identified by GC-MS as dimethyl nepetalinates. Comparisons of linear retention indices with those for authentic specimens of the dimethyl nepetalinates (Cavill and McDonald, unpublished data) show the presence of the cis,trans-, trans,trans-

[5]Relative stereochemistry only is implied by these formulae. They are represented as corresponding in absolute configuration to the known iridoids of insect origin.

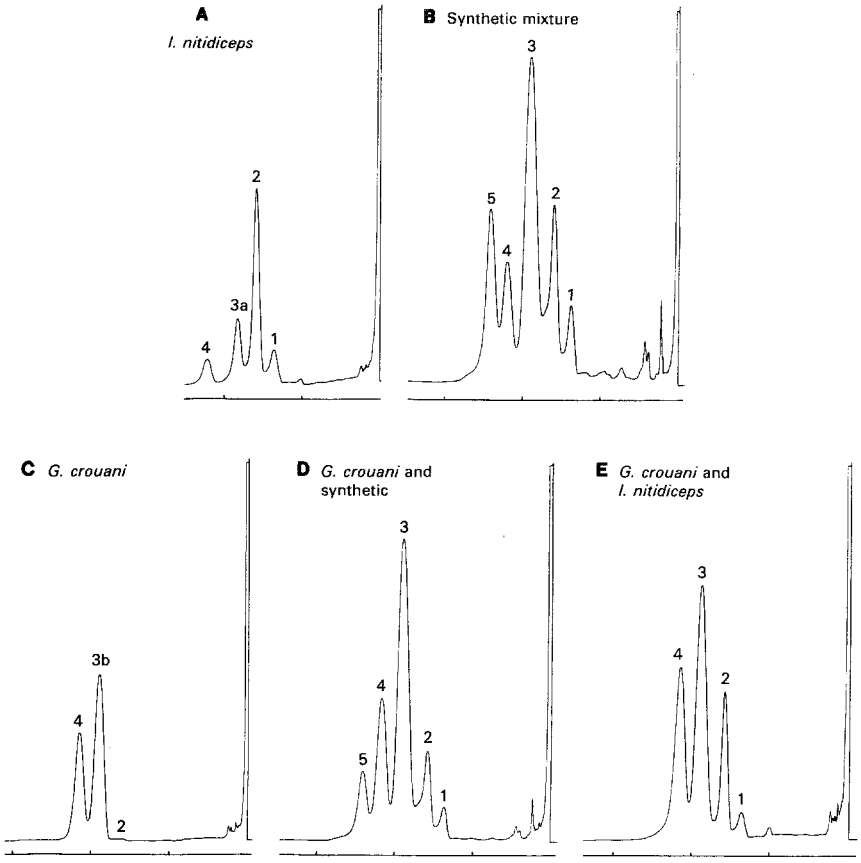

FIG. 2. Comparative gas-liquid chromatograms for the iridodials. Conditions column (4) isothermal 145°. Peak numbers correspond to the following iridodials: 1 and 2, epimers of cis,trans-; 3a and 4, epimers of trans,cis-; 3b, unresolved trans,trans epimers; 5, C_{10} acyclic dial. In 2C-E nepetalactone is also present in peak 4.

and trans,cis isomers (IV, R = CH_3) in the approximate ratio 1:30:10. Peak enhancement experiments confirm the presence of the two major isomers in the secretion from *G. crouani*. Methylation of the original secretion with diazomethane established that the acids were not present initially. Peaks 1 and 2 (Figure 1) are thus characterized as the *trans,trans-* plus *cis,trans-*, and the *trans,cis*-iridodials respectively.

Peak 3, on GC-MS, shows a strong molecular ion, M^+, *m/e* 166 (85%), and peaks at *m/e* 151 (10%), 138 (35), 137 (15), 123 (90), 121 (15), 110 (20), 109 (50), 107 (15), 95 (80), 81 (100), 69 (90), 67 (75), 55 (40), 53 (30), 43 (45), 41 (75). The spectrum differs from that reported for dolichodial (Cavill et al.,

1976), but closely corresponds to that of nepetalactone (Regnier, 1972). This assignment was confirmed by direct comparison of the above GC-MS data, and of linear retention indices on two columns, with that for an authentic specimen of nepetalactone from the catmint plant, *Nepeta cataria*. This nepetalactone was shown to contain the cis,trans and trans,cis-isomers (Bates and Sigel, 1963, and references therein).

The remaining compound, represented by peak 4 (Figure 1) is more polar. It did not react with diazomethane, nor was a derivative isolated on treatment with 2,4-dinitrophenylhydrazine. Peak 4, on GC-MS, does not show a molecular ion. The largest ion detected was at *m/e* 153 (35%), shown on high resolution to be $C_9H_{13}O_2$. Additional fragments were present at *m/e* 125 (13), 109 (5), 107 (5), 95 (13), 81 (30), 67 (12) and 43 (100%). This minor constituent would also appear to be an iridoid.

DISCUSSION

The defensive secretion of the coconut stick insect *G. crouani*, has as its major constituents the *trans,trans*-VI and *trans,cis*-VII iridodials and nepetalactone (III). The nepetalactone was shown to correspond, on gas chromatography, with naturally occuring nepetalactone from the catmint plant, *Nepeta cataria*, and hence is considered to be the cis,trans and/or trans,cis isomer (cf. Bates and Sigel, 1963). Only one species of a stick insect, *Anisomorpha buprestoides*, has been examined previously (Meinwald et al., 1962) from which anisomorphal (dolichodial) was characterized. Stereochemically anisomorphal has been shown to correspond to the trans,cis isomer of dolichodial (II) (Pagnoni et al., 1976).

Isolation of the known *trans,cis*-iridodial (VII), and a small proportion of the cis,trans-V isomer, from the defensive secretion of *G. crouani* is not unexpected in the light of the previous studies on *A. buprestoides*. The isolation of *trans,trans*-iridodial (VI), and nepetalactone (III), are reported for the first time from an insect source.

Recently *trans,trans*-dolichodial (type II) was characterized as a major constituent of the anal gland secretion of the Argentine ant, *Iridomyrmex humilis* (Cavill et al., 1976,) in this case in association with the known *cis,trans*-iridomyrmecin (VIII). The present isolation of three iridodials (V, VI, and VII) in association with nepetalactone (III) is consistent with the original biosynthetic scheme proposed for the iridoids (Clark et al., 1959; see also Cavill and Robertson, 1965). Nepetalactone may arise by enzymic oxidation of an enol-lactol tautomer of iridodial. An earlier attempt to achieve this oxidation using manganese dioxide in light petroleum was unsuccessful (Cavill and Ford, 1960).

Iridodial isomer variation has been reported for three species of

American dolichoderine ants (McGurk et al., 1968). Each of the three species studied contained more than 80% of a single cis,trans or trans,cis isomer, the remaining cis,trans- and trans,cis isomers being minor constituents. Two of the species also produced an iridolactone-iridomyrmecin (VIII) and isoiridomyrmecin (IX), respectively, of the same configuration as the major iridodial isomer. The biological significance, if any, of such iridodial isomer variation and of the present data for the predominant *trans,trans*- and *trans,cis*-iridodials in the coconut stick insect is not known.

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PERINEAL SCENT GLAND OF WILD AND DOMESTIC GUINEA PIGS

A Comparative Chemical and Behavioral Study

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Abstract—Wild and domestic male guinea pigs (*Cavia aperea* and *Cavia porcellus*) prefer the perineal secretion from males of the same species to that of males of the other species. Gas chromatographic-mass spectroscopic analyses of the volatile components of the secretions show complex mixtures comprised primarily of fatty acids, alcohols, and ketones. Interspecies differences in the composition of the volatiles are evident. The possible role of bacteria in odor production is discussed.

Key Words—Chemical communication, guinea pig, skin secretion, scent mark, *C. aperea*, *C. porcellus*.

INTRODUCTION

Many mammalian species exhibit scent-marking behaviors (Doty, 1979; Johnson, 1973; Ralls, 1971). In the domestic guinea pig (*Cavia porcellus*) both male and female animals draw or drag their anogenital area across a substrate leaving behind odorous substances. This behavior has been termed perineal drag. Males, who mark more frequently than females, place material from the well-developed perineal glands (Grosz, 1905) as well as urine on a substrate.

The perineal gland area is located in a pocket between the penis and

[5]Camille and Henry Dreyfus Teacher-Scholar, 1978–1983.

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the anus. Opening into the pocket are several sebaceous gland ducts. From these, sebum presumably flows into the pocket (Grosz, 1905). The pocket normally remains folded shut. However, during scent marking as well as when a male is engaged in courtship and aggressive activities, this area is exposed. At these times, perineal odors are either placed on a substrate or are exposed to surrounding animals.

Previous studies have demonstrated that in an established group of guinea pigs high-ranking males mark more frequently and produce more secretion than low-ranking males (Beauchamp, 1974). Although the stimuli which elicit marking are not thoroughly understood, observations on social groups suggest that marking frequently is high during periods of excitement (e.g., during sexual and/or aggressive encounters). Intact male guinea pigs mark more frequently on clean bedding or on bedding soiled by another male than on their own bedding, suggesting that chemical signals also influence frequency of marking (Beauchamp et al., 1977).

While the functional significance of scent marking in most mammalian species remains unclear (e.g., Daly, 1977), it is presumed that the deposited material serves to transmit information among conspecifics. This supposition raises several basic questions; for example (1) what kinds of information are available in the scent-marking material; (2) how do conspecifics make use of that information, and (3) what substances are responsible for information transfer?

It has been demonstrated that males discriminate between their own perineal gland secretions and those of other males, preferring to investigate the latter. This suggests that individual identity is coded within this material (Berüter et al., 1974). Other experiments (Beauchamp, unpublished), using a variation of the adaptation techniques (Schultz-Westrum 1965), further support this conclusion. Thus a male's own scent mark, or that of a known animal, could serve to identify an area as familiar, whereas the mark of a strange male could alert the male to the presence of a possible antagonist. Furthermore, although we have no experimental evidence as yet that females respond to perineal gland odors, it is possible that this material could play a role in female recognition of, and preference for, certain individual males (see Jacobs, 1976).

It has often been suggested that information on species or subspecies identity is contained in mammalian chemical signals. This information may serve to limit interspecies interactions and thus prevent interspecies mating (see Doty, 1973; Godfrey, 1958; Moore, 1965; Nevo et al., 1976; Beauchamp et al., 1979). Rood (1972) observed that in mixed groups of domestic and wild guinea pigs (*C. porcellus* and *C. aperea*) interspecies interactions were less frequent than intraspecies interactions, suggesting that the two species discriminate each other.

In the study reported here, we examined whether the constituents of perineal gland secretion provides information on the species (*C. aperea* or *C. porcellus*) of the donor. We predicted that conspecific secretion would be more attractive than heterospecific secretion. If this were the case, we then would want to examine whether there were observable differences in the chemical constituents of the secretions of the two species.

To this end, we have extended the chemical analysis of the volatile components of secretion from the domestic perineal gland (Berüter et al., 1974) to higher molecular weight (mol wt 200–400) components. In addition we have completed a comparative study of the volatile components of the perineal secretions of the wild species.

METHODS AND MATERIALS

Animals. All animals were sexually mature domestic (*Cavia porcellus*) and wild (*C. aperea*) male guinea pigs. The domestic animals were bred at the Monell Center. The wild animals were second- and third-generation offspring of animals trapped in Argentina in 1974. Animals used for perineal gland secretion collections were housed in individual wire-bottom cages ($65 \times 25 \times 18$ cm high). Test animals were housed in solid-bottom cages ($50 \times 55 \times 35$ cm high) with sawdust chips. All domestic test males ($N = 9$) and the majority of the wild test males ($N = 10$) had been weaned at three weeks and housed individually thereafter. All animals were fed Wayne guinea pig chow and water ad libitum.

Behavior Assay. Secretions were collected on cotton swabs from the perineal sacs of 4 domestic and wild animals. For behavioral analysis a small sample of the secretion from 2 animals of the domestic species was smeared (into an area of approximately 1 cm^2) on a clean glass plate (7.5×15.0 cm) and that from 2 wild animals on another plate. The plates were presented simultaneously to the animals in their home cages for 2.0 min, and the amount of the time the test animal spent with his nose within 1.0 cm of each sample was recorded. Each animal was tested twice with 48 hr separating the two tests. General observations of the test animals' behavior were recorded. (For a more complete description of the assay see Beauchamp, 1973, and Beauchamp and Berüter, 1973). For the behavioral studies employing sebum expressed directly from the gland duct, the following collection procedure was used: Four wild and four domestic males were anesthetized with halothan. The gland area was unfolded and cleaned three times with 95% ethanol on cotton swabs and then rinsed three times with H_2O. Sebum was then expressed from the ducts of the sebaceous glands lining the perineal pocket and collected on cotton swabs. The material was tested as described above.

Instrumentation. Gas chromatographic (GC) analyses were performed on a Perkin Elmer model 990 chromatograph equipped with a flame ionization detector. Gas chromatographic fractions were collected in glass capillary tubes (30 cm × 2 mm), utilizing a thermal gradient collector (Brownlee and Silverstein, 1968). Low-resolution mass spectra (MS) were obtained on a Hitachi/Perkin-Elmer RMU-6L mass spectrometer interfaced with a Perkin Elmer 990 gas chromatography via a Watson-Biemann separator (Watson and Biemann, 1965). All mass spectra were recorded at an ionization potential of 70 eV.

Reagents. Mallinckrodt nanograde solvents were used for all extractions. The NaOH pellets used to prepare solutions for extraction were preextracted with CH_2Cl_2 overnight in a soxhlet extractor and dried in a vacuum oven before dilution, Anhydrous Na_2SO_4 used for drying solvents was treated in the same manner.

Isolation of Perineal Secretions. The contents of the perineal sac were collected with a spatula by unfolding the gland pocket. The waxy material from 4 domestic animals was pooled and stored in a vial at −76°C until analysis. Material collected in a similar manner from 4 wild animals was also pooled and stored. Collections were made twice a week. The average amount of secretion obtained per collection was 45.7 and 20.8 mg/animal, respectively, for the domestic and wild guinea pigs.

Chemical Analysis of Volatiles of Pooled Sample of Perineal Secretion at 80°/0.2 torr. Approximately 1 g of secretion was accurately weighed into a round-bottom flask. Hexadecane and *t*-butylacetic acid were added as internal standards. The mixture was diluted with CH_2Cl_2 and distilled (80°C at 0.2 torr) for 5 hr. The volatile components were collected at −75°C and then the distillate was dissolved in 5 ml of 1.0 N NaOH and extracted into CH_2Cl_2 with a micro continuous extractor. The CH_2Cl_2 extract was dried over Na_2SO_4 and carefully concentrated in preparation for GC analysis. The neutral components were analyzed with a 1/8-in. (OD) × 10-ft 2% Carbowax 20 M column operated at He flow of 25 ml/min and at 100°C for 4 min, followed by temperature programing to 230°C at 3°C/min. Acidification of the aqueous layer of the extraction liberated the volatile organic acids in the secretion which were then extracted into CH_2Cl_2 with a micro continuous extractor. This CH_2Cl_2 extract was dried over Na_2SO_4 and concentrated. The small organic acids (C_1 to C_9) were analyzed directly by GC with a 1/8-in. × 10-ft 2% Carbowax 20M column at 100°C for 4 min, temperature programed to 230°C at 3°C/min, He flow 25 ml/min.

Structural assignments were tentatively based on mass spectral data and confirmed by comparison of GC retention times and mass spectral data with authentic samples for components in which the concentration was greater than 0.050 mg/g. Location of the position of unsaturation in several of the unsaturated alcohols were determined by microozonolysis of their *t*-butyl-

dimethlysilyl ether derivatives. The alcohols were purified by GC with a 2% Carbowax 20 M column and derivatized by reacting with 2 μl of *t*-butyldimethylsilyl chloride-imidazole reagent in DMF (Applied Science) (Corey and Venkateswarlu, 1972). The ethers were diluted with 50 μl CH_2Cl_2 and microozonolyzed as described by Beroza and Bierl (1966, 1967). The resulting silyl ether aldehydes were analyzed by GC/MS on a 1/8-in. × 10-ft OV 101 column operated at the flow 25 ml/min and at 90°C for a min followed by temperature programing to 230°C at 3°C/min.

Quantitative Analysis of Samples from Individual Animals. Approximately 15 mg of perineal secretion collected from one animal was accurately weighed into a 5-ml pear-shaped flask and 50 μl of hexane were added. The resulting mixture was stirred on a vortex stirrer. Utilizing the method of Zlatkis et al. (1973), the flask was attached to a nitrogen line and to a preconditioned 6 cm × 3 mm (OD) stainless-steel tube filled with Tenax (Applied Science Laboratory, Inc.). The volatiles from the secretion were collected on Tenax with a nitrogen flow of 60 ml/min for 2 hr as the flask was maintained at 25°C or 80°C. The collected material was transferred from the Tenax tube to a 1/8-in. × 10-ft 2% Carbowax 20 M chromatographic column by heating the Tenax tube to 200°C at 35°/min and held at 200° for 10 min. The first 10-cm section of the GC column was cooled to −76°C. The volatiles were eluted by heating the GC column to 85°C for 4 min followed by temperature programing at 3°/min to 220°C. Constituents were identified by GC/MS. For quantitative analysis, aliquots of standard solutions of tetradecane and *t*-butylacetic acid were added before vortex stirring and collection. GC response factors were determined with a standard solution of tetradecane, 9-decen-1-ol, 1-decanol, 2-pentadecanone, 2-piperidon, *p*-cresol, *p*-ethylphenol, and indole. It was assumed that all unsaturated alcohols, saturated alcohols, and ketones would have response factors equal to those determined for the representative of each group. GC response factors for the acids were determined with a standard solutions of acetic, propanoic, isobutyric, butyric, isovaleric, valeric, 4-methylvaleric, hexanoic, *t*-butylacetic acids and phenol.

RESULTS

Behavioral Tests. Males of both species discriminated between wild and domestic gland secretions and preferred those of their own type (Figure 1). After the data were transformed ($\sqrt{x+1}$), a two-factor analysis of variance (test animal type × donor type) with repeated measures indicated that only the interaction was significant ($F = 15.04$; $df = 1, 17$; $P < 0.01$). Simple effects analyses showed that wild males preferred wild male secretions ($F = 10.53$; $df = 1, 17$; $P < 0.01$) while domestic males preferred domestic male secretions ($F = 4.93$; $df = 1, 17$; $P < 0.05$).

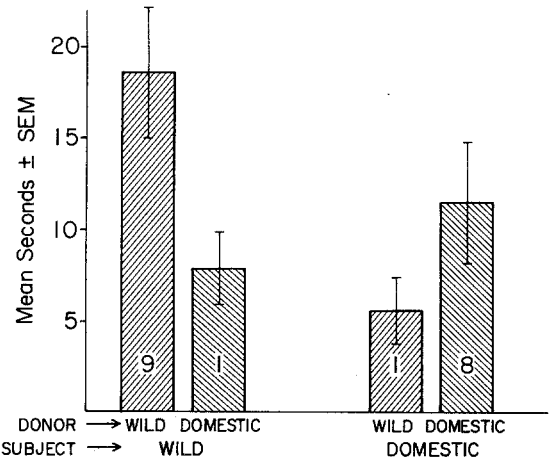

FIG. 1. Responses of wild and domestic male guinea pigs to perineal gland secretion. The height of the bars indicates the mean time spent investigating a sample during a 2-min choice test. As indicated by the numbers written in the bars, 9 of 10 wild subjects preferred wild secretion whereas 8 of 9 domestic subjects preferred domestic secretion. SEM = standard error of the mean.

A further series of choice tests was conducted to evaluate responses to gland material (secretion) collected in the perineal pocket compared to material collected by squeezing the gland area (sebum) after the gland pocket had been thoroughly cleaned. Presumably, the former is exposed to considerable bacterial activity whereas the latter material is reasonably free of metabolic action by resident microflora. Relatively large amounts (approximately 30 mg) of both secretion and sebum were obtained from 1 to 3 animals and tested in choice tests. Three choice tests were conducted with wild males and two with domestic males comparing wild sebum to domestic sebum (Table 1). Domestic males failed to demonstrate a preference, unlike the case where secretion was used (Figure 1). Wild males on the other hand did prefer wild sebum to that of domestic sebum, but the values were low, suggesting that sebum was much less attractive than secretion (Table 1). This was explored in the last two choice tests. Here, wild males demonstrated no preference for wild sebum compared to silicone oil. Furthermore, secretion from wild males was strongly preferred to sebum from wild males (Table 1).

Chemical Analysis. GC and GC/MS analysis of the acids in the volatiles collected from the pooled sample of domestic secretion showed the presence of acetic, propanic, isobutyric, butyric, isovaleric, 4-methylvaleric, and hexanoic acids as well as phenol. These short-chain aliphatic acids were also found in the volatiles of the wild secretion. 9-Decen-1-ol, 14-methylpentadecan-1-ol, 13-methyltetradecan-2-one, 2-pentadecanone, 15-methylhexadecan-2-one, 2-heptadecanone, 2-piperidone, and indole were identified

TABLE 1. RESPONSE OF DOMESTIC AND WILD MALE GUINEA PIGS TO PERINEAL GLAND MATERIAL

Subjects	No. of tests	Choice (X sec)	Probability[a]
Domestic males	2	domestic sebum (4.0) vs. wild sebum (3.2)	N.S.
Wild males	3	domestic sebum (3.6) vs. wild sebum (8.0)	<.025
	1	silicone oil (5.1) vs. wild sebum (4.7)	N.S.
	1	wild secretion (14.8) vs. wild sebum (5.6)	<.025

[a]As determined by Wilcoxon sign-rank matched-pairs test, 2 tail. For each test with domestic male subjects, $N = 9$. For each test with wild males, $N = 10$.

in the volatiles collected from the pooled sample of domestic perineal secretion.

The pooled sample of wild perineal secretion contained nonen-1-ol, 3-decen-1-ol, 1-decanol, 4-decen-1-ol, 9-decen-1-ol, decadienol, undecen-1-ol, (two isomers), dodecen-1-ol (six isomers), 2-undecanone, 3-dodecanone, 2-pentadecanone, 2-hexadecanone, 2-heptadecanone, 2-piperidone, and indole. The C_{12} unsaturated alcohols were not sufficiently separated by the packed Carbowax 20M column for GC/MS. A GC fraction which contained these alcohols was collected from the packed column, diluted with hexane, and the mixture analyzed by GC/MS on a Carbowax 20M glass capillary column (160 ft $\times$ 0.03 in.). Only C_{12} unsaturated alcohols were evident in the mass spectra.

The perineal secretions also contained a higher-molecular-weight ($M^+ = 346$) compound whose mass spectrum suggests a novel structure possessing an aromatic ring. Studies to elucidate the structure of this component (unknown C) are in progress and will be reported in due course.

Table 2 gives the quantitative results for the volatiles collected at 80°C for the perineal secretions of 4 individual wild males and 4 individual domestic males. Table 3 gives the concentration of the volatiles in the headspace over the wild and domestic secretions of the same individuals at room temperature. Due to losses in our colony, these are not the same animals used for the pooled samples, and differences in the composition of the secretions are evident.

DISCUSSION

The major volatile components of the perineal secretion of domestic and wild guinea pigs are listed in Table 2. This is the first reported analysis of the perneal secretion of the wild species *Cavia aperea*. In addition, this report extends the analysis of Berüter et al. (1974) for secretions from *C. porcellus* to

TABLE 2. VOLATILE COMPONENTS OF GUINEA PIG PERINEAL SECRETIONS COLLECTED AT 80°C

	Concentration[a] (mg/g secretion)							
	C. porcellus				*C. aperea*			
	D 732	D 804	D 827	D 723	W 10	W 334	W 344	W 57
Acids								
Acetic[b]	0.60		1.31	0.59		0.94		
Propanoic[b]	1.05	0.54	0.50	1.38	0.76			
Isobutyric[b]	1.01	0.69	1.96	0.82	1.30	0.30		0.30
Butyric[b]				1.06	1.41			
Isovaleric[b]	2.32	0.88	2.51	2.38	3.50	0.89	3.43	0.54
4-Methylvaleric[b]	0.53	0.38	2.00	0.084				
Hexanoic[b]			0.31	0.13				
Phenol[b]	0.13	0.069	0.13	0.095				
Neutrals								
Nonen-1-ol							1.08	
3-Decen-1-ol[c]					0.113	0.072	0.157	0.059
1-Decanol[b] + decen-1-ol	0.035				0.333	0.074	0.448	0.081
4-Decen-1-ol[c]					0.285	0.050	0.441	0.069
9-Decen-1-ol[c]		0.059			0.145		0.198	

Undeden-1-ol					0.106	0.035	0.221	0.031
Dodecen-1-ols (6 isomers)	0.043	0.108	0.068		0.843	0.177	1.24	0.257
Pentadecan-1-ol	0.030	0.030	0.055		0.093		0.099	0.031
Pentadecan-1-ol		0.066		0.047				
2-Undecanone[b]						0.039		
2-Pentadecanone[b]	0.065	0.073	0.039		0.162		0.310	0.034
2-Heptadecanone[b]	0.149	0.261	0.145	0.055	0.464	0.102	0.838	0.186
p-Cresol[b]					0.083		0.137	0.058
p-Ethylphenol[b]			0.039		0.241	0.038	0.113	0.056
2-Piperidone[b]	1.06	0.53	1.55	1.45	0.232		0.992	
Indole[b]	0.047		0.071	0.039	0.115		0.220	0.050
Unknown A[d] + *methylnaphthalene*[d]		0.037		0.036	0.091		0.121	0.035
Unknown B[d]					0.084		0.030	
Unknown C (aromatic)[d]	0.040	0.033			0.345	0.041	0.351	0.087
Unknown D[d]	0.073	0.036	0.094	0.044	0.091	0.053	0.286	0.050
Unknown E[d]	0.063	0.042						
Unknown F[d]	0.037	0.034	0.061					

[a]Compounds present at concentration > 0.030 mg/g secretion.
[b]Structures verified by comparison of GC retention times and mass spectra of authentic samples.
[c]Structure elucidated by GC/MS and microozonolysis. The assignment of 3-decen-1-ol is tentative.
[d]Unknowns were quantified based on a response factor = 1.

TABLE 3. CONCENTRATION OF VOLATILES IN HEADSPACE OVER GUINEA PIG PERINEAL SECRETIONS AT 25°C

	Concentration[a] (mg/g secretion)							
	C. porcellus				*C. aperea*			
	D 732	D 804	D 827	D 723	W 10	W 334	W 344	W 57
Acids								
Propanoic	0.081	0.025	0.035					
Isobutyric	0.199	0.069	0.117	0.072				
Butyric				0.129				
Isovaleric	0.434	0.081	0.189	0.437	0.057			
4-Methylvaleric	0.082							
Phenol[b]	0.024	0.019	0.033	0.046	0.055	0.022	0.054	0.012
Neutrals								
Nonen-1-ol					0.013	0.010	0.024	
Nonen-1-ol					0.013	0.011	0.011	
3-Decen-1-ol					0.024	0.042	0.022	
1-Decanol + decen-1-ol					0.049	0.051	0.116	0.021
4-Decen-1-ol					0.015	0.024	0.152	0.020
9-Decen-1-ol							0.028	
Dodecen-1-ols						0.013	0.018	
2-Undecanone					0.014	0.011		
2-Pentadecanone							0.011	
Methylnaphthalene							0.014	
Methylnaphthalene							0.016	
p-Cresol					0.032	0.020	0.012	0.016
p-Ethylphenol					0.011		0.012	

[a]Compounds present in headspace at concentration >0.010 mg/g secretion.
[b]Quantified based on tetradecane standard.

include the neutral components, the most abundant of which is 2-piperidone. The volatile acids isolated from the domestic perineal secretion in our study agree well with those reported by Berüter et al. (1974); quantitative differences are most likely due to differences in the acid profiles of individual animals and/or isolation techniques used. The volatiles of the secretions obtained from wild animals contain a series of short-chain aliphatic acids (C_2 to C_5), a series of saturated and unsaturated alcohols (C_9 to C_{15}), ketones (C_{11} to C_{17}), *p*-cresol, *p*-ethylphenol, 2-piperidone, and indole.

The secretions from wild and domestic animals differ primarily in the composition of the neutral components. Table 2 lists the concentrations of components present at greater than 0.030 mg/g secretion in the secretions of 4 individual wild males and 4 individual domestic males. Several components (3-decen-1-ol, 1-decanol and decen-1-ol, 4-decen-1-ol, undecen-1-ol, the dodecon-1-ols, and the unknown aromatic compound) were consistently detected in higher concentrations in the volatiles isolated from wild individuals. In addition, 4-methylvaleric acid and phenol were found in higher concentrations in the domestic secretions.

Coelution of several components complicates these results. The compounds 4-decen-1-ol, 4-methylvaleric acid, and 9-decen-1-ol all eluted within a period of 1/2 min under the GC conditions used. The same is true for phenol and one of the dodecen-1-ols. The mass spectra were examined carefully for traces of each compound. The wild samples appeared free of 4-methylvaleric acid but contained traces of phenol. One domestic sample contained traces of C_{10} unsaturated alcohol coeluting with 4-methylvaleric acid.

The total amount of volatile material isolated varied considerably from sample to sample. Therefore the concentration of each component was calculated relative to isovaleric acid to see if a consistent species pattern was present. Isovaleric acid was chosen because it was present in all samples, and its mean concentration was similar for both groups. 3-Decen-1-ol, 1-decanol and decen-1-ol, 4-decen-1-ol, undecen-1-ol, *p*-ethylphenol, and the unknown C (aromatic) had consistently higher relative concentrations in the wild samples. In addition, *2-piperidone*, 4-methylvaleric acid, and phenol showed consistently higher relative concentrations in all domestic samples.

The probability of there being no overlap in concentration of a substance in the wild and domestic animals is 0.028 (Mann-Whitney test, 2-tailed; Siegel, 1956). Therefore, by chance, less than 1 compound in 30 should exhibit no overlap. Thus the observed differences between wild and domestic samples cannot be attributed to random interanimal variations.

Under natural conditions, guinea pigs sniff perineal secretion which has been deposited in the environment as well as that in the anogenital region of other individuals (Beauchamp, 1974; Kunkel and Kunkel, 1964). With this fact in mind and with concern about possible changes in the secretion on

heating, we have analyzed the headspace above the perineal secretion at room temperature. The results of this analysis indicate even greater species differences than did the previous analyses; there was almost no overlap between the two species. The headspace above the perineal secretion isolated from 4 individual domestic males contains fatty acid and phenol, while the headspace above the secretion isolated from 4 individual wild males contains primarily neutral components (Table 3). The low amount of acids observed in the headspace above the wild secretion is somewhat suprising when one compares the room-temperature headspace data (Table 3) with the data collected at higher temperatures from the same animals (Table 2). One particular animal (number W 10) had a total acid concentration of 6.79 mg/g secretion (as determined at 80°C), which is higher than three of the four domestic animals, yet the total acid concentration in the headspace above this animal's secretion at 25°C is considerably less than all domestic animals.

For room temperature collections, the recovery of GC standard from a sample was reasonably consistent. For example, 3 samples from animal W 10 resulted in recovery of 16.1, 15.1, and 16.3% of tetradecane and 5.4, 6.0, and 3.7% of *t*-butylacetic acid. There was considerable variation in the percent recovery of standards between animals however. The recovery of *t*-butylacetic acid standard appears to be species dependent with a range from 12 to 33% for the domestic samples and <1% to 6% for the wild. To explain these somewhat peculiar results, we suggest that there is a weak base present in varying amounts in the perineal secretion. This base reacts with the added acid standard as well as the perineal acids to form salts which dissociate to the acids and base on heating. For example, vapor density studies of ammonium acetate indicate that the compound is completely dissociated to acetic acid and ammonia on vaporization (Rây and Jânâ, 1913). Under our collection conditions at 80°C, 2 mg of ammonium acetate are completely vaporized and acetic acid is trapped by the Tenax. Other glandular sacs have been found to contain both fatty acids and bases. Albone and Perry (1975) reported the presence of short-chain fatty acids, as well as putrescine, cadaverine, and ammonia in the anal sac of the red fox, *Vulpes vulpes*. They measured the pH of their aqueous samples and found that the total volatile acid composition was linearly dependent on pH. Thus the pH controls the odor of a given mixture. Studies are currently in progress in this laboratory to determine whether volatile bases are present in the perineal secretions of the guinea pig, and whether the concentration of these correlates with our acid recovery data at room temperature.

Our behavioral studies are in agreement with the chemical studies in that differences exist between gland secretions obtained from wild and domestic cavies. For example, wild males investigate wild secretion more than domestic secretion, while the converse is true for domestic males (Figure 1). This, and

parallel work with urine (Beauchamp et al. 1979), is the first demonstration of olfactory divergence with domestication. Analogous data have, however, been reported for closely related undomesticated species. (e.g., Doty, 1973; Godfrey, 1958; Moore, 1965; Nero et al., 1976). Behavioral studies in cavies by Rood (1972) indicated that wild and domestic animals could discriminate amongst each other when living in social groups; animals of the same species were much more likely to interact than animals of different types. Behavioral results reported here and elsewhere (Beauchamp et al., 1979) indicate that olfactory characteristics of secretions and excretions could account for much of this discriminatory behavior.

Studies comparing attractiveness of whole secretion with that of freshly expressed sebum suggest that microflora may play an important role in producing chemical communicants. Male subjects were much less attracted to fresh sebum than to secretion which had been allowed to build up in the gland pocket (Table 1). In fact, domestic males showed no preference for domestic over wild sebum, as would be expected; wild males, while showing a preference for wild sebum, actually were not very attracted to either sample. It is possible that this small preference could be due to incomplete cleaning of the gland area prior to sebum collection or to bacterial activity in the gland duct. Clearly (Table 1, comparison 4), the native secretion is more attractive than fresh sebum.

The differences in attractiveness (or differences in information content) cannot be attributed to the lack of a characteristic odor for sebum. To human observers both substances clearly have odor but with considerably different character. In addition, histological studies by Grosz (1905) and by us (unpublished) show only sebaceous glands present in the perineal area and no apocrine glands. Thus the attractiveness of the secretion is not due to mixing of attractants from other gland sources.

Preliminary tests indicate that there is considerable bacterial activity within the perineal pocket. Interestingly, several research groups have suggested that bacteria play an important role in the production of chemical communication (Albone, 1977). In particular Gorman et al., (1974) demonstrated that the acids present in the anal sacs of the Indian mongoose were the products of bacterial metabolism of sebum and apocrine secretions. In addition, Leon (1974) has shown that the material which attracts young rat pups to their lactating mother is the result of bacterial activity in the digestive tract of the mother. If bacteria do play a role in producing the substances which distinguish wild from domestic cavies, then two hypotheses could be put forth to account for the mechanism. The two species could differ in the types of bacteria found in the perineal region and/or the substrate on which the bacteria work could be species specific. Studies are currently in progress in our laboratories to evaluate these hypotheses.

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CHEMISTRY OF THE MANDIBULAR GLAND SECRETION OF THE INDIAN BEE *Pithitis smaragdula*

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Abstract—The mandibular glands of the stem-nesting Indian bee *Pithitis smaragdula* contain a mixture of salicylaldehyde, citronellyl acetate, geranyl acetate, pentadecane, heptadecane, ethyl tetradecanoate, and ethyl hexadecanoate. Salicylaldehyde is reported for the first time from a hymenopterous source. The secretion, emitted when the bee is disturbed, is rubbed with the legs over the bee's body and the disturbing object. The reactivity of some of the components in combination with the bee's behavior make the glandular exudate an effective defensive secretion.

Key Words—*Pithitis*, Hymenoptera, Ceratininae, mandibular glands, defensive secretion, salicylaldehyde, citronellyl acetate, geranyl acetate, hydrocarbons, ethyl hexadecanoate.

INTRODUCTION

Pithitis smaragdula (F.) is a small, green, polylectic, stem-nesting ceratinine native to southern Asia (Batra, 1976a, 1977) that has been successfully managed in India for alfalfa pollination (Atwal, 1970; Sandhu et al., 1976). This bee was first introduced into the United States in 1968, where it proved to be an efficient pollinator of alfalfa in greenhouses (Batra, 1976b). After

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an apparently unsuccessful attempt to establish it in California (Daly et al., 1971), it was recently reintroduced from Punjab, India, into Florida (Batra, 1979).

In nature, both sexes of this stem-nesting bee guard their nest entrances by blocking them with the abdominal terga. According to Malyshev (1912), nest-guarding females of the closely related *Ceratina* species protect their nests against ants and earwigs by emitting a lemon-like odor, accompanied by a buzzing sound. Recently the mandibular gland secretions of three *Ceratina* species have been analyzed (Wheeler et al., 1977), and several of the identified terpenes appear to be the source of the lemony odor observed earlier (Malyshev, 1912). The present study was undertaken in order to elucidate the chemistry of the mandibular gland secretion of a species in the Asian genus *Pithitis,* a taxon closely related to *Ceratina.*

METHODS AND MATERIALS

Hibernating bees were field-collected with their nests during January 1978 at Chohla Sahib Village, Punjab, India. The nests, containing bees, were chilled during storage and air-shipped to the United States. All nesting material and parasites were destroyed in quarantine. The bees that died as a result of handling to remove mites were preserved in ethanol and later their heads were transferred to methylene chloride. The heads were split with microscissors in order to expose the mandibular glands.

Samples were analyzed on an LKB 9000 gas chromatograph-mass spectrometer at 70 eV. Separation of the different components was achieved using a 3% OV-17 on Gas Chrom Q 70/80 column (Applied Science) programed from 70° to 200°C at 5°C/min. The different components of the glandular exudate were identified by their mass spectra. In each case the spectrum was compared with that of an authentic standard. The identity of the glandular components was further checked by comparing their isothermal retention times with those of authentic samples and by coinjecting standards with the extracts.

RESULTS

During preintroductory processing of the bees, it was noticed that both sexes orally secreted a pungent substance. This copious blood red to deep orange secretion was wiped by the prothoracic tarsi from the base of the mandibles onto the bees' heads and thoraces, and onto the forceps with which they were restrained. The secretion was of a paler orange in males than in females, and when deposited on filter paper (Figure 1), it faded to a light

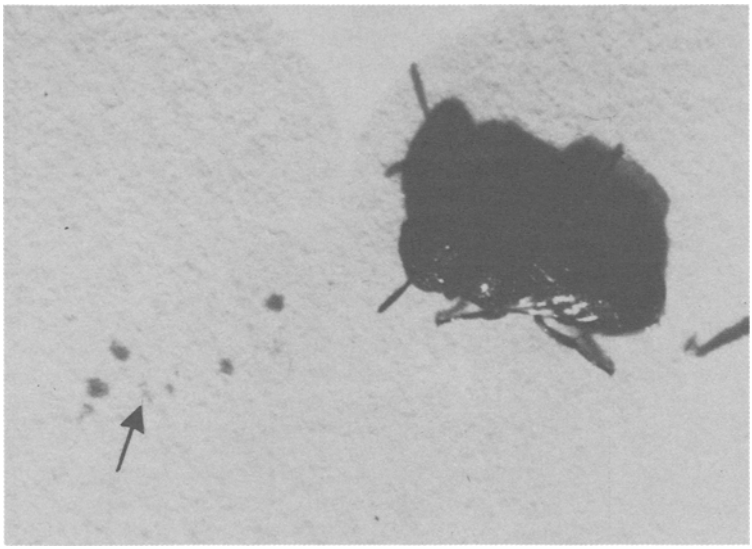

FIG. 1. *Pithitis smaragdula,* releasing, in defense, the contents of its mandibular glands onto a paper substrate (arrow). All the spots were produced by a single bee.

tan within an hour. Dissection of the bees revealed a cluster of enlarged cells comprising the mandibular glands, the probable source of the odorous part of the secretion.

Analysis of the glandular extracts revealed a chemically diverse blend composed of an aldehyde, terpene esters, ethyl esters, and hydrocarbons (Figure 2). The first component eluted at 97°C (peak 1) and was identified as salicylaldehyde by its fragmentation pattern. The mass spectrum showed a strong molecular ion at m/e 122 which was also the base peak and an almost equally strong M-1 ion at m/e 121. The terpene esters in the extracts were identified as citronellyl acetate (peak 2) and geranyl acetate (peak 4), eluting at 115° and 120°C, respectively. The components eluting as peak 3 and peak 5 showed typical hydrocarbon spectra and were identified as pentadecane and heptadecane, respectively. The mass spectra of the additional volatiles, peaks 6 and 7, on the other hand, had major ions at m/e 88 and 101, characteristic of ethyl esters, and the compounds were identified as ethyl tetradecanoate ($M^+ = 256$) and ethyl hexadecanoate ($M^+ = 284$). None of the components identified in the glandular exudate is apparently responsible for its red color. This suggests that the red chromophore is of enteric origin and is cosecreted with the mandibular gland contents when the bee is disturbed.

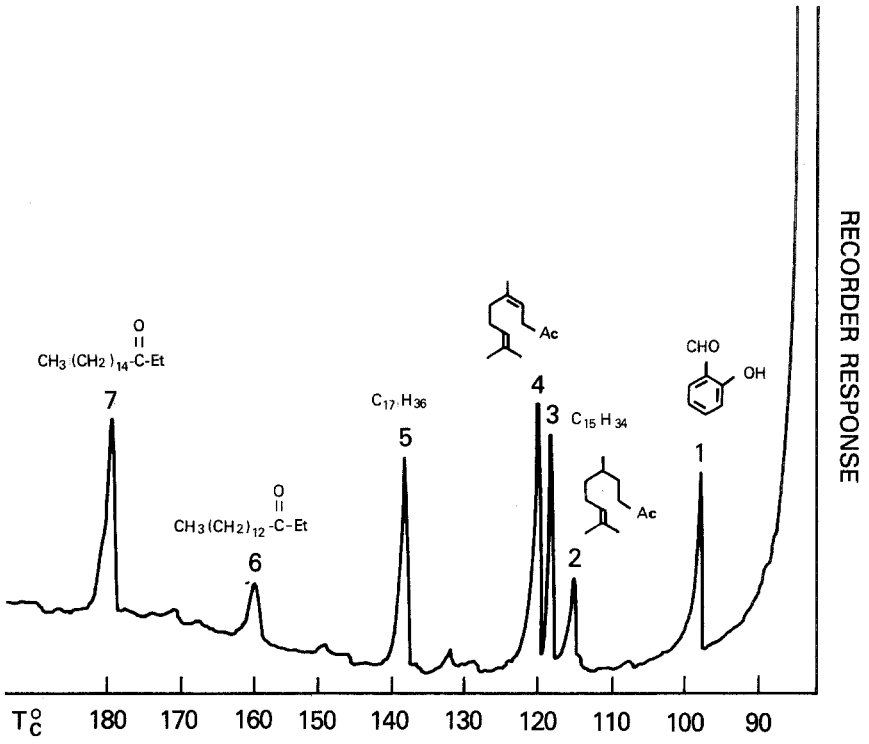

FIG. 2. Gas chromatogram of the mandibular glands exudate of *P. smaragdula*. Mandibular glands were extracted in methylene chloride and analyzed on a LKB 9000 combined GLC-mass spectrometer, utilizing a 3% OV-17 on Gas Chrom Q 70/80 mesh column. Compounds were identified by their fragmentation patterns and comparison with authentic samples: peak 1, salicylaldehyde; peak 2, citronellyl acetate; peak 3, pentadecane; peak 4, geranyl acetate; peak 5, heptadecane; peak 6, ethyl tetradecanoate; peak 7, ethyl hexadecanoate.

DISCUSSION

The mandibular gland secretion of *Pithitis smaragdula* has proven to be diverse and unique in composition. The presence in the secretion of pentadecane, heptadecane, and geranyl acetate demonstrated that it is similar to those of species in the closely related genus *Ceratina* (Wheeler et al., 1977), but that of *P. smaragdula* is clearly distinguished from those of *Ceratina* spp. in having, in addition, citronellyl acetate and salicylaldehyde. These three groups of compounds clearly originate from three different biosynthetic pathways and constitute another example of the diverse biosynthetic capabilities of an exocrine gland.

Whereas citronellol has been identified in several ant species (Blum and Hermann, 1978), citronellyl acetate has only been reported once in an arthropod secretion (Kullenberg et al., 1970). The latter, accompanied by geranyl acetate, is responsible for the lemon-like odor of the secretion. Salicylaldehyde was reported earlier from chrysomelid larvae (Coleoptera: Chrysomelidae), and from adult ground beetles (Coleoptera: Carabidae) (Weatherston and Percy, 1978), but not from Hymenoptera. Interestingly this aldehyde is employed by the bees, as well as the immature and adult beetles, in defense, emphasizing the biochemical parallelism of defensive secretions.

The appearance of the ethyl esters in the glandular exudates could possibly represent an artifact due to the extraction procedures. Since the bees were kept in ethyl alcohol prior to methylene chloride extraction, it is conceivable that free tetradecanoic acid and hexadecanoic acid in the glands formed the ethyl esters, but we consider this possibility unlikely. This point will be further investigated as soon as more fresh material is available.

In nature, both sexes guard their nest entrances by blocking them with the abdominal terga and probably by utilizing their sting in defense. The exudate of the mandibular glands provides an additional defensive mechanism since salicylaldehyde is a highly effective deterrent to insects (Wallace and Blum, 1969) as is geranyl acetate (Wheeler et al., 1977). On the other hand, this diverse secretion may possess a pheromonal role as well that could be important in the bee's management for pollination. Investigations of the functions of the compounds in the mandibular gland secretion of *P. smaragdula* are now being undertaken.

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CHEMICAL CONVERSION OF 9-TETRADECEN-1-OL ACETATES TO 3,13-OCTADECADIEN-1-OL ACETATES, SEX ATTRACTANTS FOR MALE CLEARWING MOTHS (LEPIDOPTERA: SESIIDAE)

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Abstract—Males of many species of clearwing moths are attracted by one of the geometrical isomers of 3,13-octadecadien-1-ol acetate or by a mixture of isomers. The synthesis of (*E,Z*)-, (*E,E*)-, and (*Z,E*)-3,13-octadecadien-1-ol acetate is described starting with the (*Z*)- and (*E*)-9-tetraceden-1-ol acetates, which are commercially obtainable.

Key Words—Sex pheromone, attractants, 9-tetradecen-1-ol acetate, 3,13-octadecadien-1-ol acetate, clearwing moth, Lepidoptera, Sesiidae, synthesis.

INTRODUCTION

Males of many species of clearwing moths (Lepidoptera: Sesiidae) are attracted by a 3,13-octadecadien-1-ol acetate (3,13-ODDA) or by a mixture of two or more of its geometrical isomers (Nielsen et al., 1975, 1978; Nielsen and Purrington, 1978; Barry et al., 1978; Karandinos et al., 1977; Yaginuma et al., 1976; Tumlinson et al., 1974). Recently we found that commercially obtained (*Z,Z*)-3,13-ODDA containing small amounts of its geometrical isomers is very attractive to the male clearwing moth *Synanthedon myopaeformis* (Borkhausen) (Voerman et al., 1978).

In order to study the influence of the *E,Z*, *Z,E*, and *E,E* isomers on the attractivity of (*Z,Z*)-3,13-ODDA for *S. myopaeformis*, we had to synthesize them.

A synthetic route to all four isomers was indicated by Tumlinson et al. (1974) and was described in detail by Doolittle et al. (1978). They used

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octamethylene chloroiodide as starting material. Underhill et al. (1978) prepared (*Z,Z*)-3,13-ODDA beginning with the condensation of 1-bromo-9-tetradecyne with 2-(3-butynyloxy)tetrahydropyran. No details were given. Uchida et al. (1978) described the synthesis of (*Z,Z*)- and (*E,Z*)-3,13-ODDA. Their overall yields were rather low.

In this paper, the conversion of (*Z*)-9-tetradecen-1-ol acetate to (*E,Z*)-3, 13-ODDA and that of (*E*)-9-tetradecen-1-ol acetate to (*E,E*)- and (*Z,E*)-3, 13-ODDA is described. The 9-tetradecen-1-ol acetates are attractive to several species of moths, singly or in combination with another compound (Mayer and McLaughlin, 1975) and are commercially obtainable.

METHODS AND MATERIALS

The progress of all reactions was followed, and all products checked, by gas-liquid chromatography (Voerman and Rothschild, 1978). The end-products were ultimately purified by liquid chromatography through a column packed with a silver-loaded resin (glass column, 200 × 1.6 cm, packed with Lewatit SP 1080, 170-200 mesh, Ag^+ form, eluent methanol, temperature 25-28°C) (Houx et al., 1974). Purity was also checked with HPLC (Houx and Voerman, 1976; Voerman, 1978).

The starting compounds (*Z*)-9- and (*E*)-9-tetradecen-1-ol acetate were bought from Farchan Division,Chemical Samples Co. (Willoughby, Ohio 44094), purity 95%, estimated by HPLC. The reaction schemes are shown in Figures 1 and 2.

Synthesis of (E,Z)-3,13-Octadecadien-1-ol Acetate

(Z)-9-Tetradecen-1-ol (I). Overnight, 17.8 g (*Z*)-9-tetradecen-1-ol acetate (0.070 mol) was stirred at room temperature in 100 ml of a 2% solution

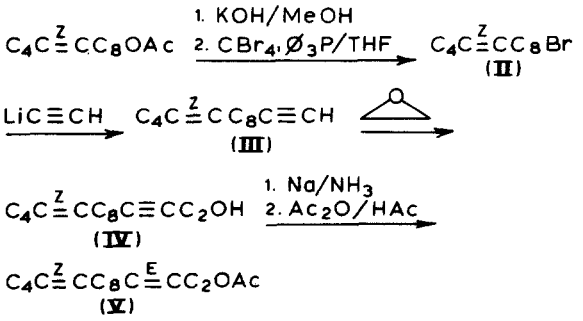

FIG. 1. Chemical conversion of (*Z*)-9-tetradecen-1-ol acetate to (*E,Z*)-3,13-octadecadien-1-ol acetate (V).

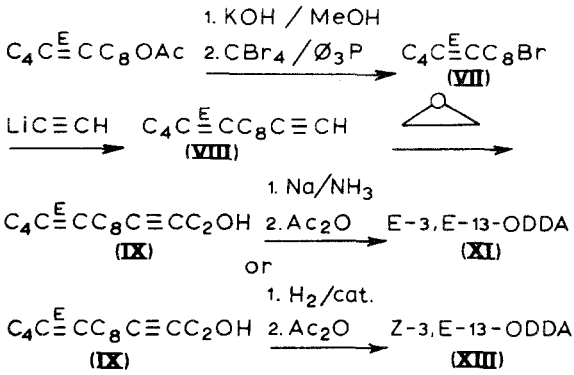

FIG. 2. Chemical conversion of (*E*)-9-tetradecen-1-ol-acetate to (*E,E*)- and (*Z,E*)-3,13-octadecadien-1-ol acetate (XI and XIII).

of KOH in MeOH. Then most of the solvent was removed at reduced pressure in a rotary evaporator; 200 ml 20% NaCl solution was added, and the water layer extracted with 5 × 50 ml hexane. The hexane extract was washed with 20% NaCl solution in water until neutral and dried over Na_2SO_4, giving I quantitatively after evaporation of the hexane in a rotary evaporator.

(Z)-9-Tetradecenyl Bromide (II). To 14.8 g (0.070 mol) of crude I in 20 ml dry THF were added 29.9 g (0.090 mol) CBr_4 in 15 ml dry THF and then slowly 23.6 g (0.090 mol) triphenylphosphine in 50 ml dry THF. The mixture was stirred mechanically. The slightly exothermic reaction was complete after about 1 hr (Hooz and Gilani, 1968). Stirring was continued for 3/4 hr after addition of 10 ml MeOH to destroy excess reagents. Then the solvent was removed by rotary evaporation and the residue chromatographed through Al_2O_3 (column 2 × 40 cm, packed with 30 g Al_2O_3) with hexane as mobile phase. The eluate was distilled, main fraction 17.5 g (90.9%), bp 93°C/0.08 mm, n_D^{25} 1.4695.

(Z)-11-Hexadecen-1-yn (III). Over 3/4 hr, 17.5 g (0.064 mol) of II in 60 ml HMPT was added to 6.9 g (0.08 mol) lithium acetylide ethylene diamine complex in 40 ml dry THF. During addition, the mixture was well stirred and the temperature was kept below 17°C. After stirring overnight at room temperature, it was poured into a mixture of 300 ml ice water and 60 g NaCl. The water layer was extracted with 5 × 50 ml hexane, washed with 20% NaCl until neutral, and dried over Na_2SO_4. Distillation gave 10.9 g (77.9%) of III, bp 73°C/0.03 mm, n_D^{25} 1.4530 [Doolittle et al. (1978) found bp 70°C/0.005 mm, n_D^{25} 1.4526.]

(Z)-13-Octadecen-3-yn-1-ol (IV). To 10.9 g (0.050 mol) of III in 80 ml dry THF was added, at 0°C under a weak stream of N_2, 37 ml of a hexane solution of butyllithium (1.6 M). After stirring was continued 1 hr at room temperature, 7 ml ethylene oxide (6.2 g, 0.141 mol) dissolved in 40 ml

cold HMPT was added at 0°C in a cold room. After stirring overnight at room temperature, the mixture was poured into a mixture of 400 ml ice water and 100 g NaCl. The resulting mixture was extracted with hexane, the extract washed with 20% NaCl until neutral, and dried over Na_2SO_4. Distillation after removing of the solvent by rotary evaporation gave a yellow oil, 10.5 g (80.2%), bp 124°C/0.03 mm, n_D^{25} 1.4730 [Doolittle et al. (1978) report bp 132–140°C/0.04 mm; Uchida et al. (1978) report bp 148–150°C/0.6 mm, n_D^{25} 1.4708.]

(E,Z)-3,13-Octadecadien-1-ol Acetate (V). A solution of 10.5 g (0.040 mol) of IV in 200 ml dry THF was slowly added to 3.7 g (0.161 mol) sodium dissolved in 300 ml NH_3. After stirring overnight, 14% of IV was still present. Another 100 ml dry THF was added and stirring was continued for 4 days. From time to time, some sodium dissolved in NH_3 was added to keep the mixture blue. Then the NH_3 was allowed to evaporate, 100 ml saturated NH_4Cl and 100 ml 20% NaCl were added, and the aqueous layer was extracted with hexane. The extract was washed with 20% NaCl and dried over Na_2SO_4. After removing the solvent, 12.4 g of an oil remained, still containing 2% of IV.

To this oil, 10 ml HAc and 10 ml Ac_2O were added. After standing for two days, the mixture was warmed for a few hours on a water bath and poured into 100 ml ice water. The mixture was stirred for a while and then extracted with hexane. The hexane extract was washed until neutral, dried over Na_2SO_4 and distilled. Main fraction 10.0 g (81.3%), bp 121°C/0.01 mm, n_D^{25} 1.4575 [Doolittle et al. (1978) found bp 135–140°C/0.03 mm, n_D^{25} 1.4569; Uchida et al. (1978) found n_D^{24} 1.4570.]

The overall yield from I to V was 46.2%.

Pure V was obtained by liquid chromatography through a column packed with a silver-loaded resin, n_D^{25} 1.4574. Calculated for $C_{20}H_{36}O_2$: C 77.9; H 11.8. Found: C 78.3; H 11.9.

Synthesis of (E,E)- and (Z,E)-3,13-Octadecadien-1-ol Acetate

(E)-9-Tetradecen-1-ol (VI). As described for the preparation of I, 48.3 g (0.19 mol) (*E*)-9-tetradecen-1-ol acetate was treated with a 2% solution of KOH in MeOH, giving VI quantitatively.

(E)-9-Tetradecenyl Bromide (VII). To a mechanically stirred solution of 40.3 g (0.19 mol) of VI in 50 ml dry THF were added 81.3 g (0.245 mol) CBr_4 dissolved in 40 ml dry THF and then dropwise a solution of 64.2 g (0.245 mol) triphenylphosphine in 110 ml dry THF. After the reaction was complete, excess reagents were destroyed by adding 5 ml MeOH, and stirring was continued for a few hours. The mixture was worked up as described for II. The resulting crude oil was chromatographed a second time through 15 g Al_2O_3 before distillation. Main fraction 47.6 g (91%), bp 92°C/0.03 mm, n_D^{25} 1.4690.

(E)-11-Hexadecen-1-yn (VIII). A solution of 59.9 g (0.22 mol) of VII in 210 ml HMPT was added to 24.8 g (0.27 mol) lithium acetylide ethylene diamine complex in 140 ml dry THF at a temperature of 10–15°C. The reaction mixture was worked up as for the preparation of III. Distillation gave a main fraction of 42.7 g (92.4%), bp 70°C/0.02 mm, n_D^{25} 1.4520.

(E)-13-Octadecen-3-yn-1-ol (IX). To 42.7 g (0.194 mol) of VIII in 250 ml dry THF was added under nitrogen at −5°C 140 ml of a hexane solution of butyllithium (1.6 M). After stirring some time at room temperature, 22.5 ml (20 g, 0.455 mol) ethylene oxide dissolved in 125 ml HMPT was added at 0°C in a cold room. Then the mixture was allowed to warm up and stirring was continued overnight at room temperature. The mixture was worked up as described for synthesis of IV. Distillation gave 43.2 (84.4%) of IX, bp 124°C/0.02 mm, n_D^{25} 1.4703.

(E,E)-3,13-Octadecadien-1-ol (X). A solution of 21.6 g (0.082 mol) of IX in 300 ml dry THF was added to 600 ml NH_3. Small pieces of sodium were added (6.9 g, 0.3 mol). This mixture was stirred for 7 days. From time to time, some sodium was added to keep the solution blue and some NH_3 to compensate for evaporation. Then it was worked up as described for V, giving 21.2 g of the oil (X), still containing about 10% of IX. Half of this oil was distilled, main fraction 8.8 g (80.7%), bp 116°C/0.01 mm, n_D^{25} 1.4663. The 10% IX in the distillate could be removed by argentation chromatography. Pure X has n_D^{25} 1.4660.

(E,E)-3,13-Octadecadien-1-ol Acetate (XI). The other half of the crude X, 10.6 g (0.040 mol), was acetylated with 10 ml HAc and 10 ml Ac_2O as described for V. Distillation gave 9.0 g (71.4%) of XI, bp 118°C/0.01 mm, n_D^{25} 1.4560 [Doolittle et al. (1978) report bp 129–132°C/0.07 mm.]

The overall yield from VI to XI was 50.7%.

Pure XI was obtained by liquid chromatography, n_D^{25} 1.4567. Calculated for $C_{20}H_{36}O_2$; C 77.9; H 11.8; found: C 78.1; H 11.7

(Z,E)-3,13-Octadecadien-1-ol (XII). To 2.5 g $Ni(OAc)_2 \cdot 4H_2O$ in 90 ml EtOH was added, under H_2, 10 ml of a $NaBH_4$ solution (prepared by filtering the solution resulting from 1 g $NaBH_4$, 24 ml EtOH, and 1.25 ml 2 N NaOH) (Brown and Ahuja, 1973a,b). After the hydrogen had ceased to evolve, 1.3 ml 1,2-diaminoethane and 21.6 g (0.082 mol) of IX were added. With vigorous stirring, 1887 ml H_2 was taken up. Then the reaction stopped. The mixture was filtered into 700 ml 20% NaCl, the water layer extracted with ether, the extract washed with 20% NaCl, and dried over Na_2SO_4. After removing the ether with a rotary evaporator, 21.3 g oil remained, of which 10.6 g was distilled, main fraction 9.5 g (87.2%), bp 115°C/0.005 mm, n_D^{25} 1.4667, which was further purified by argentation chromatography, giving pure XII, n_D^{25} 1.4668.

(Z,E)-3,13-Octadecadien-1-ol Acetate (XIII). The other half of crude XII, 10.6 g (0.040 mol) was acetylated as for XI. Distillation gave XIII,

9.8 g (79.9%), bp 115°C/0.005 mm, n_D^{25} 1.4560 [Doolittle et al. (1978) report bp 133–135°C/0.25 mm, n_D^{25} 1.4572.]

The overall yield from VI to VIII was 56.7%.

The product was further purified by liquid chromatography giving pure XIII, n_D^{25} 1.4574. Calculated for $C_{20}H_{36}O_2$: C 77.9; H 11.8; found: C 78.3; H 11.9.

RESULTS

Purified commercial (*Z,Z*)-3,13-ODDA (from Farchan Division) had n_D^{25} 1.4580. The four geometrical isomers could be separated on a stainless-steel column (25 × 0.46 cm) packed with 10-μm Nucleosil 10 SA (Ag^+). Mobile phase: methanol, 0.8 ml/min. Pressure: 32 kg/cm^2. Column temperature: 7°C. (Houx and Voerman, 1976; Voerman, 1979).

Compounds V, XI, and XIII had consistent mass spectra.

[^{1}H]Nuclear magnetic resonance (NMR) spectra (Hitachi Perkin-Elmer R-24B and Varian XL-100-15 spectrometers, $CDCl_3$ solutions, tetramethylsilane as internal reference) of these acetates were very similar. Chemical shifts, δ: 0.89 broad t (CH_3); 1.29 broad s (16 H); 1.70–2.40 broad (6H); 2.01 s (CH_3—$\overset{O}{\overset{\|}{C}}$—); 2.30 q [2H, J = 6 Hz: CH_2 group at position 2, also coupled with H at carbon atom number 3 (proved by decoupling experiments)]. In XIII this CH_2 group absorbs at 2.36; 4.05 t (2H, J = 6 Hz: —CH_2—O); 5.00–5.80 m (4H: olefinic H, slightly different patterns in V, XI, and XIII).

The [^{1}H]NMR spectra of the alcohols X and XII agreed with those of the acetates V, XI, and XIII except the chemical shifts of the CH_2 group at position 2, δ ppm: 2.25 q (in X); 2.30 q (in XII) and —CH_2—O— 3.60 t (2H, J = 6 Hz).

Infrared spectra (Hitachi EPI-G3 spectrometer, solutions in $CHCl_3$) of the acetates V, XI, and XIII showed typical absorption bands at 1735 cm^{-1} (CO stretching) and 971 cm^{-1} (CH deformation, most intense in XI). The alcohols X and XII showed typical bands at 3600 cm^{-1} (OH stretching) and 971 cm^{-1} (CH deformation, most intense in X).

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LABORATORY EVALUATION OF ZANTHOPHYLLINE AS A FEEDING DETERRENT FOR RANGE CATERPILLAR, MIGRATORY GRASSHOPPER, ALFALFA WEEVIL, AND GREENBUG

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Abstract—Choice and no-choice tests were used to evaluate zanthophylline as a feeding deterrent for range caterpillar, migratory grasshopper, alfalfa weevil, and greenbug. Range caterpillar, migratory grasshopper, and alfalfa weevil exhibited reduced feeding on zanthophylline-treated foliage in choice tests. Only range caterpillar was deterred by zanthophylline in no-choice tests; greenbug host preference was not affected by zanthophylline.

Key Words—Zanthophylline, alkaloids, feeding deterrent, range caterpillar, migratory grasshopper, alfalfa weevil, greenbug.

INTRODUCTION

Chemicals play a major role in the food-plant selection processes of phytophagous insects. Chemical feeding inhibitors and deterrents may prevent insects from coming into contact with the host plant or may limit the amount of feeding. Chemicals which deter feeding could be useful for the management of phytophagous insect pests if they are an integral part of the host plant or if they can be applied to the plant in the same manner as other agricultural chemicals.

The principal properties required of a practical feeding deterrent are: it should be persistent, it should be translocated to untreated parts of the plant, and it should have no harmful effects on nontarget organisms (Chapman, 1974). Most recent research in the area of feeding deterrents has concentrated in the use of organic salts or plant extracts. Some plant extracts,

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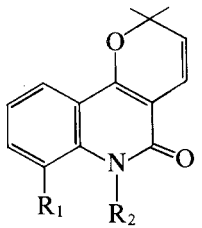
such as azadirachtin, a triterpenoid from the neem tree, *Azadirachta indica* A. Juss., show elements of persistence, within-plant mobility, and specificity. Thus, chemical deterrents that can be extracted from one plant and applied to another to confer protection seem worthy of further investigation. Alkaloids may be especially useful because many are believed to have evolved specifically as feeding deterrents for herbivorous animals (Fraenkel, 1969). We evaluated zanthophyllin (I), an alkaloid recently isolated from *Zanthoxylum monophyllum* Lam. (Stermitz and Sharifi 1977), as a feeding deterrent because of the structural similarity to *N*-methylflindersine (II), a compound known to possess antifeedant properties (Chou et al., 1977).



I: Zanthophylline: $R_1 = OCH_3$; $R_2 = CH_2OAc$
II: *N*-Methylflindersine: $R_1 = H$; $R_2 = CH_3$

A great variety of 2-quinolone alkaloids such an zanthophylline and *N*-methylflindersine are known, and it would be of interest to establish whether or not substituent changes would have an effect on the level or type of antifeedant activity.

METHODS AND RESULTS

Zanthophylline was extracted from the stems and branches of *Z. monophyllum* by the technique of Stermitz and Sharafi (1977). The crystalline extract was dissolved in ETOH for application to plant material. Activity of zanthophylline was evaluated by presenting insects with a choice between zanthophylline-treated plant material and control foliage treated with ETOH, or by presenting only one of these treatments, and recording the feeding activity associated with each. The insects tested are all significant crop pests and represent four major economically important insect orders. The insect and host plant combinations evaluated were range caterpillar, *Hemileuca oliviae* Cockerell (Lepidoptera: Saturniidae) and corn, *Zea mays* L.; migratory grasshopper, *Melanoplus sanguinipes* (F.) (Orthoptera: Acrididae) and barley, *Hordeum vulgare* L.; alfalfa weevil, *Hypera postica* (Gyllenhal)

(Coleoptera: Curculionidae) and alfalfa, *Medicago sativa* L.; and greenbug, *Shizaphis graminum* (Rondani) (Homoptera: Aphididae) and barley.

Range Caterpillar. Preference studies were conducted with two sections of corn foliage 1-cm wide and 5-cm long placed in a slit cut in a 2-cm-diam. foam stopper such that 50% of the foliage extended from one end of the stopper and ca. 1 cm extended from the opposite end. A stopper containing foliage was inserted into a 1.5-cm-diam. hole in the bottom of a 30-ml transparent cup. The 1-cm leaf portion protruding from the bottom of the cup was inserted into water to keep the foliage fresh. Each of the two sections of foliage was weighed prior to insertion into the cup and painted with either zanthophylline or ETOH. A 4th instar caterpillar larva was inserted into a cup, and the cup was capped with a paper lid. After a standard time interval caterpillars were removed and the zanthophylline and/or ETOH-treated foliage reweighed. Data were expressed as percent foliage weight loss relative to the first weighing. If the foliage section was not fed upon, the weight frequently increased between weighings and was expressed as 0% consumption. Data were transformed to arcsin $\sqrt{X}$ for analysis by Student's t test. Twenty-five larvae were used in each test, but frequently some larvae did not feed within the test period.

Range caterpillar larvae allowed to feed for 5 hr were strongly deterred from consuming corn foliage treated with 500 ppm zanthophylline if untreated foliage was available. Larvae ($N = 14$) consumed significantly more control foliage, 29.0 ± 12.1%, than treated, 12.1 ± 14.1% ($P = 0.005$). At 200 ppm larvae ($N = 13$) were also deterred, consuming 27.7 ± 15.5% of the control foliage and 16.0 ± 17.0% of the treated ($P = 0.025$).

Larvae were similarly presented with foliage in a no-choice test for 16 hr. Given no alternative but to feed on 500 ppm zanthophylline-treated foliage, larvae ($N = 24$) were significantly deterred, consuming only 9.3 ± 14.8% of the treated plant material while similar larvae ($N = 24$) consumed 33.7 ± 18.7% of the control foliage ($P = 0.001$). Larvae were slightly deterred by a 200-ppm level, consuming 43.2 ± 21.5% of the control ($N = 25$) and 24.3 ± 23.5% of the treated foliage ($N = 24$; $P = 0.01$).

Migratory Grasshopper. Adult migratory grasshoppers were presented with barley foliage in a choice situation in the aforementioned manner. Allowed a 5-hr feeding period, the grasshoppers ($N = 16$) exhibited a slight avoidance of the 500-ppm zanthophylline, consuming 33.2 ± 12.4% of the control and 25.2 ± 17.7% of the zanthophylline-treated foliage ($P = 0.1$). The grasshoppers ($N = 12$) consumed slightly more of the 200-ppm-treated foliage, 36.2 ± 6.4%, than the control, 34.0 ± 8.7%. This difference was not significant statistically.

In no-choice tests conducted for 5 hr, *M. sanguinipes* consumed 22.2 ± 18.6% of the 500-ppm zanthophylline-treated foliage ($N = 22$), 30.1 ±

17.1% of the 200-ppm treatment ($N = 24$), and 31.4 ± 19.9% of the control ($N = 25$). These differences are not significant statistically.

Observation of grasshopper feeding behavior indicated that the sporadic nature of feeding was probably masking much of the zanthophylline effect. Many of the grasshoppers in the choice tests initially avoided feeding on the zanthophylline-treated foliage, but after the control foliage was consumed the grasshoppers fed on the treated foliage, often consuming all the available plant material. To establish that the grasshoppers were actually deterred by zanthophylline in a choice situation, grasshoppers were provided both 500-ppm-treated and control foliage and allowed to feed until ca. 50% of any one foliage was consumed. Some of the grasshoppers consumed 50% within a few minutes while others required several hours. Thus, we obtained a more sensitive test of preference, and the grasshoppers ($N = 17$) consumed significantly more control foliage, 33.5 ± 8.8%, than 500-ppm-treated foliage, 22.1 ± 11.8% ($P = 0.005$).

Alfalfa Weevil. Fourth instar alfalfa weevil larvae were tested in the aforementioned manner except that alfalfa leaflets were used. In a 16-hr choice test, larvae ($N = 22$) consumed significantly ($P = 0.001$) more control foliage, 31.9 ± 13.6%, than 500-ppm zanthophylline-treated foliage, 13.6 ± 19.8% consumption.

A 5-hr no-choice test with 200 ppm ($N = 23$) and 500 ppm ($N = 24$) zanthophylline-treated and control foliage ($N = 23$) resulted in 17.2 ± 13.9, 14.6 ± 14.5%, and 19.8 ± 15.7% consumption respectively, and no statistically significant differences.

Greenbug. Insects with sucking mouthparts do not cause a detectable short-term weight loss of host-plant foliage. Hence, location of feeding aphids was used to evaluate the deterrent properties of zanthophylline. Choice tests ($N = 50$) with barley foliage were established in the aforementioned manner, except that 10-25 greenbugs were placed in each cage. After 16 hr the location of aphids was recorded. Barley foliage with 500 ppm zanthophylline supported 44.2 ± 22.1% of the aphids while control foliage supported 55.8 ± 22.3%. This difference was not statistically significant.

DISCUSSION

Zanthophylline-treated foliage was frequently rejected by range caterpillar, migratory grasshopper, and alfalfa weevil in choice tests. In no-choice tests, zanthophylline resulted in reduced feeding by range caterpillar larvae. Thus, zanthophylline appears to offer potential for crop protection, deterring herbivory by a wide variety of insect taxa.

The negative responses of insects to the 200–500 ppm concentrations tested indicate that zanthophylline is about as active as isoboldine (Wada

and Munakata, 1968) and *N*-methylflindersine (Chou et al., 1977). However, quinine sulfate inhibits feeding by larvae of *Pieris brassica* (L.) at a concentration of 0.35 ppm (Ma, 1972; cited by Chapman, 1974) and azadirachtin deters locusts from feeding at 0.04 ppm (Butterworth and Morgan, 1971). While zanthophylline is not active at very low concentrations, it may be effective against insects not affected by more active compounds because of the specificity of many deterrents, including azadirachtin.

Since both xanthophylline and *N*-methylflindersine are active, it seems likely that 2-quinolone alkaloids as a group will prove to be effective antifeedants. The activity levels of both are quite similar. Zanthophylline, however, appears to be a more general deterrent than *N*-methylflindersine, as Chou et al. (1977) reported the latter alkaloid active only against a single species. Hence, molecular modification may be of value in design of antifeedants.

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SYNTHESIS OF (*E*)-3,9-DIMETHYL-6-ISOPROPYL-5,8-DECADIEN-1-YL ACETATE, THE SEX PHEROMONE OF THE YELLOW SCALE[1]

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Abstract—Synthesis of a *definable* mixture of the racemic *Z* and *E* isomers of 3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate has been achieved. Comparison of these isomers with the natural pheromone of the yellow scale, *Aonidiella citrina* (Coquillett) resulted in the identification of the pheromone as (*E*)-3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate.

Key Words—Synthesis, sex pheromone, yellow scale, *Aonidiella citrina*, Homoptera, Diaspididae, isomers (*E*)- and (*Z*)-3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate.

INTRODUCTION

The yellow scale, *Aonidiella citrina* (Coquillett), is a pest of citrus and of ornamental plants, and can co-occur with the closely related California red scale, *Aonidiella aurantii* (Maskell). The sex pheromone of the California red scale has been identified as a mixture of two components, 3-methyl-6-isopropenyl-9-decen-1-yl acetate (I) and (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate (II) (Roelofs et al., 1977, 1978). More recently, the sex pheromone of the yellow scale was isolated and identified as 3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate (Gieselmann et al., 1979). Since the stereochemistry of the trisubstituted olefin could not be established from the spectral data of the material isolated from the insect, we chose, in a collaborative effort with the Geneva group, to prepare a definable mixture of the racemic *Z* and *E* isomers which could be separated and used

[1] *Aonidiella citrina* (Coquillett) (Homoptera: Diaspididae).
[2] Contribution No. 69 from the Research Laboratory of Zoecon Corporation.

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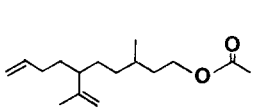

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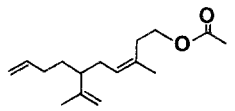
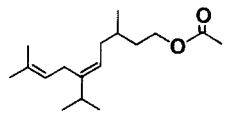


II

to confirm the structural assignment and to establish the stereochemistry of the 5-ene double bond in the natural pheromone. We now report the details of the synthetic portion of this collaboration which has resulted in the identification of the pheromone of the yellow scale as (*E*)-3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate (III).



III

METHODS AND MATERIALS

Preparative thin-layer chromatography was, in general, carried out on 1-m × 20-cm glass plates coated with 1.3 mm of Merck (Darmstadt) silica gel PF-254. NMR spectra were determined on a Varian T-60 spectrometer. Infrared spectra were measured on a Unicam SP 200G spectrophotometer. Mass spectra were measured on a Hewlett-Packard model 5984A GC/MS/DS with an all-glass jet separator at 70 eV ionization potential. Gas-liquid chromatographic analyses were performed on model 402 Hewlett-Packard instrument equipped with hydrogen flame ionization detectors. All solvents were dried over activated molecular sieves.

5-(2-Methoxyethoxymethoxy)-3-methyl-1-pentanol (*IV*). To 3.54 g (30 mmol) of 3-methyl-1,5-pentanediol in 60 ml of dry tetrahydrofuran (THF) at 0° under N_2 was added 19.2 ml of 1.56 M *n*-butyllithium (30 mmol). After 30 min, 3.74 g (30 mmol) of 2-methoxyethoxymethyl chloride (MEM-Cl) was added, and the reaction mixture was stirred overnight. The reaction mixture was then poured into a mixture of ether and water. The phases were separated, and the aqueous phase was extracted twice with ether. The combined ether fractions were washed with brine and were dried ($CaSO_4$). After solvent removal *in vacuo*, 6.3 g of crude product was obtained which was applied to seven 1-m × 20-cm preparative silica plates impregnated with Rhodamine 6G (developed with 40% ethyl acetate in hexane). The monoether was isolated in 47% yield (2.9 g, 14 mmol). IR (CCl_4) 3630 and 3500 cm^{-1} (OH); NMR ($CDCl_3$, δ) 4.73 (s, 2H), 3.40 (s, 3H) and 0.92 ppm (d, 3H, $J = 6$ Hz).

5-(2-Methoxyethoxymethoxy)-3-methyl-1-pentanal (*V*). Alcohol IV (1.78 g, 8.65 mmol) was added at room temperature to a suspension of 3.2 g (14.7 mmol) of pyridinium chlorochromate and 246 mg (3 mmol) of sodium acetate in 24 ml of dichloromethane. After 2 hr, the black suspension was diluted with ether and poured onto a 2.5 × 10 cm column of Florisil. The solvent from the eluate was removed *in vacuo* to give 1.44 g (7.1 mmol, 82% yield) of aldehyde V. NMR ($CDCl_3$, δ) 9.63 (t, 1H, $J = 2$ Hz), 4.73 (s, 2H), 3.40 (s, 3H), and 0.98 ppm (d, 3H, $J = 6$ Hz).

Ethyl 2-isopropyl-7-(2-methoxyethoxymethoxy)-5-methyl-2-heptenoate (*VI*). Sodium hydride (380 mg of 57% NaH in oil dispersion) was washed free of oil with pentane under a N_2 atmosphere (theoretical yield, 9 mmol) and was then suspended in 20 ml of THF. To this suspension was added 2.4 g (9 mmol) of diethyl 1-ethoxycarbonyl-2-methylpropylphosphonate [prepared from commercially available diethyl ethoxycarbonylmethylphosphonate by treatment with 1 equiv of NaH and then with 1 equiv of isopropyl iodide in 10% hexamethylphosphoramide (HMPA)-dimethylformamide]. After 45 min, 1.5 g (7.35 mmol) of aldehyde V was added, and the reaction was stirred for 1.25 hr. Water was added to the reaction, and it was poured into ether. The organic phase was washed with brine and was dried ($CaSO_4$). The residue obtained after solvent removal *in vacuo* was purified on three 1-m × 20-cm preparative silica plates (developed with 30% ethyl acetate in hexane) to give 1.34 g (4.25 mmol, 58% yield) of the *Z* and *E* esters VI in a 4:1 ratio, respectively. IR (CCl_4) 1715 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 6.57 (t, $J = 7$ Hz, C=CH *E* isomer), 5.70 (t, $J = 7$ Hz, C=CH *Z* isomer), 4.72 (s, 2H), 4.22 (q, 2H, $J = 7$ Hz), 3.40 (s, 3H), 1.28 (t, 3H, $J = 7$ Hz), 1.05 (d, 6H, $J = 6.5$ Hz), and 0.88 ppm (d, 3H, $J = 6$ Hz). MS (70eV) *m/e* (relative intensity) for each isomer 240 (10), 89 (100).

2-Isopropyl-7-(2-methoxyethoxymethoxy)-5-methyl-2-hepten-1-ol (*VII*). To 1.68 g (5.3 mmol) of esters VI in 20 ml of dry benzene under a N_2 atmosphere was added 6.7 ml (12 mmol) of 1.8 M diisobutylaluminum hydride in heptane. An additional 1.5 ml of reducing agent was added after 1.5 hr. Saturated ammonium chloride was carefully added to the solution after another hour, and then the mixture was poured into ether and water. To facilitate solution of aluminum salts, the pH of the aqueous layer was adjusted to pH 4 with 2% aqueous HCl. The aqueous layer was extracted twice more with ether, and the combined ether fractions were washed with brine and were dried ($CaSO_4$). Removal of solvent *in vacuo* gave 1.28 g (4.7 mmol, 90% yield) of allylic alcohols VII. IR (CCl_4) 3600 cm^{-1} (OH); NMR ($CDCl_3$, δ) 5.33 (br t, 1H, $J = 7$ Hz), 4.72 (s, 2H), 4.12 (br s, 2H), 3.40 (s, 3H), 1.05 (d, 6H, $J = 7$ Hz), and 0.88 ppm (d, 3H, $J = 6$ Hz).

2-Isopropyl-7-(2-methoxyethoxymethoxy)-5-methyl-2-hepten-1-yl acetate (*VIII*). Allylic alcohols VII (100 mg, 0.36 mmol) in 0.15 ml of acetic anhydride and 0.25 ml of pyridine were stirred overnight at room temperature under N_2 atmosphere. Ice was added to the mixture, and after 30 min the

reaction was poured into ether and 5% aqueous HCl. The organic layer was washed with 2 M Na_2CO_3 and brine and was dried (Na_2SO_4). Removal of solvent *in vacuo* gave 114 mg (0.36 mmol, 100% yield) of allylic acetates VIII. IR (CCl_4) 1740 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 5.43 (br t, 1H, J = 7Hz), 4.70 (s, 2H), 4.60 (br s, 2H), 3.40 (s, 3H), 2.05 (s, 3H), 1.02 (d, 6H, J = 7 Hz), and 0.87 ppm (d, 3H, J = 6 Hz).

3,9-Dimethyl-6-isopropyl-5,8-decadien-1-yl 2-methoxyethoxymethyl ether (IX). To 190 mg (1 mmol) of cuprous iodide suspended in 4 ml of dry ether at −25° under a N_2 atmosphere was added 3.3 ml (1.98 mmol) of 0.60 M 2-methylpropenyllithium (prepared from 2-methyl-1-bromo-1-propene and lithium—1% sodium wire in ether). After 20 min, an aliquot gave a negative Gilman test (Gilman and Schulze, 1925), and 105 mg (0.33 mol) of allylic acetates VIII in 1 ml of ether was added. After 4 hr, saturated $(NH_4)_2SO_4$ was added, and then the reaction was poured into ether and additional $(NH_4)_2SO_4$ solution. The ether layer was washed with water and was dried (Na_2SO_4). Solvent was removed *in vacuo* and the residue was purified on one 1-m × 20-cm preparative silica plate impregnated with Rhodamine 6G to give 62 mg (0.20 mmol, 60% yield) of diene ethers IX. NMR ($CDCl_3$, δ) 5.27–4.90 (m, 2H), 4.73 (s, 2H), 3.40 (s, 3H), 2.72 (br d, 2H, J = 7 Hz), 1.67 (br s, 6H), and 1.00 ppm (d, 6H, J = 7 Hz). MS (70 eV) m/e (relative intensity) 312 (M^+, 0.2), 69 (100).

3,9-Dimethyl-6-isopropyl-5,8-decadien-1-yl acetate (III and X). A solution of 56 mg (0.18 mmol) of diene ethers IX in 5 ml of ethanol and 1 ml of water containing 75 mg of trichloroacetic acid was heated at 70° for 72 hr under a N_2 atmosphere. The reaction was cooled and poured into a mixture of ether and 2 M Na_2CO_3. The organic layer was washed with brine and was dried (Na_2SO_4). After solvent removal, the desired alcohols were purified by preparative thin-layer chromatography (developed with 30% ether in hexane) to give 27 mg of product. NMR ($CDCl_3$, δ) 5.30–4.90 (m, 2H), 3.70 (t, 2H, J = 6 Hz), 2.73 (br d, 2H, J = 6 Hz), 1.70 (br s, 6H), and 1.00 ppm (d, 6H, J = 7 Hz).

The alcohol isomers were stirred in 0.10 ml of acetic anhydride and 0.15 ml of pyridine for several days. Ice was added to the mixture, and after 30 min the reaction was poured into ether and 5% aqueous HCl. The organic fraction was washed with 2 M Na_2CO_3 and brine, and was dried (Na_2SO_4). After solvent removal *in vacuo*, the product was filtered through a small column of Florisil with ether-pentane (1:4) to give 30 mg (0.12 mmol, 65% yield) of the acetates III and X in a 4:1 ratio, respectively. IR (CCl_4) 1740 cm^{-1} (C=O); NMR (C_6D_6, δ) 5.33–5.03 (m, 2H), 4.07 (t, 2H, J = 6 Hz), 2.78 (br d, 2H, J = 6 Hz), 1.72 (s, 3H), 1.62 (br s, 6H), 1.05 (d, J = 7 Hz, $(CH_3)_2CH$ for major E isomer), 0.98 (d, J = 7 Hz, $(CH_3)_2CH$ for minor Z isomer) and 0.82 ppm (br d, 3H, J = 6 Hz). MS (70eV) m/e (relative intensity) for both isomers 266 (M^+, 14), 43 (100). The diene acetate isomers III and X were

analyzed and resolved by gas-liquid chromatography (4m 3% PDEAS, 145°). The faster eluting *E* isomer had a retention time of 14.6 min, while the minor *Z* isomer was retained on the column for 15.4 min. The isomers were preparatively separated on an XF-1150 column (Gieselmann et al., 1979).

RESULTS AND DISCUSSION

The synthetic scheme outlined in Figure 1 was designed to give a definable mixture of (*Z*)- and (*E*)-3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetates. The stereochemistry of the olefin in the natural pheromone could then be assigned by comparison with the synthetic mixture. Thus, the monomethoxyethoxymethyl (MEM) ether IV of 3-methyl-1,5-pentanediol, obtained by treating the diol with 1 equiv each of *n*-butyllithium and MEM chloride (Corey et al., 1976) followed by chromatography, was oxidized to the aldehyde V in 82% yield with 1.7 equiv of pyridinium chlorochromate and 0.35 equiv of sodium acetate in dichloromethane (Corey et al., 1975). Treatment of aldehyde V with the anion of diethyl 1-ethoxycarbonyl-2-methylpropyl phosphonate (prepared by isopropylation of diethyl ethoxycarbonylmethylphosphonate) in THF gave, in 58% yield, a mixture of isomeric esters in a 4:1 ratio. Inspection of the olefinic hydrogen region of the PMR spectrum of the esters VI allowed the following assignment of stereochemistry (Jackman and Wiley, 1960). For the major isomer (*Z*), the olefinic hydrogen resonance occurred at δ ($CDCl_3$) 5.70 ppm, while for the minor isomer (*E*), this same resonance occurred at δ ($CDCl_3$) 6.57 ppm. This mixture of esters was then reduced with 2.8 equiv of diisobutylaluminum hydride in benzene to give allylic alcohols VII in 90% yield, which were quantitatively converted to their acetates VIII with excess acetic anhydride and pyridine. Reaction of the allylic acetates VIII in ether with 3 equiv of lithium bis(2-methyl-1-propenyl)cuprate (Anderson et al., 1970, 1972) (prepared from isobutenyl bromide; Farrell and Bachman, 1935) at −10° for 4 hr gave the desired diene ethers IX in 60% yield. Cleavage of the ethers IX (trichloroacetic acid in refluxing ethanol, 12 hr) to their corresponding alcohols followed by acetylation with excess acetic anhydride and pyridine gave, in 65% yield, the diene acetates III and X in a ratio of 4:1, respectively. In the PMR spectrum of this mixture in deuterobenzene the isopropylmethyl resonances were resolved, i.e., δ 1.05 ppm (d, $J = 7$ Hz) for the major isomer (*E*) and δ 0.98 ppm (d, $J = 7$ Hz) for the minor isomer (*Z*). Since the isopropylmethyls in the natural pheromone absorb at δ 1.08 ppm, the stereochemistry of this pheromone was established as *E*.

The racemic *Z* and *E* isomers were separated by preparative GLC on an XF-1150 column. The faster eluting *E* isomer had the same retention

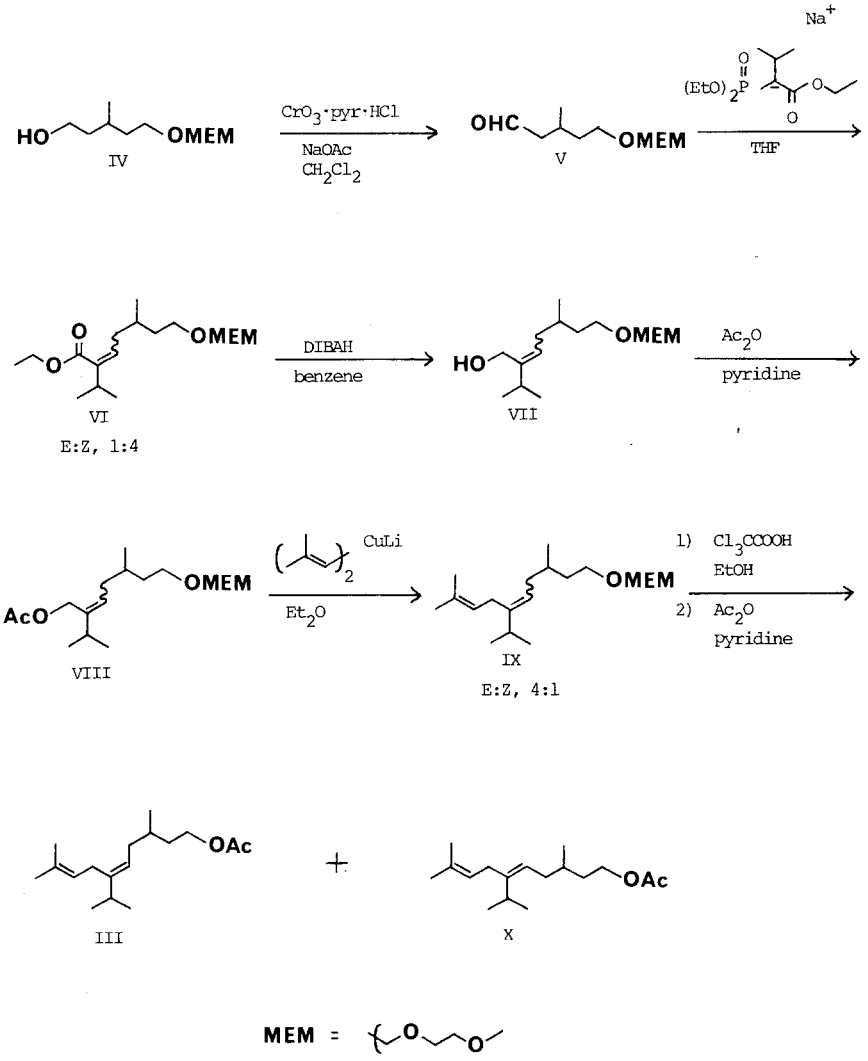


FIG. 1. Synthesis of (*E*)- and (*Z*)-3,9-dimethyl-6-isopropyl-5,8-decadien-1-yl acetate.

time as the natural pheromone and had an identical PMR spectrum. In greenhouse bioassays the *E* isomer was found to be attractive to the male yellow scale, whereas the *Z* isomer was inactive. The absolute configuration of the naturally occurring pheromone has not been determined, and the effect of enantiomeric purity on the attraction of males to synthetic (*E*)-III also has as yet not been established.

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ATTRACTION OF EUROPEAN ELM BARK BEETLES, *Scolytus multistriatus*,[1] TO PHEROMONE-BAITED TRAPS[2]

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Abstract—Newly emerged *Scolytus multistriatus* reared in the laboratory were marked, released in the field, and recaptured on sticky pheromone-baited traps at various distances. Four groups of beetles were conditioned before release by providing food, flight exercise, both food and flight, or by withholding food and flight for 24 hr to determine effect of treatment on pheromone response. Average catches per trap for untreated beetles and the respective treatments were 5.5, 2.1, 1.1, 1.0, and 0.6. Overall recovery ranged from 3 to 8% of 58,421 marked beetles released in four experiments. Combined feeding and flight exercise resulted in low trap response but flight to distant traps increased. Beetles held with no food or flight showed the lowest response. Endemic *S. multistriatus* responded to all traps with catches ranging from 13 to 17 times the number of marked beetles recaptured.

Key Words—Smaller European elm bark beetle, *Scolytus multistriatus*, Coleoptera, Scolytidae, aggregation pheromone, Multilure, Dutch elm disease, trapping.

INTRODUCTION

Knowledge of the flight dispersal habits of the smaller European elm bark beetle, *Scolytus multistriatus* (Marsham), from its breeding site in elm trees has been considered of great importance ever since this insect was discovered

[1]Coleoptera: Scolytidae

[2]The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture or the Forest Service of any product or service to the exclusion of others that may be suitable.

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to be the vector of Dutch elm disease (DED). Conflicting estimates of flight distance of these beetles were derived from early studies. Reported distances, for example, ranged from 30–150 m (Welch and Matthysse, 1956), to 5–6 km (Wolfenbarger and Jones, 1943). Some beetle dispersion attributed to flight may actually be the result of transport on infested elm wood carried by rivers in flood stage (Craighead, 1950).

Methods of investigating beetle dispersion have been improved greatly by the development of better marking techniques and by better recapture procedures such as the use of pheromone-baited traps. Multilure, the synthetic aggregation pheromone for *S. multistriatus* (Pearce et al., 1975), simulates the chemical attraction of a beetle breeding site. Multilure-baited traps have been used effectively in surveys to delineate the distribution of this beetle, to detect its presence in new locations, and to monitor its emergence patterns (Lanier et al., 1976). The present study used these improved methods to show flight distance and recovery rate of identifiable beetles released from a designated source.

Large numbers of elm bark beetles have been attracted to and captured at Multilure-baited traps. It is unknown if the response to the pheromone occurred immediately upon flight of the beetle from the brood tree or whether flight exercise, feeding, or both, were significant or necessary preconditions to this response. If preconditions do exist, the potential for reducing DED by pheromonal suppression of bark beetles is somewhat diminished. I investigated the influence of several preconditioning regimes on the response of *S. multistriatus* to Multilure-baited traps.

METHODS AND MATERIALS

Studies employing two distinct release-recapture systems were made on a 40-hectare field in a farm area 7 km north of Delaware, Ohio. The first study, in 1973, determined the pheromone-response pattern of laboratory-reared beetles that were marked and released almost immediately after emergence (i.e., with no conditioning). A second study, in 1975, determined the effect on pheromone response of several food or flight regimes imposed on different groups of marked beetles before their release.

Beetle Rearing, Collecting, and Counting

The building used for mass rearing of *S. multistriatus* is a large-scale adaption of the refrigerated emergence cabinets described by Browne (1972). The beetles were retrieved each day in groups of several thousands. Frequent sampling of females isolated on elm bolts showed, by the absence of progeny, that they had not mated. Bartels and Lanier (1974) found no mated females

among several thousand beetles emerging from the type of chamber used in this study. Any flight that may have occurred in the darkened rearing room was considered slight as the beetles do not fly at night (Wollerman, unpublished data). However, some maturation processes may occur. Beetle vitality was demonstrated in preliminary experiments by the survival to 29 days of individual beetles fed on elm twigs in the laboratory, and by the capture of marked beetles in field traps for 3 days after their release. The number of beetles produced by the rearing room was estimated volumetrically by a ratio of 275/ml.

Beetle Marking for Recognition

The radioisotope ^{32}P obtained as orthophosphate in dilute HCl was used to mark beetles in the first study. A 0.5% aqueous ^{32}P solution was poured into a large Erlenmeyer flask containing the beetles plus a handful of styrofoam fragments. These fragments provided a surface for the beetles to cling to during the 15-min treatment period, during which time the stoppered flask was rotated slowly to ensure repeated contact of the beetles with the marking solution. At the field release point, the liquid was decanted and the beetles were shaken from the flask onto absorbent paper, which removed excess marking solution. Earlier laboratory studies had established that this treatment produced a mark that lasted at least 2 weeks and that the mortality of beetles so treated did not exceed that of controls. Beetles that had not flown by 12 hr after the field-marking treatment were counted and removed from the study. The marking on beetles was verified in the field by use of a survey meter, and beetles recaptured in the study were identified in the laboratory by use of a gas-flow detector (Nuclear Chicago, model 1042).

In the second study, five groups of beetles were subjected to different treatment regimes before being released in the field. This study required five kinds of identifying marks; distinctive colors of fluorescent powder were used. Each group of beetles to be marked with the powder (Dayglo fluorescent pigments, Dayglo Color Corp., Cleveland, Ohio) was placed in a cylindrical 8-liter cardboard food container covered with a piece of window glass. Inserted through the side of the container was a plastic tube connected by flexible tubing to a rubber bulb. Only as much powder as could be held on the end of a flat toothpick was placed in the tube. A wire screen, placed in the tube between the powder and the lumen of the container, helped to disperse the powder in the air stream. With beetles in the chamber and the glass cover in place, a quick squeeze on the bulb dispersed the powder as an almost invisible haze. The uniformity of the mark on the beetles was verified by viewing them under UV light (Sylvania blacklight blue, F15T8-BLB). Immediately after the marking, the containers were taken to the center of the field, uncovered, and left on a table for about an hour for the beetles to take flight.

Conditioning of Beetles

In the first study, beetles were removed from the refrigerated collection flask and kept at room temperature until they became active. They were then marked with ^{32}P, taken to the field, and released within an hour.

In the second study, four groups of about 9000 emerged beetles each were subjected to different conditioning regimes for 24 hr. At the end of this period, a fifth group of 9000 was obtained from the rearing room. Beetles in each group were mass marked with an identifying color, taken to the field, and released. The five conditioning regimes were: (1) food and flight exercise for 24 hr; (2) food, but no flight exercise, for 24 hr; (3) flight exercise, but no food, for 24 hr; (4) 24 hr without food or flight exercise; and (5) no time lapse after emergence, no food or flight exercise.

Procedures for Conditioning

Feeding. Beetles in regimes 1 and 2 were placed on bundles of fresh elm twig bark confined in a plastic wrapping. After a 24-hr feeding period, the bundles were opened and the beetles were brushed into the marking container. Beetles receiving both food and flight conditioning (regime 1) received food for 12 hr, then flight exercise of 15 min in each hour for 12 hr.

Flight. Beetles in regimes 1 and 3 were stimulated to fly in a transparent plastic cage 61 × 61 × 122 cm by long-wave UV ($\lambda > 360$ mm, Ultra-Violet Products, Inc., San Gabriel, California). The UV lamp was placed above the cage and a time switch turned it on for 15 min each hour for 12 hr. Moist filter paper was always present in the cage.

Aging. Beetles that were aged 24 hr after emergence, but denied food and flight exercise (regime 4), were held in a moisturized container too small for flight. Beetles for regime 5 (not aged, no preconditioning) were collected 15 min before being marked and released in the field.

Trap Design

In the first study, the trap was a 30-cm-diameter cylindrical metal screen of 0.64-cm mesh coated with Stikem Special® (Michel and Pelton, Emeryville, California) and held upright between a base and a cover. The attractant was produced from elm bolts infested with virgin female beetles actively excavating galleries under the bark (Peacock et al., 1971). The bolts were enclosed in screen fabric and placed inside the trap supported 2 m above the ground.

In the second study, the traps and attractants used were based on the work of Lanier et al. (1976), which indicated that two-dimensional sticky surfaces were the best traps for *S. multistriatus* and that contrast between dark and light was a very important factor. In my studies I used black posterboard,

35 × 56 cm, coated with Stikem Special®. The trap supports were 2.5-m-high wood timbers. Plywood boards, 49 × 61 cm, fastened to the timbers served as a backing for the traps.

Multilure (Pearce et al., 1975) was dispensed from laminated plastic Hercon® baits (Health-Chem Corp., New York) (Lanier et al., 1976). Small pieces of bait, about 3 × 5 cm, were cut from impregnated plastic sheets and stapled inside a single fold of cardboard, which was then hung from a wire in front of the sticky trap.

Field Plot Designs

The first study used a design with eight equally spaced radii on which a total of 40 traps was spaced at increments of 80.5 m. Where field boundaries were limiting, some of the traps were placed between radii (Figure 1).

The second study was begun with a trap pattern of a grid with 64 traps

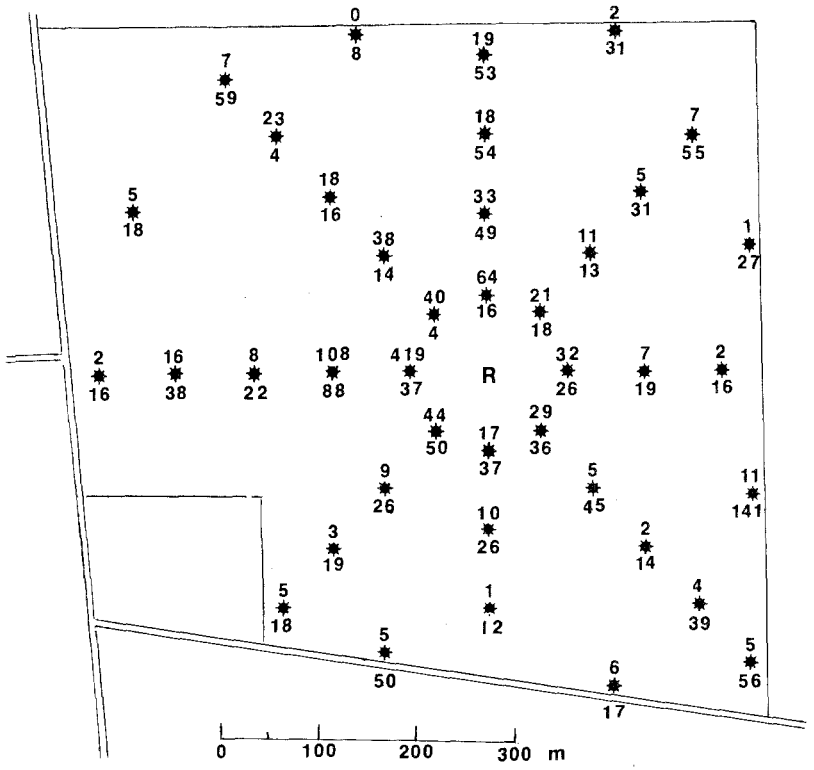

FIG. 1. Location and number of *S. multistriatus* trapped August 13-16, 1973, after 14,114 marked beetles had been released. Top numerals show marked beetles, bottom numerals show endemic beetles. R is release point.

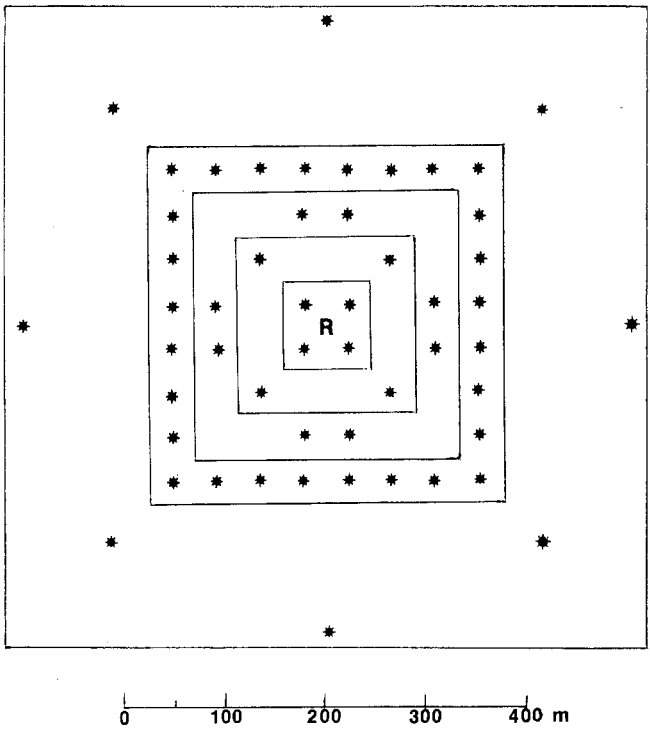

FIG. 2. Modified 8 × 8 grid design of 52 field traps, with 20 of the original traps removed and 8 traps added at 305 meters. Release of beetles at R.

spaced at 46 m over an area of 10 hectares. After this pattern had been used in two beetle releases, it was modified (Figure 2) for use in a beetle release on August 19. This 52-trap modification of the original 8 × 8 grid design was made by removing 20 traps and adding an outer circle of 8 traps on a radius of 305 m, thereby enlarging the total area to 37 hectares.

Techniques for Release and Recapture of Beetles

The physical operations of trap retrieval and beetle counting were designed to coordinate with beetle releases. In the first study, there was only one release (on August 13) of 14,114 beetles marked with ^{32}P. After one week all 40 traps were collected and taken to the laboratory. The beetles were picked from each screen, counted, and sorted for those marked with ^{32}P.

In the second study, there were three beetle releases: June 30, July 28, and August 19. In most cases, traps were changed hourly from 5 PM to 10 PM for 3 days after each release. The schedule was extended to include

the period between July 29, 10 PM, and July 30, 6 AM, to reveal any night flight. The number of beetles released varied from 7975 to 9767, as the production of beetles varied.

RESULTS AND DISCUSSION

In the first study, 40 traps baited with beetle-infested elm wood attracted both ^{32}P-marked beetles and endemic beetles. The distance from the point of release of marked beetles to the farthest traps in the 40-hectare experimental area was 402.6 m (Figure 1). Fewer marked beetles were caught as the distance of the trap from the release point increased (Table 1). The number decreased from 666 at 80 m to 21 at 402 m.

It is difficult to explain why only 1062 of the 14,114 marked beetles released were trapped. Because all the traps at a height of 2 m are in a single plane, the beetles may have flown above and beyond them. Or the low recovery may have resulted from the handling, and subsequent reduction in vigor, of these laboratory-reared beetles. Another explanation is that a period of feeding, flight exercise, or both, is required before beetles will exhibit a maximum response to an attractant (Meyer and Norris, 1964; Lanier et al., 1976).

The data on beetles trapped in all releases in the second study are shown as the average number per trap in relation to their conditioning treatment and distance flown from the release point (Table 2). The percent recovery of flying beetles in the first, second, and third releases were 0.2-16.4%, 2.7-4.7%, and 0.1-9.8% respectively.

Initial plans were to sample all parts of the plot equally, using the grid design, or to sample by placing traps in concentric circles at increasing distance from the release point. However, neither of these two-dimensional plans could provide an accurate measure of any differences in flight of

TABLE 1. CAPTURES OF MARKED BEETLES AT INCREASING DISTANCES FROM THEIR RELEASE POINT IN TRAPS BAITED WITH LIVE VIRGIN FEMALES

Distance of trap from release point	Number of beetles captured (% recovery)	Average catch per trap
(meters)		
80.5	666 (4.7)	83
161	221 (1.6)	28
241.6	57 (0.4)	7
322	97 (0.7)	10
402.6	21 (0.2)	4
	Total 1062 (7.6)	

TABLE 2. AVERAGE BEETLE CATCHES RELATED TO CONDITIONING TREATMENT AND TRAP DISTANCE IN THREE RELEASES

Treatments	Flyers		Distance of traps (meters)					Catch (%)
	No.	%	32	72-97	117-162	162-227	305	
1st release (6/30)							No data	
No treatment	1087	99	3.3	2.4	1.2	2.2		16.4
Food only	1100	67	3.8	1.2	0.8	0.8		7.4
Flight only	1100	67	3.3	0.8	0.6	0.3		5.1
Held 24 hr	1100	67	0	0.5	0.6	0.5		3.6
Food and flight	990	60	0	0.1	0	0.1		0.2
2nd release (7/28)							No data	
No treatment	4957	99	13.5	4.3	1.6	3.5		4.7
Food only	4250	85	3.8	1.5	1.7	3.8		4.1
Flight only	3750	75	1.5	1.0	0.9	1.5		2.1
Held 24 hr	4375	87	1.0	0.7	0.8	1.1		1.4
Food and flight	3875	77	0.8	1.0	0.8	2.6		2.7
3rd release (8/19)								
No treatment	3723	99	44.0	8.0	5.6	3.4	1.1	9.8
Food only	4000	80	12.5	1.3	0.9	1.1	0.5	2.5
Flight only	3750	75	5.3	1.0	1.4	1.4	0.4	2.0
Held 24 hr	2500	50	0.3	0	0	0.3	0	0.6
Food and flight	3750	75	0	0	0	0.03	0.4	0.1

variously preconditioned beetles in distances as short as the 46 m between traps, given the interactions in three dimensions of fluctuating air currents, odor plumes of Multilure, and flight. Therefore, the data were tabulated from traps set in four successively larger squares, beginning with the smallest with four traps at the center (Figure 2). Because the number of traps per square varied, catches were calculated as the average number of beetles per trap.

In general, the number of beetles recovered in this experiment showed this ranking: no treatment > food only > flight only > no food or flight for 24 hr > food and flight. There was a pattern of fewer beetles per trap as distance of the trap from the release point increased, regardless of treatment.

The marking of beetles was shown to have little effect on behavior or mortality in laboratory tests. When groups of marked and unmarked beetles were placed on elm twigs all beetles were very active and immediately began feeding and tunneling. After one week the survival was 51% for marked beetles and 58% for controls. Twig dissection revealed eggs and larvae in both groups. The influence of treatment was seen to be greater than the marking technique in field releases of large numbers. In all of the releases 99% of nontreated marked beetles flew upon release. Flight among treated-marked beetles was less and ranged from 50% to 87% (Table 2).

The manipulations involved in handling all groups of beetles, including those not conditioned, beginning with the rearing room procedure, included such unnatural conditions as use of a refrigerated collecting flask and crowding of beetles. To minimize the effect of these conditions, the only beetles used were those active after being handled and marked.

When the results of all three releases were combined (Table 3), the no-treatment beetles recaptured (8% recovery) was twice that of the group given food only, and between 3 and 6 times that of the groups treated in other ways. Captures over all treatments was 2.3% (captured, 777; released, 34,540). Two groups of beetles, those given no treatment and those given food only, showed greater response to traps at all distances than the starved beetles. The ratio of the no-treatment beetles caught at 32 m compared to 305 m was about 4:1, that for beetles given food only was about 3:1, and that for beetles given flight exercise only was about 4:1. The weakest beetles—those held 24 hr—responded to the nearest and most distant traps in about equal numbers.

In this experiment, it was assumed that released beetles would be caught in large numbers on traps baited with Multilure in sufficient numbers to permit comparison of the responses of beetles subjected to various prerelease conditioning treatments. The data obtained validated this assumption, but also raised some questions and revealed new information on beetle behavior in the field. The maximum recovery of 8% leaves unknown the fate of the remainder of those released. They may have flown above and beyond the traps, or may have fallen to the ground and died after a short flight; it would be impossible to find these very small beetles in irregular ground with weed cover. The attraction of elms in a nearby nursery could also have drawn beetles out of the trap area.

The chronology of flight of both marked and endemic beetles shows that,

TABLE 3. COMPARISON OF CONDITIONING EFFECTS IN ALL RELEASES BY AVERAGE BEETLES PER TRAP AT INCREASING DISTANCE FROM RELEASE

Distance (meters)	No treatment	Food only	Flight only	Food and flight	Held 24 hr
32	15.6	5.2	2.7	0.6	0.8
72-97	3.9	1.0	0.7	2.2	0.7
117-162	1.5	0.8	0.6	0.2	0.3
162-227	3.0	1.7	0.9	0.8	0.5
305	3.6	1.8	0.7	1.2	0.7
Totals:					
Released	9767	9350	8600	8615	7975
Trapped	783	352	209	108	108
Percent	8	3.8	2.4	1.3	1.4

of the marked beetles recovered, 70% were trapped in the first 6 hr after release. Within this time, the maximum catch was 209 in the first hour after release, followed by 102 at 5–7 PM, and 66 at 7–9 PM. Some marked beetles not caught the first evening remained in the area. Catches of these in the following 3 days were 141, 5, and 18.

Large numbers of endemic *S. multistriatus* were captured in all of the traps, mostly between 7 and 9 PM. Overnight trapping between 10 PM, July 29, and 6 AM, July 30, caught only one endemic beetle and no marked beetles.

The decrease in numbers trapped on successive days occurred in both marked beetles and the endemic population. The population of marked beetles was fixed, but endemic beetles could continue to fly into the field. However, the total of 3213 endemic beetles trapped from 4 to 10 PM July 28 decreased to 2356 between 4 to 10 PM July 29, with a further drop to 2104 trapped mostly from 4 to 10 PM July 30, but not collected until 10 AM July 31. The observed decrease in numbers of endemic beetles may reflect a depletion of the local population by trapping, a reduction in wind-aided immigration, or lower rates of emergence from local brood sources.

A close examination of the data suggests that strong and variable winds cause only brief interruptions of the attraction of the beetles to the Multilure-baited traps. For example, consider the 5–10 PM beetle catches of July 28. These catches were separated into four areas from the marked beetle release-point outward (Table 4). The highly variable winds on that day did not prevent endemic beetles from reaching every trap; furthermore, only one trap had a zero count for marked beetles. The marked beetles released at the plot center responded to Multilure in all directions. The number per trap decreased to the edge of the plot, where the catch increased, probably because there were no emitters of attractants (i.e., pheromone, baits, diseased trees) beyond that line. The reverse of this situation is seen in the trapping of endemic beetles, which were most numerous at the periphery of the plot.

Although about half of the marked beetles captured were on the most distant traps at 162–227 m, the largest catch per trap was at 32 m. This indicates that some beetles responded to the pheromone immediately upon detecting it. Similarly, most of the endemic beetles flying into the area responded to the first traps they encountered as shown by the highest per trap average. These results are in partial agreement with those reported by Lanier et al. (1976); in their studies, indigenous beetles were captured in greatest numbers at an outer circle of traps, and the largest number of beetles emerging from elm logs were recaptured at 400 and 600 m from the emergence site. They concluded that *S. multistriatus* undergoes a dispersal flight after emergence that is a prerequisite to pheromone response. A dispersal flight requirement has been noted for other scolytids (Gara, 1963; Bedard,

TABLE 4. CAPTURES IN 6 HR OF MARKED AND ENDEMIC BEETLES ON MULTILURE-BAITED TRAPS IN FOUR AREAS OF THE 8 × 8 LATTICE DESIGN PLOT[a]

Marked beetles		Distance from release at plot center (m)	Endemic beetles	
Total	Avg./trap		Total	Avg./trap
76	19.0	32 (4 traps)	65	16.3
80	6.7	72-97 (12 traps)	162	13.5
91	4.6	117-162 (28 traps)	505	25.3
238	8.5	162-227 (20 traps)	2486	88.8
485			3218	

[a]Marked beetles released at plot center.

1966; Hertel et al., 1969; Hosking and Knight, 1975), and a dispersal flight probably accounts for the higher catches of released beetles at the more distant traps in this study.

Flight studies have shown that beetles were caught at distances ranging from 20 to 600 m (Lanier at al., 1976). Variability in response to pheromone has been noted for other scolytidae (Vité and Gara, 1962; Bedard, 1966), so it is not surprising that variable responsiveness would be recorded for *S. multistriatus*. Catches at both near and far traps (Table 4) could be the result of both beetle variability and treatment. The no-treatment beetles show a wider range of trap response in three releases than is seen in any of the treated beetle groups, although the generally lower catches of the latter could account for the smaller ranges of response. Low recovery of marked insects released in other studies is often reported. In my studies only 485 beetles were caught during 6 hr after release of 21,207. However, in this time, 3218 endemic beetles that flew into the area responded to the Multilure-baited traps.

CONCLUSIONS

Difficulty has been encountered in appraising the efficiency of pheromone-baited traps because of the variation in beetle behavior. Beetles released with no treatment responded as if they had two patterns of behavior: dispersing and nondispersing. About half flew to the nearest traps, the remainder flying to all other traps at distances to 305 m. They also differed in numbers caught at the nearest traps in three releases.

The treated beetles showed that feeding was not necessary before the beetles responded to Multilure. This finding agrees with those by Lanier et al. (1976), whose analysis of guts showed that most beetles had not fed in twig crotches prior to reaching pheromone-baited traps. This information

has practical value, because it can be assumed that, upon emergence, beetles may fly to a pheromone source (such as baited traps) without first feeding in healthy trees.

Flight exercise is probably essential for beetles to respond to pheromone, as indicated by the flight of a large portion of the released beetles beyond traps that were relatively close to the point of release. The effect of differential air currents on molecules of the pheromone and a direct effect of fluctuating winds on beetle flights may influence responses to the pheromone. These factors would also affect endemic beetles approaching the plot, causing some to fly beyond the first trap encountered.

The very low recovery of released beetles is attributed to their free flight above and beyond the traps that were in a fixed location and in a relatively small area. In the first 2 days of one release the recaptures were 385 and 141, whereas the numbers of endemic beetles caught were 3213 and 3055. The decrease in recaptures of released beetles implies a dispersal flight. The consistent captures of endemic beetles suggests that the efficiency of Multilure-baited traps in catching endemic beetles cannot be determined because their population size is not known. An estimate would be possible when results of continuous trapping were compared with the incidence of Dutch elm disease in the area.

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IDENTIFICATION OF THE MAJOR COMPONENTS IN THE SECRETION FROM THE RECTAL PHEROMONE GLANDS OF THE QUEENSLAND FRUIT FLIES *Dacus tryoni* AND *Dacus neohumeralis* (DIPTERA:TEPHRITIDAE)

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Abstract—The secretion from the rectal pheromone glands of male *Dacus tryoni* and *Dacus neohumeralis* is largely a mixture of six aliphatic amides. In order of decreasing quantity these are *N*-3-methylbutylpropanamide, *N*-3-methylbutylacetamide, *N*-(3-methylbutyl-2-methylpropanamide, *N*-2-methylbutylpropanamide, *N*-2-methylbutylacetamide, and *N*-(2-methylbutyl)-2-methylpropanamide. The proportions of the various amides in the two species are similar.

Key Words—Queensland fruit fly, *Dacus tryoni*, *D. neohumeralis*, Diptera, Tiphritidae, rectal pheromone gland, aliphatic amides, sex pheromone.

INTRODUCTION

The common name, Queensland fruit fly, is used for two very closely related species of *Dacus*, *D. tryoni* (Froggatt) and *D. neohumeralis* (Perkins), which breed in a wide range of native and introduced fruits in the eastern part of Australia (May, 1963). In the northern part of their range they occur sympatrically and infest a similar range of hosts. However *D. neohumeralis* only occurs as far south as northern New South Wales whereas the range of *D. tryoni* extends to East Gippsland in northern Victoria.

In the laboratory the two species produce fertile hybrids, but in the wild they seem to be reproductively isolated (Vogt, 1977); the main barrier to cross mating appears to be a difference in mating times (Gibbs, 1965). *D.*

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neohumeralis normally mates at high light intensities during the middle of the day whereas *D. tryoni* will only mate under a low light intensity at dusk (Tychsen and Fletcher, 1971). In both species sexually excited males emit an oily secretion with a sweet pungent odor which acts as a sex pheromone. The source of this secretion is a glandular complex with an associated reservoir which opens into the rectum (Fletcher, 1969). Similar gland structures are known to occur in some other species of this economically important genus (Fletcher, 1969; Schultz and Boush, 1971).

We examined the composition of the rectal pheromone gland secretion of *D. tryoni* and *D. neohumeralis* to determine whether they are the same or different. Any differences might act as a further barrier to mating between the two species. In addition, it is possible that pheromone components might be of use in control programs for the two species, particularly in view of the successful use of the synthetic attractants methyl eugenol and 4-*p*-acetyloxyphenylbutan-2-one in monitoring and suppression programs for fruit flies (Steiner et al., 1965; Bateman et al., 1966).

METHODS AND MATERIALS

The infrared spectra were measured in CCl_4 solution with a Perkin Elmer model 221 spectrophotometer. The ultraviolet spectra were determined in ethanol solution on a Perkin Elmer model 402 spectrophotometer. Mass spectra were measured with a GEC-AEI MS-902 mass spectrometer to which was connected a Philips Research model PV4000 gas chromatograph. For high-resolution measurements the output from the mass spectrometer was fed through an A/D converter to an online Raytheon 706 computer.

Preparative gas chromatography was conducted on a Varian 1200 instrument which had been modified to incorporate an annular splitter (Brownlee and Silverstein, 1968). Samples were collected in glass capillaries with the collection zone cooled with dry ice.

Kováts retention indices were measured on a Varian 2100 gas chromatograph. Retention times were measured with a Hewlett-Packard model 3370A integrator. Calculations were made on a CDC 3600 computer with use of the program KLINDEX (Bellas, 1975). Unless otherwise indicated the gas chromatography support was Gas-chrom Z of 80/100 mesh, the phase loading was 5%, columns were made of stainless steel 2 m × 2.3 mm and the carrier gas was helium at a flow rate of about 20 cm^3/min.

Collection of Pheromone Gland Secretion and Separation of Components for Spectrophotometry. The secretion was collected from the pheromone glands of mature male specimens of *D. tryoni* and *D. neohumeralis* which had been reared on standard artificial diet (Bateman, 1968)

and fed as adults on protein hydrolysate, sugar, and water. Some samples were also obtained from field-collected mature male *D. tryoni* for comparison with the laboratory stocks. After dissection of the reservoir, the pheromone gland secretions were collected in micropipettes and were stored as solutions in ether. The ether phase was withdrawn from the small quantity of water also present and filtered through anhydrous $MgSO_4$. The dried solutions were concentrated by slow distillation of the solvent through a short fractionating column.

Samples for spectrophotometry were collected by preparative gas chromatography. Injections of an ether solution containing about 8 male equivalents of the secretion from *D. tryoni* onto a column containing OV-225 at 140° afforded the major components as two peaks with retention times of 12.0 and 14.4 min. These components were collected in one tube and were washed from the tube with the appropriate solvent. UV (ethanol), end absorption; IR ν_{max} (CCl_4 solution, 5× scale expansion) 3460, 2960, 2930, 2875, 1680 cm^{-1}.

The columns used for GC-MS were made of stainless steel and were 2 m or 4.2 m × 2.3 mm. The stationary phases were OV-1 (on Gas-chrom Q) and Carbowax 20M. Conditions were chosen so that each peak took at least one minute to elute.

Determination of Ratios of Components in Individual Flies. The gland content of each fly was collected in a micropipette and dissolved in about 100 mm^3 of acetone. Each sample was injected onto two columns: OV-225 at 140° which resolved the amides into two peaks, and terephthalic acid-terminated Carbowax 20M at 130° which resolved them into three peaks. The carrier gas was helium at a flow rate of 20 cm^3/min in each case. For each peak a relationship between the peak height and the area under the curve was derived using solutions of mixtures of the synthetic amides. The areas were measured with a planimeter on eightfold time-expanded traces. The relative areas for the fly material were then determined by measuring the peak heights on the two traces for the secretion from each animal.

Synthesis of Amides. The amides were prepared by one of two methods. Propanamides were prepared by heating the amine with propanoic anhydride. Acetamides and 2-methylpropanamides were prepared from the amines and the acid chlorides. The amides were purified by gas chromatography before measuring the mass spectra. The mass spectra of the synthesized amides are listed in Table 1.

Measurement of Retention Indices. The columns and conditions used for determining the retention indices were A: 2 m × 4 mm glass, Carbowax 20M at 125°, B: same column at 140°, C: 2 m × 3 mm glass, EGSS-X at 125°, D: 3.7 m × 3 mm glass, Carbowax 20M at 130°, E: same column at 150°. The carrier gas was nitrogen at a flow rate of 25 cm^3/min.

TABLE 1. MASS SPECTRA OF SYNTHESIZED AMIDES[a]

N-3-Methylbutylacetamide: $M^{+\cdot}$ 129.11574, calculated for $C_7H_{15}NO$, 129.11537: *m/z* 129(11%), 114(4), 86(11), 73(36), 72(31), 60(17), 44(25), 43(25), 30(100).

N-3-Methylbutylpropanamide: $M^{+\cdot}$ 143.13107, calculated for $C_8H_{17}NO$, 143.13102: *m/z* 143(13%), 128(8), 114(4), 100(10), 87(44), 86(31), 74(20), 57(44), 44(41), 43(23), 30(100).

N-(3-Methylbutyl)-2-methylpropanamide: $M^{+\cdot}$ 157.14669, calculated for $C_9H_{19}NO$, 157.14667: *m/z* 157(18%), 142(13), 114(13), 101(37), 100(11), 88(17), 72(25), 71(67), 44(47), 43(100), 41(18), 30(34).

N-Pentylacetamide($C_7H_{15}NO$): $M^{+\cdot}$ 129.11573: *m/z* 129(13%), 114(2), 100(7), 73(21), 72(29), 60(10), 44(16), 43(24), 30(100).

N-Pentylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13108: *m/z* 143(22%), 128(1), 114(22), 100(7), 87(35), 86(33), 74(11), 57(49), 44(32), 43(25), 30(100), 29(14).

N-Pentyl-2-methylpropanamide($C_9H_{19}NO$): $M^{+\cdot}$ 157.14689: *m/z* 157(23%), 142(3), 128(21), 114(16), 101(24), 100(12), 88(12), 72(29), 71(75), 44(34), 43(100), 30(40).

N-2-Methylbutylacetamide($C_7H_{15}NO$): M^{+} 129.11550: *m/z* 129(10%), 114(<1), 100(10), 73(20), 72(35), 60(24), 43(20), 30(100).

N-2-Methylbutylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13111: *m/z* 143(9%), 128(<1), 114(9), 87(18), 86(32), 74(31), 58(12), 57(36), 44(8), 43(10), 30(100), 29(29).

N-(2-Methylbutyl)-2-methylpropanamide($C_9H_{19}NO$): $M^{+\cdot}$ 157.14645: *m/z* 157(29%), 142(2), 128(9), 114(5), 101(19), 100(23), 88(67), 72(49), 71(88), 58(20), 44(10), 43(100), 41(21), 30(56).

N-1-Methylbutylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13089; *m/z* 143(6%), 114(5), 101(10), 100(23), 74(10), 72(14), 57(15), 45(28), 44(100), 43(11), 29(17).

N-1-Ethylpropylpropanamide($C_8H_{17}NO$): $M^{+\cdot}$ 143.13105: *m/z* 143(7%), 114(19), 74(7), 58(100), 57(11), 29(12).

[a] The high-resolution spectra were measured in the Mass Spectrometry Unit of the Division of Entomology. Only the major and diagnostic ions are given for the low-resolution spectra but high-resolution data are available for most ions above *m/z* 38 of relative abundance greater than 1% of the base peak. The complete low resolution spectra have been submitted to the Mass Spectrometry Centre, Aldermaston, U.K.

RESULTS

Gas chromatography on four phases (OV-225 at 140°, OV-1 at 110°, PEGA at 160°, and Carbowax 20M at 120°) indicated the presence in the secretion collected from *D. tryoni* of at least four components which had similar retention characteristics. The mixture of these components collected from the gas chromatograph showed only end absorption in the UV. Mass spectrometry-gas chromatography showed molecular ions at *m/z* 143 and 129 for the major components of the two large peaks that were resolved on the OV-1 column used. A high-resolution spectrum of the major component gave the molecular ion as *m/z* 143.13029. $C_8H_{17}NO$ requires 143.13102 and the majority of the fragment ions contained both N and O. These results and the presence in the infrared spectrum of absorptions at 1680 cm^{-1} and 3460 cm^{-1} suggested that the compound was an *N*-substituted primary amide.

One freshly prepared Carbowax 20M 4.2m column resolved the four

major components in the rectal pheromone gland secretion of *D. tryoni* and these four were estimated to form more than 95% of the total volatile material from the glands (Figure 1). The separation of the amides obtained on Carbowax columns deteriorated rapidly with age and use. Within several days of use the major propanamide and acetamide (3P and 3A in Figure 1) became coincident. It was on an aged column that the ratios of components in individual flies were measured. The retention times at 140° for these four peaks were 21.2, 23.0, 24.4, and 27.2 min. The molecular ions were at m/z 157, 143, 129, and 143. The spectra of these four components suggested that they were all amides, and a fragment ion attributable to the protonated primary amide ($RCONH_3^+$) appeared at m/z 88, 74, 60, and 74, respectively. The amine moiety in each component thus contained five carbon atoms.

The fragmentation patterns for the three components of greatest amount were similar and included a substantial loss of methyl with subsequent loss of C_3H_6. The lack, in the spectrum of the component of highest

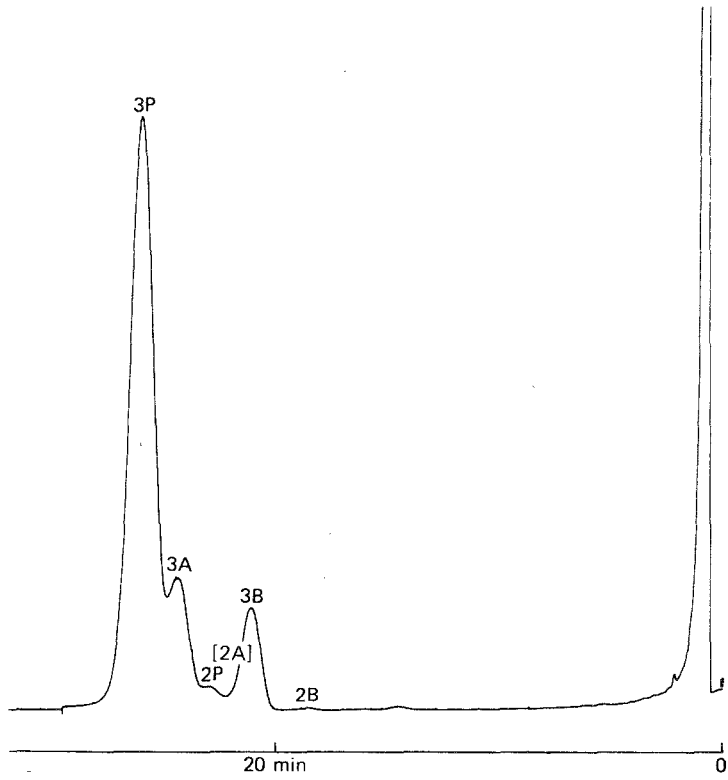

FIG. 1. Gas-chromatograph trace of volatiles from the rectal gland secretion of *Dacus tryoni*. Conditions: 4.2-m column containing 5% Carbowax 20M at 120°.

molecular weight, of a peak corresponding to a McLafferty rearrangement to give an ion at M-28 showed that the 4-carbon acyl fragment was derived from 2-methylpropanoic acid. These three components were therefore in order of abundance *N*-3-methylbutylpropanamide (3P), *N*-3-methylbutylacetamide (3A), and *N*-(3-methylbutyl)-2-methylpropanamide (3B).

The smallest peak of the four, some 3% of the total, with a molecular ion at m/z 143, was a propanamide. The mass spectrum lacked the prominent loss of CH_3 shown by the *N*-3-methylbutyl series of amides but did show a loss of C_2H_5. This indicated either a straight chain or a 2-methylbutyl substituent. The spectrum of this component corresponded to that of *N*-2-methylbutylpropanamide (2P) but not to those of the propanamides derived from 1-, 2-, or 3-pentanamine. The presence of this propanamide prompted a search for the corresponding *N*-2-methylbutylacetamide (2A) and *N*-(2-methylbutyl)-2-methylpropanamide (2B) which were found to be present in even smaller amounts.

Coinjection of synthetic 2A and 2B with the rectal gland secretion showed that 2B was coincident with a small component which in Figure 1 is at 18.4 min, while 2A appeared between 2P and 3B. The mass spectrum of this small component was very similar to that of synthetic 2B. The first peak (retention time 12.0 min) to emerge from an OV-225 column at 140° was collected and reinjected for GC-MS onto a 4.2 m Carbowax 20M column at 150°. Two peaks, in a ratio of about 1:15, emerged at retention times of 30.3 and 34.3 min. The mass spectrum of the first component was very similar to that of 2A. The second component was 3A.

The secretion from *D. neohumeralis* was analyzed by gas chromatography and mass spectrometry and the same six amides were identified. In this species too they formed the major part of the volatiles of the rectal gland secretion.

The identity of the six amides was confirmed by a comparison of the retention indices (Wehrli and Kováts, 1959) and mass spectra with those of authentic materials. With the exception of the minor 2-methylpropanamide (2B), the retention index of which was measured using the fly secretions directly, the amides from the secretions were first isolated using gas chromatography by first separating the acetamides from the other four amides on OV-225 at 140° and then separating these two fractions into their components on Carbowax 20M at 110° before determining the retention indices.

Retention indices (compound, conditions, index of natural compound, index of synthetic compound) were as follows:

Dacus tryoni: (3A) A, 1875,1874; (3P) A, 1884,1884; (3B) A, 1839,1838; (2P) A, 1859,1857; (2A) E, 1831,1831; (2B) E, 1797,1796.

Dacus neohumeralis: (3A) B, 1878,1880, C, 2192,2188; (3P) B, 1890,1891, C, 2192,2191; (3B) B, 1843,1841, C, 2131,2131; (2P) B, 1862,1862, C, 2174,2172; (2A) D, 1896,1896; (2B) D, 1852,1853.

Determination of Ratios of the Six Amides in Individual Males. The OV-225 column separated the amides into two peaks: the two acetamides (2A+3A) with a retention time of 6.5 min and the other four amides (3P+2P+2B+3B), which eluted as a single peak, with a retention time of 7.8 min. The Carbowax 20M column separated the amides into three peaks; (2B+3B) with a retention time of 10.8 min, (2A+2P) 11.9 min, and (3A+3P) 13.5 min.

The percentages of acetamides and 2-methylpropanamides were obtained directly by measurements of their peak size, and the figure for the propanamides was then obtained by subtraction of these from the total. These data are given in Table 2.

The secretion obtained from mature *D. tryoni* males caught in the wild contained the same compounds in about the same proportions as the laboratory-reared males.

DISCUSSION

Simple amides have not previously been reported from insects. *N*-3-Methylbutylacetamide has been identified in a Californian sherry (Webb et al. 1966) and in tobacco (Demole and Berthet, 1972). Both of the acetamides have been isolated from wines (Schreier et al., 1975).

The presence of 3-methylbutyl and of 2-methylbutyl residues parallels the presence of the corresponding amyl alcohols in fermentation products, where it has been shown, for example, in brewer's yeast (Ayrapaa, 1967), that the alcohols are derived from the amino acids leucine and isoleucine. Tracer experiments also demonstrated that the 3-methylbutanamine found in the ergot fungus *Claviceps purpurea* is formed from the leucine in the substrate, although 2-methylbutanamine was not found (Hartmann, 1965). It, therefore, appears likely that the ultimate sources of the two amine residues in *D. tryoni* and *D. neohumeralis* are also leucine and isoleucine, although

TABLE 2. PROPORTIONS OF 2-METHYLPROPANAMIDES (B), ACETAMIDES (A), AND PROPANAMIDES (P) IN INDIVIDUAL MALE FLIES OF *D. tryoni* AND *D. neohumeralis*

	3B + 2B[a]	3A + 2A	3P + 2P
D. tryoni (10 flies)	5.2 ± 2.2[b]	20.8 ± 3.5	74.0 ± 3.9
D. neohumeralis (14 flies)	3.4 ± 1.4[b]	19.4 ± 6.0	77.1 ± 6.7

[a]3 indicates a 3-methylbutyl and 2 indicates a 2-methylbutyl substituent.
[b]Mean percentage with standard deviation for the amide pair.

it is not known whether the decarboxylation is accomplished by the fly or whether the amines are derived from the food. The aliphatic acyl residues are well known from biological systems (Kahn, 1969). The source of the food appears to be unimportant, at least for the amide components, since both the laboratory-reared and the wild males of *D. tryoni* have very similar secretions.

Since both species have the same amides in about the same proportions, it would appear that these compounds are unimportant in the reproductive isolation of the two species. Behavioral studies (Fletcher and Bellas, unpublished) indicate that the mixture of amides may act as a short-range pheromone which increases the sexual excitement of the female once she has arrived in the vicinity of the males but does not act as an attractant acting over a distance. It is known, however, that other volatile compounds are present in the male pheromone gland secretion, since none of the amides has the characteristic sweet odor of the male flies, and that the total extract acts as an attractant over several meters (Fletcher, 1977). It is not yet possible to say, therefore, whether the pheromones from the male rectal gland play any role in the isolation of the two species. However, because of the differences in mating times and the associated rhythmic response of the females to the pheromone (Fletcher and Giannakakis, 1973), it is quite possible that the males of the two species do use exactly the same blend of chemicals in their pheromone. A preliminary gas chromatographic survey of the secretions of the rectal glands of *D. musae* (Tryon), *D. cucumis* French, *D. jarvisi* (Tryon), and *D. absonifacies* May, which all live in the same area and mate at about the same time as *D. tryoni* (i.e., around dusk), has shown that each species has a quite different pattern of components in its secretion.

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EVIDENCE FOR A SEX PHEROMONE IN THE HIDE BEETLE, *Dermestes maculatus* (DE GEER) (COLEOPTERA:DERMESTIDAE)[1]

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Abstract—Adults of the hide beetle, *Dermestes maculatus* (De Geer), were shown to have a female-produced sex pheromone which excited males. Male response was positively correlated with increasing age, but females produced a higher level of pheromone at 6–8 days of age than at younger or older ages. Extractable female pheromone and male response varied over the photophase with peak values that occurred during the latter hours. Quantitative bioassay indicated that the 50% male response level (RD_{50}) would be elicited by a pheromone exposure of 0.01 female equivalents (FE). Females extracted 24 hr after mating had a lower level of extractable pheromone than did virgin females of the same age.

Key Words—*Dermestes maculatus*, Coleoptera, Dermestidae, pheromone, behavior, bioassay, circadian rhythm.

INTRODUCTION

In the Dermestidae, Bar-Ilan (Finger) et al. (1965), and Burkholder and Dicke (1966) first demonstrated the existence of sex pheromones in the genera *Trogoderma* and *Attagenus*, respectively. The first-identified dermestid pheromone was that of the black carpet beetle, *Attagenus megatoma* (F). (Silverstein et al., 1967). The compound was found to be the conjugated fatty acid (*E,Z*)-3,5-tetradecadienoic acid. Since then several other dermestid

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pheromones have been demonstrated and identified in the genera *Anthrenus*, *Attagenus* and *Trogoderma*.

Despite the economic importance of the hide beetle, *Dermestes maculatus* (De Geer), in certain parts of the world (Hinton, 1945; Davey, 1965; Mushi and Chang, 1974), nothing is known about the existence of a pheromone communication system. The present study was designed to investigate the possible existence of a sex pheromone and factors influencing the production of, and response to, such a compound.

METHODS AND MATERIALS

Beetles for this study were obtained from a laboratory culture maintained in the Stored Product and Household Insects Laboratory, SEA, at the University of Wisconsin-Madison. The insects were reared on Ken-L-Ration moist dog food. One ca. 28-g package was used per culture. The cultures were contained in ca. 1-liter wide-mouth canning jars maintained at $30 \pm 1°C$ and $60 \pm 10\%$ relative humidity with a photoperiod regimen of LD 12:12.

At intervals of 7 days after the insects had begun to pupate (ca. 5 weeks), the pupae were separated and sexed according to the structure of the genital lobes. Sexed pupae were held in 9-cm-diam. petri dishes lined with Whatman No. 1 filter paper. Female pupae were held in the rearing room and male pupae in a separate female-free incubator under identical conditions. Both pupal groups were examined at 2-day intervals and adults were removed, held in petri dishes, and given an ascribed age of 0–2 days.

Solvent extracts of male and female beetles were prepared by extracting 10 virgin adults of known age and sex in 10 ml of distilled hexanes in a 3-dr screw-top vial. The tops of the vial caps were foil-lined to avoid solvent loss or contamination. The solutions were held 24 hr in a refrigerator, after which time the insects were removed. Extracts were stored at $-20°C$ when not in use. A vial of hexane held under the same conditions served as a control.

The olfactometer used in this study was a vial type, similar to that used by Burkholder (1970), except that larger vials (22×85 mm) were used to accommodate the larger *D. maculatus*. One day before a bioassay was to be conducted, the beetles were placed in individual olfactometer vials which had been actone washed and oven dried to reduce contamination. Male-containing vials were stored in a female-free incubator until needed. Bioassays were conducted using a technique similar to Burkholder (1970).

Insects were exposed to beetle extracts for 1 min. A positive response was recorded if the insect became aroused and showed locomotory movements. Only restive insects were used in the bioassay. Unless otherwise indicated, the concentration of the female extract was 0.01 female equivalents (FE) per 10-μl aliquot per replicate. Ten beetles were tested per replicate.

Evidence for a female-produced pheromone was obtained by bioassaying males against male and female extracts and a control, and females against male and female extracts and a control. For each comparison 5 replicates of 10 beetles each were used. Assays were conducted between 1300 and 1500 hr unless otherwise indicated.

To study the influence of age on male response to female extract, males of various ages from 0–2 to 12–14 days old were bioassayed against an extract of 6 to 8-day-old virgin females. For each age group 10 replicates were tested.

To determine the effect of female age on pheromone content, female extracts were prepared from virgin females of various ages from 0–2 to 30–32 days old. The extracts were tested against 5 replicates of 10 to 14-day-old males for each female age group.

Diurnal variation in female pheromone content was determined by extracting 4 to 8-day-old females at 2-hr intervals over the photophase beginning at 0700. The extracts were then tested against 5 replicates of 8 to 12-day-old males for each extract.

Male response during the photophase was observed by bioassaying 5 replicates of 8 to 12-day-old males at each 2-hr interval over the photophase against an extract of 6 to 8-day-old females.

A quantitative relationship between FE concentration and male response was determined by bioassaying 5 replicates each against various concentrations of female extract ranging between 1.0×10^{-2} and 6.5×10^{-4} FE. The extract was prepared from 6 to 8-day old virgin females.

To determine the effect of mating on extractable pheromone content, ten 6-day-old females were mated individually and extracted 1 hr after the mating. A second group of ten 6-day-old females was mated and extracted 24 hr later. As controls, 6- and 7-day old virgin females were extracted. The extract was bioassayed against 3 or 5 replicates of ten, 10 to 12-day-old males each. Matings and extracts were made between 1300 and 1500 hr.

All bioassays were conducted in a separate room maintained at 24°C and 50% relative humidity.

RESULTS

Male Response Behavior. The characteristic male response to a source of female odor was similar to that reported in several other dermestids. The males exhibited a sequence of events which included antennal protraction, leg extension, bobbing up and down, and running in a zig-zag manner beneath the pheromone source.

Evidence for a Female-Produced Pheromone. The data are presented in Figure 1. The response of female beetles to male extract, female extract, or controls was not significantly different at a level of probability of 0.95. Male

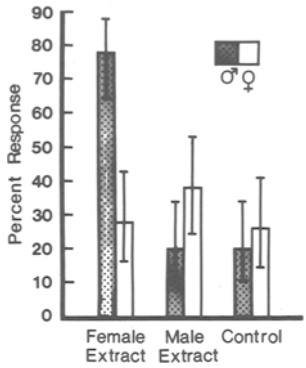

FIG. 1. Response of virgin *D. maculatus* males and females to extracts of virgin males and females. Vertical lines indicate the 0.95 binomial confidence intervals about each value.

response to male extract or control was not significantly different at a level of 0.95, but males responded at a significantly higher level (78% at $P = 0.95$) to female extracts. At the concentration tested (0.01 FE) it is obvious that female extracts were clearly exciting to male insects.

Response of Aged Males to a Pheromone Source. The data of Figure 2 show that males of 0-2 days of age responded at a level of less than 20%. As males became older, the response level increased progressively to a high of 70% with males 12-14 days of age, the oldest age tested. Males older than 14 days could not be tested in this manner because they were too active. The response of males at least 10 days old was significantly greater than males of the younger age groups.

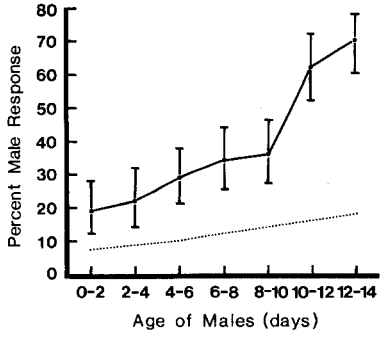

FIG. 2. Response of *D. maculatus* males of various ages to pheromone extract of virgin females 6-8 days old. Vertical lines indicate the 0.95 binomial confidence intervals about each point. Dotted line indicates control response.

FIG. 3. Response of *D. maculatus* males to pheromone extracts of females of various ages. Vertical lines indicate 0.95 binomial confidence intervals about each point. Dotted line indicates control response.

Male Response to Females of Various Ages. The data are presented in Figure 3. Females as young as 0–2 days of age contained a quantity of extractable pheromone which gradually increased and reached a peak value with females 6–8 days of age. After this time the male response gradually decreased to a low level, indicating a decline in pheromone production with age. At the age peak of pheromone production, the male percent response was significantly greater than to extracts made from females less than 2 days of age or older than 20 days.

Extractable Female Pheromone Content Over the Photophase. As estimated by percent male response, females extracted at the earlier hours of the photophase had a lower pheromone titer than females extracted at the end of the photophase. Response of males increased from 28% to female extract made at 0700 hr to 60% to female extract made at 1900 hr. The data are illustrated in Figure 4. Male response to the extracts of the first 6 hr were similarly lower than to extracts made during the last 4 hr, thus indicating that a significant increase in pheromone production took place at about 1500 hr.

Male Response Over the Photophase. The data of Figure 5 show that an increase in male response took place as the photophase progressed. At 0900 hr a minimum response level of 26% was observed. The response level increased to a maximum of 70% at 1500 hr. This corresponded to the time at which extractable female pheromone content showed an apparent increase. After this time the male response declined to the same level as at the beginning of the photophase. The increased male responsiveness during 1300–1500 hr was significantly greater than that observed during the first two and last hour of the photophase.

Quantitative Bioassay. Figure 6 illustrates the quantitative relationship between male response and log concentration of FE of the female extract.

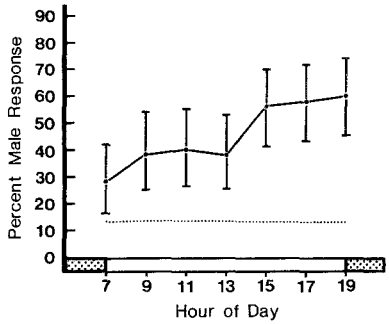

FIG. 4. Response of *D. maculatus* males to pheromone extracts made at 2-hr intervals during the photophase. Vertical lines indicate the 0.95 binomial confidence limit about each point. Dotted line indicates control response.

Probit regression of raw data resulted in a 50% response threshold (RD_{50}) of 0.0051 FE after a 1-min exposure to the pheromone source. Correction of the data by Abbott's formula for control response, $[P = (P' - c)/(1 - c)]$, resulted in a RD_{50} of 0.01 FE and a calculated 26% increase in male response for an increase in log dose of 1 order of magnitude.

Effect of Mating on Extractable Pheromone Content of Females. The data are presented in Table 1. Females that had been mated 1 hr prior to extraction did not differ significantly in extractable pheromone content from virgin females of the same age. However, females extracted 24 hr after mating had a lower extractable pheromone content than virgin females of the same age. Male response was lower ($P = 0.05$) to the extracts of females mated for 24 hr prior to extraction. After correction for control response of 14% by Abbott's formula, a response differential of 24% indicated a decrease in pheromone content of 1 order of magnitude one day after the females were mated.

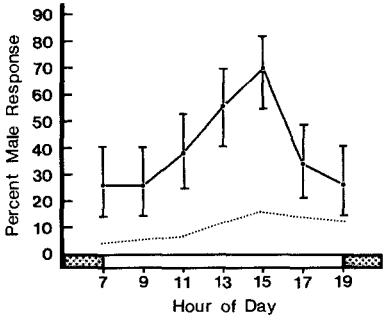

FIG. 5. Response of *D. maculatus* males at 2-hr intervals over the photophase to a single pheromone extract. Vertical lines indicate the 0.95 binomial confidence interval about each point. Dotted line indicates control response.

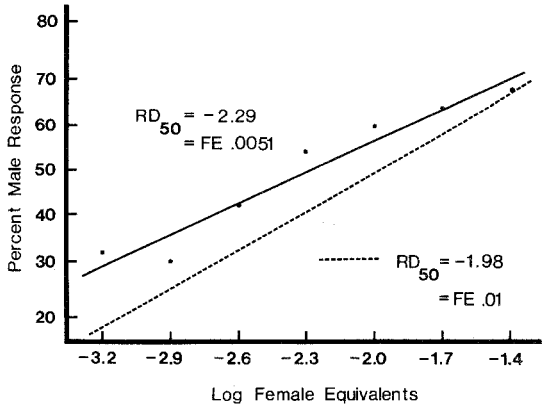

FIG. 6. Probit response of *D. maculatus* males to different concentrations of a pheromone extract of virgin females. The calculated RD_{50} was 0.0051 FE. Dashed line is probit regression after correction for control response. The calculated RD_{50} after correction was 0.01 FE, Probit = 6.45 + 0.73 × log FE.

DISCUSSION

The evidence for the existence of a female-produced sex pheromone attractive to males is here reported. As shown in Figure 1, females did not significantly respond to males or other females, but males responded significantly to female extracts as compared to male extracts or a control.

The response (antennal protraction, leg extension, and rapid zig-zag running) was similar to that reported for other dermestids (Burkholder and Dicke, 1966; Barak and Burkholder, 1977) and is a function of increasing pheromone concentration which releases specific sequences of behavior (Bartell and Shorey, 1969; Levinson, 1975).

As with some other dermestids, the female pheromone production and titer are evidenced at a younger age than the male response (Figures 2 and 3).

TABLE 1. RESPONSE OF 10 TO 12-DAY OLD MALES TO PHEROMONE EXTRACTS (0.01 FE) OF VIRGIN FEMALES AND FEMALES EXTRACTED 1 AND 24 HR AFTER MATING

	Source of female extract			
	6-day-old females		7-day-old females	
Bioassay results	Virgin	Mated 1 hr	Virgin	Mated 24 hr
Males tested[a]	30	50	30	50
Percent male response	50	40	70	50
Binomial limits	32–68	26–55	51–85	36–64

[a]Males tested in replicates of 10.

Both male responsiveness and extractable female pheromone showed significant variation over the photophase. This was expected, since Hammack and Burkholder (1976) and Hammack et al. (1976) demonstrated the circadian nature of pheromone-related female calling behavior in another dermestid, *Trogoderma glabrum* Herbst. Similar apparent circadian female rhythmicity was also demonstrated in several species of the genera *Attagenus*, *Anthrenus*, and *Trogoderma* (Burkholder et al., 1974; Barak and Burkholder, 1977, 1978; Cross et al., 1977; Ma and Burkholder, 1978). The male response cycle correlated with the female pheromone content and, as in *Attagenus* and *Trogoderma* (Vick et al., 1973), is likely also to be circadian and photoperiodically entrained.

Behavior similar to calling was observed in *D. maculatus* females. Females held in culture jars, if undisturbed, were noted at high places in a motionless state with protracted antennae and legs extended. This is not surprising, since photoperiodic variations in pheromone content and male response appears to be part of a package of behavioral adaptations which include calling and since Hammack et al. (1973) demonstrated abdominal sternite localization of pheromone-producing glandular tissues in 6 species of *Trogoderma.* Calling has been unquestionably linked to increased pheromone production and release in numerous dermestids and other Insecta.

After correction for control response by Abbott's formula, it was calculated that the pheromone content of females during the last hour of the photophase was 29 times that of females during the first hour. Similarly, male sensitivity increased 35-fold between the peak hour (1500) of response and the early hours of the photophase.

The possibility exists that a sex pheromone of *D. maculatus* could be useful in detecting populations, infestations, or the possibility of infestation in areas where this insect is of economic significance. Earlier, Barak and Burkholder (1976) demonstrated that certain other dermestid pheromones (*A. megatoma, Trogoderma inclusum* Le Conte) were useful in establishing seasonal emergence patterns of adults and indicating areas of highest insect activity in warehouse and storage facilities.

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EFFECTS OF THREE PHENOLIC ACIDS ON CHLOROPHYLL CONTENT AND GROWTH OF SOYBEAN AND GRAIN SORGHUM SEEDLINGS

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Abstract—Experiments were designed to test the hypothesis that interference with chlorophyll metabolism may be one mechanism of inhibition of plant growth in allelopathic interactions. Effects of ferulic, *p*-coumaric, and vanillic acids on soybean and grain sorghum growth and chlorophyll content were quantified and compared after seedlings were treated with these compounds in a nutrient culture. Following a 6-day treatment cycle, dry weights of soybean seedlings were reduced by both 10^{-3} M and 5×10^{-4} M treatments of ferulic, *p*-coumaric and vanillic acids. Soybean weight reductions in each case were paralleled by a significant reduction in the concentration (μg Chl/mg dry wt) of chlorophylls a and b and total chlorophyll in the unifoliate leaves. Sorghum seedling growth was also reduced by each of the compounds at the 5×10^{-4} M level, but leaf chlorophyll concentration was not below that of control plants.

Key Words—Ferulic acid, *p*-coumaric acid, vanillic acid, chlorophyll a, chlorophyll b, allelopathy, inhibitors, soybean, sorghum.

INTRODUCTION

Even though the widespread occurrence of allelopathic interference among plants is well documented (Rice, 1974), the mechanisms through which phytotoxins cause reduction in growth in susceptible species remain obscure. Those compounds which have been implicated in allelopathy are primarily phenols and some of the terpenoids (Rice, 1974; National Academy of Sciences, 1971; Whittaker and Feeny, 1971). The water-soluble phenols contribute to the inhibition of higher plants as they are leached from plant parts or are returned to the rhizosphere in the decomposition process.

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Apparently a variety of plant processes may be modified by these phenolic phytotoxins. Several coumarin derivatives, phenolic acids, and extracts from plants alter respiration rates in seeds and other plant organs, with some compounds causing inhibition and others stimulating oxygen uptake (Van Sumere et al., 1971; Koeppe, 1972; Lodhi and Nickell, 1973; Demos et al., 1975). Inhibitors may cause reduction in protein synthesis (Danks et al., 1975), lowering of tissue water content (Lodhi and Nickell, 1973), and inhibition of active absorption of ions through an increase in membrane permeability (Glass, 1973; Glass and Dunlap, 1974). Scopoletin, *p*-coumaric acid, and caffeic acid reduced net photosynthetic rate in test seedlings, and these compounds, as well as several others, caused stomatal closure (Einhellig et al., 1970; Einhellig and Kuan, 1971; Einhellig, 1971; Kadlec, 1973). While phenolic-induced inhibition of photosynthesis was demonstrated, the affected component of photosynthesis was not investigated. Photosynthetic reductions may have related to changes in chlorophyll content.

Wilson and Rice (1968) observed that test seedlings inhibited by extracts from *Helianthus annuus* L. had mottled and chlorotic leaves. Rice (1974) suggested that some allelopathic compounds may interfere with porphyrin synthesis. Walters and Gilmore (1976) noted that fescue-inhibited sweetgum were possibly deficient in chlorophyll. We have found in our own laboratory that young seedlings inhibited by known phytotoxic phenols and plant extracts often show some loss of chlorophyll (Einhellig et al., 1970; Einhellig, 1971; and unpublished observations), and in experiments where seedlings were inhibited by extracts of *Abutilon theophrasti* Medic., chlorophyll reduction was quantified (Colton, 1978).

Since chlorophyll content is often closely related to plant production, any reduction in leaf chlorophyll would limit net photosynthesis and thus diminish total plant growth. The present experiments were designed to quantify the effects of three common phenolic acids on chlorophyll content. These experiments report on approximately 600 leaf chlorophyll analyses from treated seedlings.

METHODS AND MATERIALS

Test seedlings used in experiments for determining the effects of ferulic, *p*-coumaric, and vanillic acids on chlorophyll content were soybean [*Glycine max* (L.) Merr. cv. Wells, Cert. #DDS-2915-Q] and grain sorghum [*Sorghum bicolor* (L.) Moench, Hybrid 701, Gurney's Nursery, Yankton, South Dakota]. Recent work in our laboratory showed that soybean was susceptible to inhibitors from *A. theophrasti* and previous experiments established grain sorghum to be sensitive to phenolic compounds (Einhellig and Rasmussen, 1973, 1978; Rasmussen and Einhellig, 1975, 1977). Both species are agro-

nomic crops which may be affected by interference from allelopathic weeds and/or phenols released from decomposition of crop residues.

Seeds were germinated in vermiculite for 8 days and then individually transplanted to 80-ml light-free plastic vials containing diluted Hoagland's solution (Hoagland and Arnon, 1950). The Hoagland's solution used for the growth media was 2/5 full strength and contained twice the formula iron. After 3 or 4 days acclimatization, seedlings were selected for uniformity and transferred to similar nutrient solutions containing the appropriate phenolic acids dissolved in the nutrient solution. Experimental solutions for treating soybean or sorghum seedlings were 10^{-3} M, 5×10^{-4} M, and 2.5×10^{-4} M of each phenolic acid: ferulic, *p*-coumaric, and vanillic. Each treatment group comprised 10 seedlings, and treatments lasted 6 days. All germination and growth procedures were conducted in a Percival growth chamber with a 16-hr photoperiod at 20,000 lux and 30/18°C light/dark conditions. During the 6-day treatment period, nutrient media containing the appropriate phenolic acid was added to each vial on the 3rd and 5th days for soybean, while no additions were generally needed for sorghum.

At harvest, chlorophyll was extracted from the two unifoliate leaves of each soybean and the rest of the plant was oven dried at 105°C for 24 hr. Chlorophyll extractions followed the methods of Knudson et al. (1977). This involved immersing leaves in 30 ml of 95% ethanol for 24 hr, then decanting the ethanol-chlorophyll solutions to a second container. The leaves were soaked in a similar manner for two additional 24-hr periods with fresh aliquots of ethanol each time. Solutions were accumulated from each extraction and taken to a final volume of 100 ml. All chlorophyll appeared to be extracted from the leaves, with most of the extraction occurring in the first of the three 24-hr periods. The ethanol-chlorophyll solutions were kept in darkness at room temperature and immediately following the extraction sequence absorbances (A) of each chlorophyll extract were read at 665 and 649 nm on a Varian Cary 118CX spectrophotometer. Chlorophyll was computed using the following equations (Wintermans and DeMots, 1965; Knudson et al., 1977):

$$\frac{\mu g \text{ Chlorophyll a}}{\text{ml solution}} = (13.70)(\text{A665 nm}) - (5.76)(\text{A649 nm}) \quad (1)$$

$$\frac{\mu g \text{ Chlorophyll b}}{\text{ml solution}} = (25.80)(\text{A649 nm}) - (7.60)(\text{A665 nm}) \quad (2)$$

Unifoliate leaf dry weights (24 hr, 105°C) were used to calculate μg chlorophyll/mg dry weight. The ratio of chlorophyll a to b was also determined for each extraction, and all chlorophyll values were rounded to two decimals.

Chlorophyll extractions from grain sorghum leaves were made using the second, third, and fourth leaves from the youngest leaf that was at least 2 cm long. Leaves were cut 1 cm from the stem. In plants that were badly stunted from treatment with the phenolic acids (10^{-3} M-treated seedlings) this procedure took all but the youngest sorghum leaf, whereas in controls and those treatments showing less severe effects there were still one or two older leaves not extracted. Several plants of the 10^{-3} M and 5×10^{-4} M treatments appeared dead, and no chlorophyll was extracted from these deteriorated plants.

Total plant weights were obtained from the summation of the dry weight of extracted leaves and the weight of the remainder of each plant. Data from the several treatment groups were compared to the appropriate control through use of a *t* test. All experiments were repeated three times, but due to the similarity in replications, data from only two experiments are reported in Tables 1 and 2.

RESULTS

Observations during these experiments showed 10^{-3} M treatments of each of the three phenolic acids caused severe effects on soybean growth. After 2 days of treatment, inhibition of growth was noted and plants seemed less turgid. A general paling of the unifoliates was evident after 4 days of treatment. Seedlings harvested from 10^{-3} M ferulic, *p*-coumaric, or vanillic acid growth media had significantly reduced dry weights when compared to untreated plants (Table 1). These soybeans also had significant reductions in concentrations of chlorophylls a and b and total chlorophyll. A comparison of chlorophyll content among treatments shows that 10^{-3} M vanillic acid-treated plants are somewhat more affected than 10^{-3} M ferulic or *p*-coumaric acid-treated soybeans. However, chlorophyll concentration in seedlings under 10^{-3} M vanillic acid environments was quite variable from plant to plant.

The 5×10^{-4} M phenolic acid treatments are of greater interest, since at these treatment levels soybean seedlings were inhibited, but continued some growth, and we observed only minor changes in the appearance of these plants. However, in all cases the harvest dry weights of 5×10^{-4} M-treated seedlings were significantly below the controls (Table 1). Total chlorophyll concentration in the unifoliate leaves was also significantly below that of control seedlings, and both chlorophylls a and b were reduced. In at least one experiment with each compound there is evidence that chlorophyll b was slightly more affected than chlorophyll a, as shown by the increase in the a/b ratio. However, this change in chlorophyll ratio was not consistent among experiments.

TABLE 1. EFFECTS OF FERULIC ACID (FA), *p*-COUMARIC ACID (*p*CA), AND VANILLIC ACID (VA) ON SEEDLING GROWTH AND CHLOROPHYLL (CHL) CONTENT OF SOYBEAN UNIFOLIATE LEAVES[a]

Treatment	Exp. No.	Plant dry wt (mg)	Chlorophyll (μg/mg dry wt)		Ratio a/b	Total Chl (μg/mg)
			Chl a	Chl b		
Control	1	273.0 ± 22.5	19.42 ± 0.43	6.85 ± 0.17	2.84 ± 0.05	26.30 ± 0.58
	2[e]	325.2 ± 17.6	18.72 ± 0.35	6.44 ± 0.15	2.91 ± 0.02	25.15 ± 0.49
10^{-3} M FA	1	160.4 ± 9.5[d]	15.64 ± 1.26[c]	5.67 ± 0.46[c]	2.76 ± 0.03	21.32 ± 1.72[c]
	2	121.2 ± 13.3[d]	10.83 ± 2.19[d]	3.93 ± 0.80[d]	2.74 ± 0.27[d]	14.71 ± 2.96[d]
5×10^{-4} M FA	1	186.7 ± 8.0[c]	13.11 ± 0.26[d]	3.74 ± 0.22[d]	3.64 ± 0.25[c]	16.85 ± 0.31[d]
	2	190.0 ± 12.5[d]	18.00 ± 0.18[b]	5.81 ± 0.33[b]	2.95 ± 0.04	24.11 ± 0.21[b]
2.5×10^{-4} M FA	1	279.7 ± 12.9	18.31 ± 1.66	6.11 ± 0.61	3.04 ± 0.03[c]	24.67 ± 2.42
	2	250.2 ± 13.8[c]	19.14 ± 0.17	6.64 ± 0.07	2.88 ± 0.02	25.78 ± 0.23
10^{-3} M *p*CA	1	142.3 ± 14.2[d]	11.69 ± 0.10[d]	4.12 ± 0.32[d]	2.83 ± 0.04	15.81 ± 1.30[d]
	2	133.3 ± 6.9[d]	12.57 ± 0.14[d]	4.30 ± 0.06[d]	2.92 ± 0.04	16.88 ± 0.18[d]
5×10^{-4} M *p*CA	1	159.2 ± 16.5[d]	17.18 ± 0.73[b]	5.70 ± 0.21[d]	3.13 ± 0.02[d]	23.51 ± 0.94[b]
	2	174.4 ± 11.4[d]	15.67 ± 0.30[d]	5.19 ± 0.11[d]	3.02 ± 0.03[c]	20.85 ± 0.40[d]
2.5×10^{-4} M *p*CA	1	253.9 ± 20.0	18.73 ± 0.45	6.32 ± 0.12[c]	2.96 ± 0.03[b]	25.05 ± 0.56
	2	277.5 ± 26.9	18.47 ± 0.30	6.07 ± 0.30	3.10 ± 0.17	24.55 ± 0.19
10^{-3} M VA	1	149.1 ± 18.1[d]	7.34 ± 2.99[d]	2.52 ± 1.03[d]	2.70 ± 0.23	9.69 ± 3.90[d]
	2	134.7 ± 14.2[d]	5.98 ± 1.38[d]	2.14 ± 0.48[d]	2.75 ± 0.05[d]	8.12 ± 1.86[d]
5×10^{-4} M VA	1	201.8 ± 13.3[c]	17.78 ± 0.94	5.71 ± 0.31[c]	3.13 ± 0.05[d]	23.50 ± 1.14[b]
	2	168.3 ± 10.5[d]	17.18 ± 0.32[c]	5.82 ± 0.09[d]	2.96 ± 0.03	22.99 ± 0.39[c]
2.5×10^{-4} M VA	1	257.7 ± 13.5	14.13 ± 0.26[d]	5.00 ± 0.14[d]	2.84 ± 0.05	19.13 ± 0.38[d]
	2	294.7 ± 16.9	18.63 ± 0.17	5.97 ± 0.05[c]	3.12 ± 0.02[d]	24.56 ± 0.24

[a]Each is the mean ±SE of 10 plants.
[b]Difference from the control mean at 0.05 level.
[c]Difference from the control mean at 0.01 level.
[d]Difference from the control mean at 0.001 level.
[e]Duplicate experiment.

Only in the case of the second soybean experiment with 2.5×10^{-4} M ferulic acid were phenolic acids of this concentration significantly inhibitory to growth. In addition, chlorophyll analyses generally show no differences between chlorophyll levels in 2.5×10^{-4} M-treated and control soybeans (Table 1).

Sorghum seedling growth was more affected by the phenolic acid treatments than was soybean growth. In the first experiment with sorghum seedlings treated with 10^{-3} M *p*-coumaric acid, 40% of the plants deteriorated and appeared dead at the time of harvest. In both additional experiments all plants of this treatment died. With the exception of two plants, sorghum seedlings treated with 5×10^{-4} M *p*-coumaric acid remained alive, but they were significantly inhibited in growth (Table 2). This agrees with earlier results (Rasmussen and Einhellig, 1977). During the three replications of sorghum experiments, 10^{-3} M ferulic acid treatments killed 20% of the plants. Growth of other seedlings in 10^{-3} M and all 5×10^{-4} treatments was inhibited. Sorghum was most tolerant to vanillic acid, but the 5×10^{-4} M-treated seedlings still had greatly reduced weights, as previously noted (Einhellig and Rasmussen, 1978). Plants treated with 2.5×10^{-4} M concentrations of each phenolic acid had lowered dry weights at harvest, but this reduction was statistically significant in only one ferulic acid series.

Although the chlorophyll extraction data are biased by not extracting dead plants, under the conditions of these experiments only those sorghum seedlings in the first trial with 10^{-3} M *p*-coumaric showed less chlorophyll (Table 2). Even where stunting of 5×10^{-4} M-treated plants was visually apparent, at the time of harvest there were no obvious differences in plant color, and extractions showed μg chlorophyll per mg leaf equal to, or slightly above, controls. However, it can also be calculated that there was a great reduction in the amount of chlorophyll per plant in the treated seedlings (smaller plants than controls), and the equivalence of chlorophyll concentrations does not compensate for this reduction.

DISCUSSION

The dry weight data demonstrate that soybean seedling growth is inhibited by ferulic, *p*-coumaric, and vanillic acids, and the extent of inhibition is related to the concentration of phenolic acid treatment. The three different phenolic acids caused similar growth effects in soybeans, with 5×10^{-4} M treatments of each compound apparently the threshold level at which growth was significantly stunted. Comparisons of seedling growth between sorghum and soybeans show that sorghum was more sensitive than soybean to the phenolic acids. However, growth in both species was inhibited by phenolic acid concentrations used in these investigations. Any appraisal of the ef-

TABLE 2. EFFECTS OF FERULIC ACID (FA), *p*-COUMARIC ACID (*p*CA) AND VANILLIC ACID (VA) ON SORGHUM SEEDLING GROWTH AND CHLOROPHYLL (CHL) CONTENT[a]

Treatment	Exp. No.	Plant dry wt (mg)	Chlorophyll (μg/mg dry wt)		Ratio a/b	Total Chl (μg/mg)
			Chl a	Chl b		
Control	1	200.6 ± 24.4	18.45 ± 1.28	4.58 ± 0.29	4.01 ± 0.05	23.03 ± 1.57
	2[e]	191.7 ± 24.3	19.14 ± 1.53	5.52 ± 0.46	3.48 ± 0.04	24.66 ± 1.99
10^{-3} M FA	1	64.7 ± 6.0[d]	19.07 ± 1.36	4.97 ± 0.37	3.85 ± 0.08	24.04 ± 1.71
	2	92.2 ± 8.9[d]	16.87 ± 1.17	5.29 ± 0.22	3.18 ± 0.10[c]	22.15 ± 1.39
5×10^{-4} M FA	1	90.8 ± 7.8[d]	20.95 ± 2.00	5.40 ± 0.49	3.86 ± 0.05[b]	26.35 ± 2.49
	2	68.8 ± 7.9[d]	18.84 ± 1.20	5.49 ± 0.34	3.43 ± 0.06	24.33 ± 1.53
2.5×10^{-4} M FA	1	121.4 ± 23.2[b]	22.88 ± 0.58[c]	5.69 ± 0.15[c]	4.03 ± 0.04	28.57 ± 0.72[c]
	2	168.4 ± 20.9	21.66 ± 0.69	5.86 ± 0.20	3.70 ± 0.04[d]	27.52 ± 0.87
10^{-3} M *p*CA	1	65.2 ± 9.7[d]	9.08 ± 2.64[c]	2.75 ± 0.65[c]	3.20 ± 0.23[d]	11.83 ± 3.29[c]
	2					
5×10^{-4} M *p*CA	1	91.7 ± 15.2[d]	20.45 ± 1.09	5.19 ± 0.23	3.93 ± 0.08	25.64 ± 1.30
	2	81.7 ± 9.7[d]	17.90 ± 0.57	5.20 ± 0.19	3.45 ± 0.04	23.10 ± 0.74
2.5×10^{-4} M *p*CA	1	172.3 ± 18.6	21.24 ± 1.25	5.10 ± 0.27	4.15 ± 0.04[b]	26.35 ± 1.52
	2	150.8 ± 14.7	22.72 ± 0.95[b]	6.12 ± 0.25	3.71 ± 0.05[d]	28.85 ± 1.19[b]
10^{-3} M VA	1	93.2 ± 11.9[d]	15.62 ± 1.68	4.00 ± 0.47	3.94 ± 0.11	19.62 ± 2.14
	2	83.9 ± 14.3[d]	17.87 ± 1.81	5.24 ± 0.40	3.73 ± 0.12	23.11 ± 2.21
5×10^{-4} M VA	1	109.2 ± 12.4[c]	21.64 ± 0.58[b]	5.19 ± 0.14[b]	4.17 ± 0.03[b]	26.83 ± 0.71[b]
	2	145.4 ± 15.6	23.34 ± 1.04[b]	6.41 ± 0.27	3.64 ± 0.02[d]	29.75 ± 1.31[b]
2.5×10^{-4} M VA	1	179.0 ± 16.2	19.69 ± 1.29	4.55 ± 0.25	4.31 ± 0.07[c]	24.24 ± 1.54
	2	182.3 ± 14.0	21.69 ± 0.99	6.07 ± 0.27	3.57 ± 0.02[b]	27.75 ± 1.27

[a]Each is the mean ± SE of 10 plants except where several died (see text details).
[b]Difference from the control mean at 0.05 level.
[c]Difference from the control mean at 0.01 level.
[d]Difference from the control mean at 0.001 level.
[e]Duplicate experiment.

fects of phytotoxins must also be viewed with the recognition that the several phenolic acids that are functional in allelopathic interactions may act in additive or synergistic ways (Rasmussen and Einhellig, 1977; Einhellig and Rasmussen, 1978). Thus concentrations of ferulic, *p*-coumaric, and vanillic acids below the inhibitory levels noted in these experiments may be inhibitory in plant communities.

Making comparisons based on mg leaf dry weight, soybean plants inhibited by 10^{-3} M and 5×10^{-4} M treatments of these three phenolic acids contained less of chlorophylls a and b, and thus less total chlorophyll in unifoliate leaves. Since there is a good correlation between chlorophyll content and leaf area photosynthetic rate in soybean (Buttery and Buzzell, 1977), it is possible that reduction in concentration of leaf chlorophyll caused the inhibition in growth that was noted. This chlorophyll reduction would be especially important at nonsaturation light intensities. However, the data do not separate primary from secondary effects, and it may be that chlorophyll reduction occurs after some other physiological processes are altered by these phenolic acids. The present data also do not establish whether reduction in chlorophyll concentration is due to phenolic acid-induced degradation or to a reduction in synthesis of chlorophylls. Although several antibiotics are known to inhibit the chlorophyll formation process in *Euglena* (Linnane and Stewart, 1967), no investigations have been conducted with allelopathic inhibitors of higher plants. Parks and Rice (1969) used chlorophyll content as a measure of growth reduction in blue-green algae caused by several phenols, but they did not separate cause and effect relationships.

Apparently phenolic acid-induced growth reduction in sorghum is not due to less chlorophyll per mg of leaf. Significant growth inhibition was seen in sorghum plants treated with 5×10^{-4} M ferulic, *p*-coumaric, and vanillic acids, yet these seedlings had no reduction in concentration of chlorophyll. If the mechanism of growth inhibition caused by these phytotoxins is the same in soybeans and sorghum, then it must be concluded that phenolic acid-induced chlorophyll reductions observed in soybean seedlings are a secondary response. Alternatively, it is also possible that interference with chlorophyll metabolism is a mechanism of allelopathic inhibition of soybeans and not in sorghum.

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INSECT SEX PHEROMONES

Evaporation Rates of Acetates from Natural Rubber Septa[1]

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Abstract—The half-lives ($t_{1/2}$) of acetate sex pheromones and *n*-alkyl acetates were determined on either or both West Co. and Arthur H. Thomas Co. natural rubber septa. These septa gave equal half-lives. The $t_{1/2}$ values covered a large range: e.g., $t_{1/2}$ for decyl acetate was 5 days and that for pentadecyl acetate was 1353 days. The expected linear relationship between $\ln t_{1/2}$ versus the number of carbon atoms was found for the six acetates from *n*-decyl to pentadecyl, but the $t_{1/2}$ of hexadecyl acetate was only 478 days. This nonlinearity in the plot is attributed to the presence of polymer cross-links which create molecular size cages and produce an effect similar to that produced in gel permeation chromatography wherein large molecules elute faster than smaller ones. The $t_{1/2}$ of 4 monounsaturated acetates were close to the $t_{1/2}$ for the corresponding saturated acetates, but this relationship may not be general. The half-life of one nonconjugated diunsaturated acetate was much less than that for the corresponding monounsaturated acetate, whereas the half-life of another nonconjugated diunsaturated acetate was similar to the half-life of the corresponding monounsaturated acetate. The $t_{1/2}$ values determined for the pheromones may be used to estimate evaporation rates, the length of time the evaporation rates will be within a given range, and, with multicomponent pheromones, the ratio of components in the vapor.

Key Words—Insect sex pheromones, insect sex attractants, Lepidoptera pheromones, pheromone formulations, insect population monitoring.

INTRODUCTION

Sex attractant pheromones of Lepidoptera have become important in recent years because of their use by extension entomologists and private pest management consultants who monitor insect populations in order to provide

[1]Mention of proprietary products does not constitute an endorsement by the USDA.

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guidance for their control programs. The effectiveness of a sex pheromone as a monitoring tool is dependent on its formulation. For maximum trap capture of many species, the rate of release of pheromone must be within a restricted range (Roelofs and Cardé, 1977, summarized these). Consequently, knowledge of the length of time a formulation can maintain the necessary range and the dosage needed to produce the desired rate is important. Also, many insect pheromones consist of two or more components, some of which differ in volatility, and these components must be present in a certain narrow ratio for maximum attractiveness. As a result, knowledge of the length of time the desired ratio will be maintained is important.

Gaston et al. (1971) determined the release rate of dodecyl acetate from several substrates; Kuhr et al. (1972) have reported release rates of acetate analogs of pheromones from polyethylene caps; and Kydonieus (1977) has reported release rates of pheromone analogs from laminated polymeric membranes. Because natural rubber septa (rubber sleeve stoppers with a septum) have proved to be excellent substrates for many pheromones (e.g., Maitlen et al., 1976), we determined the release rates of acetate pheromones and related compounds impregnated in this substrate. The data obtained will make it possible to use the septa with maximum effectiveness for a variety of species.

METHODS AND MATERIALS

Maitlen et al. (1976) found that the solvent used had a pronounced effect on the percentage of applied pheromone of the codling moth, *Laspeyresia pomonella* (L.), that became impregnated into the septum. Because dichloromethane deposited about 90% of the applied pheromone into the septa, and was superior to the others tested, it was also used in the present study.

Analytical Procedure. Previous work (Maitlen et al., 1976) had shown no change in $t_{1/2}$ for amounts of 0.1–4 mg/septum for *E*8,*E*10–12:OH, and 0.5–5.0 mg/septum for gossyplure (Flint et al., 1978). In the present study, initial dosages were 4–6 mg/septum.

Septa impregnated with a substrate were prepared by adding a dichloromethane solution (100–200 μl) of the substrate. After the dichloromethane had disappeared, the septa were attached to horizontal wires with paper clips in such a way that they did not contact each other. The rubber septa used were either No. 1F-66F (The West Co., Phoenixville, Pennsylvania) or 5 × 9-mm rubber septa (Arthur H. Thomas Co.). The impregnated septa were allowed to age in the laboratory (23° C), and samples of three were taken at appropriate intervals for individual analysis. Each septum was extracted with 50 ml of hexane–dichloromethane (1:1) by shaking with a mechanical shaker for 1 hr. The resultant solution was analyzed directly with a gas chromatograph equipped with a 2-mm × 2-m column packed with 3% polydimethyl siloxane (SE-30®) on 80/100 mesh Gas Chrom Q®. An external standard was used.

The pheromones were purchased from Farchan Division of Story Chemical Co., Willoughby, Ohio. The saturated acetates were prepared from the corresponding alcohols and purified by liquid chromatography. The formate was prepared as reported previously (Butler et al., 1977).

Satistical Calculations. The correlation coefficients were calculated by a standard regression analysis of ln M (logarithm of the amount remaining) versus time.

The 95% confidence limits of the slopes of the lines obtained from a plot of ln M versus time were calculated from the expression:

$$k \pm \frac{t(n-2,\ 1-\frac{1}{2}\alpha)s}{[\Sigma(X_i - X)^2]^{1/2}}$$

where $t(n-2,\ 1-\frac{1}{2}\alpha)$ is the $(1-\frac{1}{2}\alpha)$ percentage point of a t-distribution with $(n-2)$ degrees of freedom (the number of degrees of freedom on which the estimate s^2 is based); k is the slope; α is 0.05; and s is the residual variation (Draper and Smith, 1966). The average half-lives and the 95% confidence limit of the half-lives, $t_{1/2}$, were then calculated from $t_{1/2} = k^{-1} \ln 2$.

Calculation of Evaporation Rates and Amount Remaining After Given Intervals. The rate of evaporation, R, of a substrate from a septum was calculated from the equation (McDonough, 1978);

$$R = Mt_{1/2}^{-1} \ln 2 \tag{1}$$

The amount of pheromone, M, in a septum after a given time was calculated from the equation:

$$M = M_0 e^{-tt_{1/2}^{-1} \ln 2} \tag{2}$$

where M_0 is the amount present initially, and t is the time during which M_0 decreased to M. (R and R_0 may be substituted for M and M_0, respectively, in equation 2.)

RESULTS AND DISCUSSION

The data summarized in Table 1 give the half-lives ($t_{1/2}$), the 95% confidence limits of $t_{1/2}$, and the correlation coefficients for model compounds and selected acetate pheromones (and one formate). The correlation coefficients are a measure of how well the data fit a first-order relationship. Coefficients above 0.3 are considered positive, and above 0.8 are considered excellent. Compounds with the longer half-lives have larger 95% confidence limits and smaller correlation coefficients, probably because at any one time the experimental error, although it is constant for all compounds, constitutes a greater proportion of the relatively smaller loss of pheromones with the longer half-lives. It was practical to follow the loss of 10:Ac (decyl acetate) for

TABLE 1. HALF-LIVES FOR EVAPORATION OF PHEROMONES AND RELATED COMPOUNDS IMPREGNATED IN RUBBER SEPTA

Compound[a]	$t_{1/2}$, (days) Average[b]	95% confidence limit	Correlation coefficient	No. of data points	No. of days evaporation monitored
6:Ac	0.458	0.433–0.486	0.987	18	1.2
7:Ac	0.666	0.613–0.729	0.980	26	2.0
8:Ac	1.48	1.41–1.55	0.991	27	5.0
9:Ac	2.68	2.56–2.82	0.994	24	8.0
10:Ac	4.97	4.77–5.18	0.993	30	21
9–10:Ac	6.49	6.32–6.66	0.997	39	28
11:Ac	14.6	13.6–15.8	0.986	24	20
12:Ac	37.3	36.8–37.8	0.996	78	222
*Z*7–12:Ac(T)	35.9	34.0–37.9	0.988	38	141
*Z*7–12:Ac	34.8	33.8–35.7	0.991	57	141
*Z*7–12:Fo	14.8	14.5–15.1	0.998	39	68
*E*8,*E*10–12:Ac[c]	47.1	40.4–56.5	0.906	33	49
13:Ac	97.9	89.6–108	0.971	36	83

14:Ac	350	330–373	0.950	87	387
*Z*11–14:Ac	325	298–357	0.892	66	278
*Z*11–14:Ac(T)	297	273–324	0.946	66	278
*Z*11–14:Ac(W&T)[d]	310	291–331	0.919	132	278
*Z*9,*E*12–14:Ac	319	285–361	0.837	41	145
15:Ac	1,353	1109–1735	0.715	69	338
16:Ac(T)	474	396–592	0.828	54	218
16:Ac	481	418–568	0.840	52	218
16:Ac(W&T)[d]	478	424–546	0.830	106	218
*Z*11–16:Ac	635	525–801	0.774	55	229
*Z*7,*Z*11–16:Ac + *Z*7, *E*11–16:Ac(1:1)(M_0 = 5.0 mg)[e]	171	158–187	0.934	63	142
*Z*7,*Z*11–16:Ac + *Z*7, *E*11–16:Ac(1:1)(M_0 = 0.5 mg)[e]	} 159 147	137–159	0.951	63	142
*Z*3,*Z*13–18:Ac	8,664	2097–(−4068)	0.073	45	272

[a] The letters after the colon indicate the functional group: Fo, formate; 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.
[b] West septa were used for the samples designated W and for nondesignated samples, and Arthur Thomas septa were used for the samples designated T.
[c] Data from Hathaway et al., 1979.
[d] Data from West and Thomas septa were combined and then analyzed statistically.
[e] Data from Flint et al., 1978.

ca. Four half-lives (21 days; 94% loss), whereas the loss of 16:Ac (hexadecyl acetate) was followed for 218 days (less than one half-life; 27% loss).

In the three cases in which the West and Thomas septa were compared, (*Z*)-7-dodecenyl acetate (*Z*7-12:Ac) (Berger, 1966), (*Z*)-11-tetradecenyl acetate (*Z*11-14:Ac) (Roelofs and Arn, 1968), and 16:Ac, the half-lives were equivalent (Table 1), since the 95% confidence limits overlapped. However, in field tests, these septa would not necessarily produce equivalent results because the additives to the rubber, which could conceivably be repellent to insects, are different.

Effect of Chain Length on $t_{1/2}$. Often a linear relationship exists between a physical parameter such as the logarithms of the gas chromatographic retention times (ln t_r) of a homologous series versus the number of carbon atoms (Kováts, 1965). When that is the case, the contributions to the ln t_r by individual molecular features such as an acetate group or a methylene group, etc., are additive, and it is possible to predict the ln t_r of a large number of compounds by using the parameters determined for a few compounds (McReynolds, 1966). We included the saturated straight-chain compounds in this study because, even though they are generally not implicated as pheromone components, they provided a convenient homologous series to test for the mentioned relationship.

The $t_{1/2}$ is a parameter thermodynamically equivalent to the gas chromatographic retention time because both are a measure of volatility, and their logarithms are linearly related to the free energy of vaporization. A graph of ln $t_{1/2}$ versus the number of carbon atoms for the saturated acetates is shown in Figure 1. Within the 95% confidence limits, the plot is linear for 10-to 15-carbon acetates, but values for compounds possessing a greater or lesser number of carbon atoms do not fall on this line. For the 6- to 10-carbon acetates the relationship is also linear, but here there is a different slope than for the 10- to 15-carbon acetates. Pentadecyl acetate has the remarkable $t_{1/2}$ of 1353 days. In contrast, the $t_{1/2}$ for hexadecyl acetate (478 days) is both smaller than would have been predicted from the line for the 10- to 15-carbon acetates and smaller than that for pentadecyl acetate.

The deviation from linearity of the plot of ln $t_{1/2}$ versus the number of carbon atoms indicates that natural rubber does not behave as an ideal liquid as do most gas chromatographic liquid phases. We attribute this deviation to the presence of polymer cross-links in rubber. Gas chromatographic liquid phases do not contain cross-links. It is an established principle that cross-linking of a polymer decreases the rate of evaporation of dissolved materials (Cardarelli and Kanakkanatt, 1977).

We therefore propose the following hypothesis. The cross-links between polymer chains create molecular-sized cages. Small solute molecules (such as the 6- to 10-carbon acetates) are able to diffuse in and out of these cages essentially as easily as they would through non-cross-linked polymer. The $t_{1/2}$

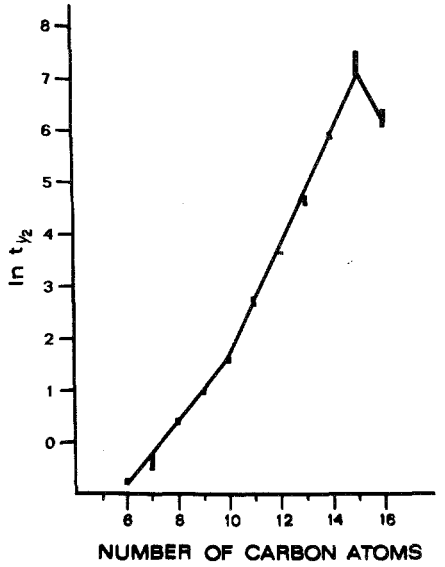

FIG. 1. Ln $t_{1/2}$ versus number of carbon atoms in the longest continuous chain for *n*-alkyl acetates. The bars define the 95% confidence limits.

will increase for the 6- to 10-carbon acetates because of increasing molecule weight. In contrast, larger solute molecules (10- to 15-carbon acetates) diffuse in and out of the cages with greater difficulty, and therefore will diffuse more slowly than in the absence of the cross-links. The $t_{1/2}$ for the 10- to 15-carbon acetates therefore increases because of increasing molecular weight and because of increasing entrapment in the cages, and the plot of ln $t_{1/2}$ versus number of carbon atoms for the 10- to 15-carbon acetates is steeper than for the 6- to 10-carbon acetates because of this additional factor. If the solute molecule is of a critical size, it may be able to just penetrate the cages, and there will be maximum retardation of diffusion (as with pentadecyl acetate). A still larger solute molecule (hexadecyl acetate) will not be able to penetrate most of the cages (a distribution of cage sizes having an average value is envisioned), and therefore would diffuse through the rest of the rubber faster and evaporate faster. Apparently the situation is similar to that of gel permeation chromatography where solute molecules below a certain size are entrapped in cages, and larger molecules elute faster because they do not become entrapped.

Effect of Double Bonds on $t_{1/2}$. For the four cases in which monounsaturated and saturated acetates can be compared, the differences in the $t_{1/2}$ were small (Table 1) compared with the differences in $t_{1/2}$ caused by differing numbers of carbon atoms. The ratios of $t_{1/2}$ for these four pairs were 1.33 for Z11-16:Ac/16:Ac, 0.89 for Z11-14:Ac/14:Ac, 0.95 for Z7-12:Ac/12:Ac,

and 1.31 for 9-10:Ac/10:Ac. In the case of 14- and 16-carbon acetates, the values for the unsaturated acetates are not different from the values for the saturated acetates at the 95% confidence limits. This similarity of $t_{1/2}$ values for saturated and unsaturated acetates in septa is also found for retention times in gas chromatography.

In gas chromatography, the effect on retention time of the second double bond of a nonconjugated diolefin acetate relative to the corresponding monoolefin acetate is similar in magnitude to the effect of the double bond of the monoolefin acetate relative to the corresponding saturated acetate. This is not true for one of the two comparisons of $t_{1/2}$ that can be made as a result of this study. The ratio of the $t_{1/2}$ of gossyplure (*Z*7,*E*11-16:Ac + *Z*7,*Z*11-16:Ac, 1:1) (Hummel et al., 1973; Bierl et al., 1974) to *Z*11-16:Ac (Chisholm et al., 1975) was 0.25 compared with 1.33 for *Z*11-16:Ac/16:Ac. However, the ratio of the $t_{1/2}$ of *Z*9,*E*12-14:Ac (Jacobson et al., 1970) to *Z*11-14:Ac was 1.03 compared with 0.89 for *Z*11-14:Ac/14:Ac. The large difference in effect of the double bonds of gossyplure relative to the double bond of *Z*11-16:Ac cannot be explained on the basis of either of the factors that determine gas chromatographic retention times, i.e., polarity and molecular weight. Instead, this difference may also be attributed to the cross-linked structure of natural rubber. Double bonds will affect the conformations a molecule can assume, and some conformations will have a smaller effective size than others. Decreasing the effective molecular size could increase or decrease the ease of diffusion within the cross-linked structure. In a molecule such as 16:Ac which is too large to fit into most of the cross-link cages, a moderate decrease in effective molecular size such as might occur as a result of the addition of one strategically placed double bond could increase $t_{1/2}$ by increasing entrapment in the cages (effective molecular size between 15:Ac and 16:Ac), whereas a greater decrease in effective molecular size due to the addition of one or two differently placed double bonds could decrease the $t_{1/2}$ by increasing the ease of diffusion through the cross-linked structure to that of an effectively much smaller molecule (e.g., effective molecular size equivalent to 13:Ac). Centrally located double bonds should have a more substantial effect on conformation than one near the end of a molecule. If a decrease in effective molecular size is the reason for the short half-life of gossyplure, then monounsaturated acetates with double bonds in the center of the molecule may have similarly short half-lives. This effect would be most pronounced for compounds that diffuse more slowly, such as the 14- and 16-carbon acetates. On this basis, then, pheromones such as *E*9-12:Ac (Smith et al., 1974), *Z*9-12:Ac (Roelofs et al., 1971), and *Z*8-12:Ac (Roelofs et al., 1969) should have half-lives similar to that of 12:Ac (about 37 days), but *Z*9-14:Ac (Jacobson et al., 1970) and *Z*7-14:Ac (Roelofs and Comeau, 1971) could have half-lives smaller than that of *Z*11-14:Ac (about 310 days).

No *E* isomers were included in this study. In gas or liquid chromatography the difference in elution volumes between *E*/*Z* isomers of monounsaturated compounds is small compared to the difference between the saturated and monounsaturated compounds. Similarly, we expect half-lives for the *E* isomers to be about the same as the *Z* isomers for the tested monounsaturated acetates. For cases such as gossyplure where the double bonds effect a large decrease in $t_{1/2}$ compared with the corresponding saturated acetate, there may be a difference in $t_{1/2}$ between *Z* and *E* isomers.

Conjugated dienes are more polar than nonconjugated dienes. On nonpolar gas chromatographic columns, nonconjugated dienes have retention times less than the corresponding saturated compound whereas a conjugated diene such as *E*8,*E*10-12:Ac (Hathaway et al., 1974; Hill et al., 1976) has a retention time between 12:Ac and 13:Ac. On rubber septa the $t_{1/2}$ of *E*8,*E*10-12:Ac is also between that of 12:Ac and 13:Ac. Also, we expect that if the $t_{1/2}$ of *Z*/*E*9, 11-12:Ac (Nesbitt et al., 1975) were known, it would be about the same as *E*8,*E*10-12:Ac.

Fujiwara et al. (1976) reported on the rate of loss of two pheromone components from rubber septa in a field test in Japan from January to March 1975. The septa were not commercially manufactured and were prepared from nonvulcanized natural rubber; dimensions were similar to those of the West Co. and Thomas Co. septa. Using their data, we calculated $t_{1/2} = 158$ days (correlation coefficient, 0.95; 95% confidence limit, 79-38,000) for *Z*9,*E*12-14:Ac, and $t_{1/2} = 172$ days (correlation coefficient, 0.94; 95% confidence limit, 82-1728) for *Z*9,*E*11-14:Ac (Tamaki et al. 1973). (The large 95% confidence limit reflects the fact that only four points were used in these determinations.) Thus, within this large 95% confidence limit, their data are consistent with ours.

Other Compounds. *Z*7-12:Fo was included in this study because in combination with *Z*7-12:Ac it is a potent sex attractant for the alfalfa looper, *Autographa californica* (Speyer) (Butler et al., 1977). The $t_{1/2}$ of *Z*7-12:Fo was very close to that of 11:Ac. Consequently, the pheromone mimic *Z*9-14:Fo (Mitchell et al., 1979) may have a $t_{1/2}$ similar to 13:Ac or possibly somewhat smaller, depending on the effect of the double bond. The $t_{1/2}$ for *Z*3,*Z*13-18:Ac (Tumlinson et al., 1974) which has the highest molecular weight of any pheromone component tested was so long as to be undeterminable after 272 days (Table 1).

Use of Data. The formulation of pheromones based on evaporation rates can be illustrated with an example. The most desirable release rates for the pheromone of the red-banded leafroller, *Argyrotaenia velutinana* (Walker), *Z*/*E*11-14:Ac (94/6), have been reported to be close to 1 μg/hr (Glass et al., 1970). Taking into account that 90% of the applied pheromone will actually become dissolved in the septum and applying equation 1, one can calculate a

desired dose of 12.0 mg. Furthermore, Roelofs et al. (1975) found that the synergistic component of the pheromone, 12:Ac, was present in female tips in a ratio of 5:4 to 11–14:Ac, and that a ratio of 1.5 or 2.0 in polyethylene caps was the most attractive in field tests. From our data, an evaporation rate of 2 μg/hr will be obtained from a dose of 2.87 mg of 12:Ac per septum. Equation 2 can then be used to determine the evaporation rate at a given future date or the ratio of 12:Ac/11–14:Ac at a given future date. Thus after 35 days the rates of evaporation of 12:Ac and Z11–14:Ac will be 1.04 and 0.92 μg/hr, respectively.

In their study of the rate of evaporation of the codling moth pheromone from rubber septa, Maitlen et al. (1976) obtained the same $t_{1/2}$ outdoors in the Yakima Valley as in the laboratory ($t_{1/2}$ = 26 days). In a similar study of the pheromone of the pink bollworm, *Pectinophora gossypiella* (Sanders), Flint et al. (1978) found $t_{1/2}$ to be 159 days in the laboratory and 106 and 133 days (average 120 days) in two separate field tests in Arizona cotton fields. Obviously, both wind and temperature will affect evaporation rates, so we anticipate differences between laboratory and field data during the season and from season to season. We expect that $t_{1/2}$ in the field will sometimes be longer as well as shorter than $t_{1/2}$ in the laboratory because some agricultural areas are cooler than the Yakima Valley. Also, $t_{1/2}$ will probably be longer for a fairly enclosed trap such as that reported by Killinen and Ost (1971) than for a more open trap such as the "wing" trap (Howell, 1972). The differences between emission rates in the laboratory and field appear to be small compared to the differences caused by structural variations of the pheromones (about 100-fold in Table 1) and small compared to the range of evaporation rates which effect maximum trap capture for various species of Lepidoptera (over 1000-fold, Roelofs and Cardé, 1977).

COMMENT

Although the present study was undertaken to obtain data to provide better formulations of pheromones for use in monitoring traps, the results have implications for formulations that might be used for control of insect infestations by male confusion with the air-permeation technique. Microcapsule formulations of pheromones presently under evaluation are very desirable because of the ease of application, but present commercial microcapsules release pheromone too fast ($t_{1/2}$ = 2–4 days; McDonough, 1978) and release only a small fraction of the pheromone (Cardé et al., 1975; Caro et al., 1977). Natural rubber spheres of the same size as the microcapsules may be a solution to these problems. Although rubber spheres of such dimensions would have shorter $t_{1/2}$ values than septa (McDonough, 1978), some pheromones (e.g., Z11–14:Ac, and Z11–16:Ac) will probably have $t_{1/2}$

values in the desirable range of 10–20 days. Further, by increasing the cross-linking density (decreasing cage size) of rubber or other hydrophobic elastomer, it may be possible to obtain adequate $t_{1/2}$ values for smaller pheromone molecules.

A study of the evaporation rates of alcohols and more acetates are in progress to provide similar data for alcohol pheromones and to test the hypothesis advanced in this paper.

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AUTOINTOXICATION MECHANISM OF *Oryza sativa*

II. Effects of Culture Treatments on the Chemical Nature of Paddy Soil and on Rice Productivity[1,2]

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Abstract—Ammonium sulfate and potassium nitrate added as a top dressing and rice straw were incorporated into soil to understand their effect on the chemical nature of the paddy soil and on the growth and yield of rice plants during two successive crop seasons. Redox potential (Eh) determination indicated that the paddy soil appeared to be the reduced form in mixture with rice residues. The amounts of ammonium nitrogen (NH_4^+—N) and nitrate nitrogen (NO_3^-—N) were significantly higher in the second crop than in the first crop, and the quantity of NH_4^+—N was about 10 times greater than that of NO_3^-—N. The incorporation of rice straw decreased both the available nitrogen and the soil cations, Zn^{2+}, Cu^{2+}, Ca^{2+}, Mn^{2+}, and Na^+. The quantity of Zn^{2+}, Cu^{2+}, Mg^{2+}, and Na^+ was significantly lower in the second crop that that in the first. The growth and yield of rice plants were significantly affected by cultural treatment; thus the ammonium sulfate dressing treatment resulted in higher yields than the potassium nitrate treatment. The NH_4^+—N treatment had an antagonistic effect on the phytotoxic nature of rice straw decomposed in soil. The phytotoxicity (primarily phenolic type compounds) of aqueous extracts of soil varied between treatments and was significantly higher in the soil which had been mixed with rice straw. This finding correlated well with the higher amounts of phytotoxic plant phenolics produced by the decomposing rice residues in the soil.

Key Words—Allelopathy, autointoxication, *Oryza sativa*, rice productivity, phytotoxicity, phytotoxic phenolics, top dressing, paddy soil chemistry.

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INTRODUCTION

Rice (*Oryza sativa*), the most important crop in Taiwan, is planted twice a year by a continuous monoculture system. For nearly a century in Taiwan, the rice yield of the second crop has been generally lower by 25% than that of the first crop (a reduction of about 1000 kg/hectare) (Wu et al., 1975). The reduction of rice productivity has been particularly pronounced in the areas of poor water drainage. Although many factors involved in this reduction have been found, their significance has not been fully understood (Chou et al., 1976, 1977; Houng et al., 1975, 1976, 1977; Lin et al., 1976; Shieh, 1976; Wu et al., 1976). For example, (1) there is about a 3-week fallowing period between the first crop season and the second crop as compared with 10 weeks between the second crop and the first crop of the following year, (2) the temperature pattern is entirely different between the two crop seasons, and (3) the farmers in Taiwan have always left rice residues in the field after harvesting. Under such circumstances, the unharvestable part of stubble submerged in the paddy field does not completely decompose within the short period of fallowing time, especially in oxygen-deficient soils. This results in a great quantity of organic compounds produced during the decomposition which can be detrimental to the growth of rice seedlings (Chou et al., 1976, 1977). This intraspecific interaction is called autointoxication and appears in many agricultural lands and in many crops (McCalla, 1971; Patrick, 1965, 1971; Rice, 1974). Chou et al. (1977) indicated that the more rice residue left in the paddy soil, the greater the phytotoxic phenolic compounds produced and the less amount of leachable nitrogen. Houng et al. (1977) studied the effect of rice straw and fertilizer on the growth and yield of rice and concluded that the application of nitrogen fertilizers during the two crop seasons are significantly different. However, the effect of different fertilizers as a top dressing on the fertility of paddy soil is not fully understood. Thus, this study was aimed at finding the influence of nitrogen application and rice straw decomposition in soil on the rice productivity and the chemical nature of paddy soil.

METHODS AND MATERIALS

Materials. After rice (Taichung 65 variety) was harvested in Nankang, Taiwan, in June 1976, the rice straw was cut into pieces about 2.5 cm long. Soil samples taken from the upper 20-cm layer of ground from rice fields were brought to Academia Sinica and allowed to air dry. The dry soil was separated from the plant residues, screened with a 2-mm sieve, thus removing all visible root fragments.

Field Experiments and Sampling. The field experiment was conducted on the experimental farm of Academia Sinica, Nankang, and consisted of a

randomized complete block design (Wu et al., 1975). The treatments in the field experiments were as follows: (1) Top dressing of ammonium sulfate and potassium nitrate was added separately around the 30th and the 70th day after transplanting. The design was described in detail by Yuan et al. (1978). The field management regime was adopted from the custom used locally. (2) Three seedlings (3 weeks old) of rice variety Taichung 65 were transplanted onto the paddy field. In the second crop season of 1976, the rice seedlings were transplanted on August 17, 1976, and rice plants and soils were sampled for determining the growth and yield of rice and for measuring the chemical nature of soil separately on September 13 (27 days after transplanting), October 4 (48 days), October 18 (62 days), November 2 (77 days), and November 25 (100 days). In the first crop of 1977, the rice seedlings and soils were sampled similarly on April 18 (32 days after transplanting), May 2 (46 days), May 23 (67 days), June 13 (88 days), and July 4 (109 days).

Greenhouse Pot Experiment. Using the chopped rice straw and dry soil, a soil-straw mixture (10 kg:100 g) was set up in each clay pot with 4 replications, in which 196 pots were used. The soil-straw mixture was flooded with tap water and allowed to decompose for 3 weeks, then 3 rice seedlings (3 weeks old) were transplanted into each pot. Soil alone treated in the same manner served as control. The transplanting dates and sampling dates in both crop seasons were the same as described above.

Soil Extraction. For bioassays and chemical analyses, aqueous extracts of paddy soil were obtained by using the method described by Chou et al. (1977). The identification of possible soil phytotoxins employed the alkaline alcoholic extraction techniques described by Chou et al. (1975, 1976).

Determination of Chemical Nature of Paddy Soil. To understand the dynamic fluctuation of some soil chemical properties, pH, Eh, leachable nitrogen, and leachable cations were determined both in the field and pot experiments at the above-mentioned sampling times. The soil pH was determined by a Chemtrix type 40 pH-meter, soil Eh determined by Kiyu Seisakusho model DM-38 Eh-meter, the contents of ammonium nitrogen (NH_4^+—N) and nitrate nitrogen (NO_3^-—N) were determined by a method described by Chou et al. (1977), the cation contents of Cu, Ca, Mg, Mn, Zn, K, and Fe were determined by an atomic absorption spectrophotometer (Perkin-Elmer, model 300), and the osmotic concentration was determined by using a Fiske G-66 osmometer.

Bioassay of Aqueous Extracts of Paddy Soil. The aqueous extracts from soil samples of paddy and pots were determined for their phytotoxicity by using the techniques described by Chou et al. (1976). Seeds of rice (*Oryza sativa* Taichung 65), and lettuce (*Lactuca sativa* var. Great Lakes 366) were used as test materials. The percentage of phytotoxicity from the soil extracts were revealed by measuring the percent inhibition of radicle growth against distilled water control.

Quantitative Determination of Phytotoxins in Soils. Although Chou et al. (1976, 1977) have previously reported seven phytotoxic substances present in the paddy soil and the decomposing rice residues in soil, the study of quantitative comparison of each compound between two crop seasons and culture treatments has not been made. Thus, the phytotoxins, namely, *p*-hydroxybenzoic, ferulic, syringic, *o*-coumaric, (*cis* and *trans*), *p*-coumaric, and *o*-hydroxyphenylacetic acids were isolated and mainly identified by paper chromatography. Quantitatively, these compounds were analyzed by a modified method of Kuwatsuka and Shindo (1973).

Statistical Analysis. The data obtained for the various treatments in the aforementioned studies were analyzed by analysis of variance and regression analysis (Snedecor, 1956).

RESULTS

Dynamics of Eh (Oxidation-Reduction Potential) in Paddy Soil. The results of Eh measurement indicated that in the paddy fields of Nankang the Eh ranged from −100 mV to +200 mV during the first (winter) crop season of 1977, and ranged from −200 mV to +100 mV during the second (summer) crop season (Figure 1A). At the experimental farm of National Chung-Hsiung University (NCHU) at Taichung the soil Eh was remarkably low reaching −500 mV to +100 mV before the tillering stage during the second crop season of 1976, and was almost in the oxidative state, ranging from +300 mV to +100 mV, during the first crop season (Wang, 1976 unpublished data). In another experiment at Nankang, when the rice residues were left in the paddy field the Eh ranged from −300 mV to −100 mV from the tillering to panicling stage during the second crop season of 1977, while the Eh increased from −200 mV to +100 mV when rice residues were removed. A similar pattern was also found on the farm of NCHU where, at the tillering stage of the second crop season of 1976, the soil Eh reached −100 mV to +200 mV in the paddy field with rice residues removed but went down to −300 mV when rice residues remained. In the pot experiment, the soil Eh ranged from +100 mV to +400 mV in the treatment with soil alone which served as control, but ranged from −300 mV to +100 mV in the treatment of the SR (rice residues) soil during the first crop season of 1977 (Figure 1B). The Eh was down to the reduction state at the tillering stage of 30–45 days after transplanting and at the panicling stage of 80–90 days after transplanting (Figure 1B). Nevertheless, the difference in the Eh was significant when the nitrogen fertilizers ammonium sulfate and potassium nitrate were applied onto the field during the tillering and panicle stage of 1977 (Figure 1A and B).

These results clearly indicate that the soil Eh was significantly low, reaching the reduced state in the early growing period of the second crop

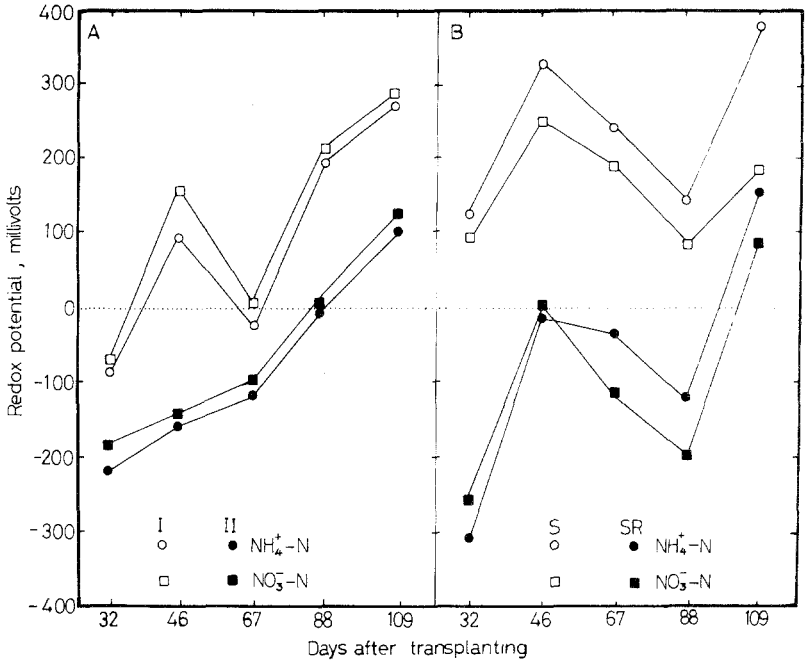

FIG. 1. The effects of different culture treatments on the redox potential of soils in field (A) and in pot (B) at five sampling times during the first crop season (I) and the second crop season (II) of 1977. The soil was separately applied with ammonium sulfate (NH_4^+—N) and potassium nitrate (NO_3^-—N) as top dressing in both the field and pot experiments. The pot was filled with 10 kg soil alone (S), and the soil mixed with 100 g chopped rice straw (SR); the mixture was allowed to decompose for 3 weeks before transplanting. Each point on the figure was obtained from the mean of at least five replications. Data for the other figures were treated in the same manner.

season which was particularly pronounced in the soil with rice residues (SR) left in the paddy field. A similar pattern was found in the pot experiment when the soil was mixed with rice residues. In fact, during the early stage of the second crop season the surface soil temperature is almost always above 30°C which expedites the decomposition of rice residues in soil.

Variation of pH and Osmotic Concentration in Paddy Soil. It was found that the pH varied with sampling times in both crop seasons ranging from pH 6.3 to 8.9. Differences in soil pH were insignificant from the two soil treatments in the two crop seasons, but were significantly different in soils between fertilizer applications. The pH values were higher in the soil receiving the ammonium sulfate (NH_4^+—N) dressing than that receiving the potassium nitrate (NO_3^-—N) dressing (Chou et al., 1977 unpublished data). The osmotic concentration in the aqueous leachate from paddy soil was zero during the

growing season in both crop seasons, different nitrogen dressings, and between S and SR soil.

Under SR soil, the pH slightly increased to neutral state from 6.7 to 7.3, a finding which suggested that during the decomposition of rice straw in soil some minerals could be released resulting in the soil becoming slightly basic. It was surprising that when the soil was applied with NH_4^+—N fertilizer as a top dressing during the panicle primordia initiation (about 70 days after transplanting) in the first crop of 1977, the soil pH went down to pH 3.5-3.8, but this did not occur on soil top-dressed with NO_3^-—N.

Osmotic concentration was significantly higher in the SR soil, suggesting that some substances were released during the decomposition of rice residues in soil. However, the osmotic concentration below 17 mosmol ($17 \times 22.4 \times 10^{-3}$ atm) in soil solution shows no detrimental effect on plant growth (Chou and Young, 1974).

Dynamics of Extractable Nitrogen in Paddy Field and Pot Experiment. The aqueous extract of paddy soil was determined for its nitrogen content, but not for all available soil nitrogen, in various sampling times and culture treatments. The results are given in Figure 2. In general, the paddy fields had significantly higher ammonium nitrogen (NH_4^+—N) content than nitrate

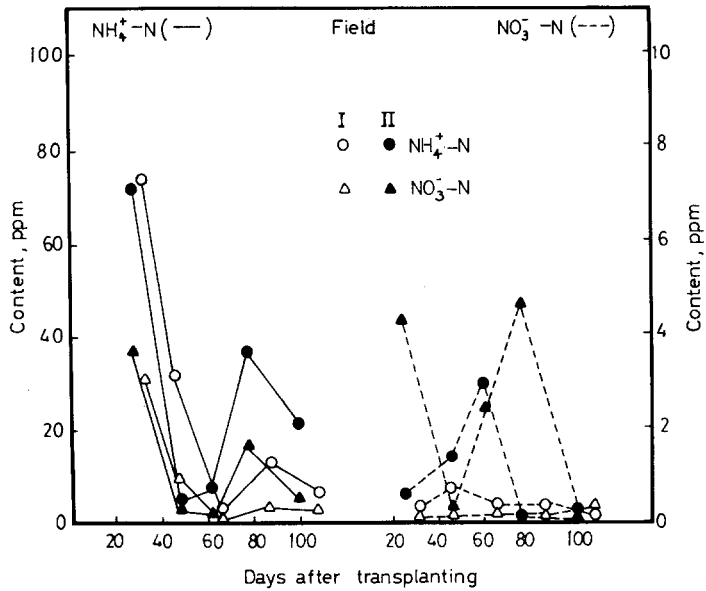

FIG. 2. The effects of nitrogen fertilizer treatments on the content of leachable soil nitrogens, NH_4^+—N (solid lines) and NO_3^-—N (broken lines) at five sampling times in the first crop season of 1977 (I) and the second crop season of 1976 (II). The description in the following figures is the same as given in Figures 1 and 2 unless otherwise stated.

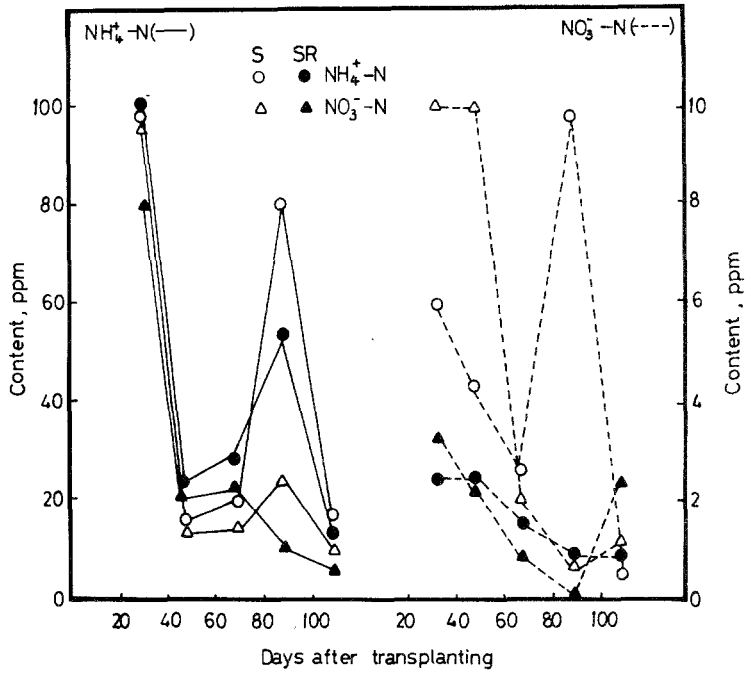

FIG. 3. The effects of culture treatments on the content of leachable nitrogen in pot soils at five sampling times during the first crop season of 1977.

nitrogen (NO_3^-—N) throughout the growing seasons. It is not surprising that the NH_4^+—N content was high at the tillering stage, because of basal placement of NH_4^+—N dressing before rice transplanting. However, this amount rapidly decreased after the maximum tillering stage, and the rate of decrease was observed to be very sharp in the treatments of the second crop season. The next high peak of NH_4^+—N content was found 77–80 days after transplanting, and this was doubtless due to the top dressing at the panicle primodia initiation stage (about 70–75 days after transplanting) in both seasons.

In the pot experiments, the water-extractable NH_4^+—N and NO_3^-—N were also determined. Regarding the NH_4^+—N content, the amount was generally higher when in the soil alone (S) than when in the soil-residue mixture (SR) except in the period 40–70 days after transplanting (Figure 3). The curves mostly agreed with the findings of field soil (Figure 2). In the NO_3^-—N analysis the amount of NO_3^-—N was about one tenth the NH_4^+—N content and was significantly higher in the S soil than in the SR soil except during the last 40–70 days after transplanting (Figure 3). This clearly indicated that the available nitrate nitrogen would rapidly decrease when the soil was

incorporated with rice residues. The results suggest that the nitrate nitrogen could be denitrified or easily leached out, resulting in its fast disappearance.

In conclusion, the amount of NH_4^+—N and NO_3^-—N was significantly higher in the second crop than that in the first crop, and the quantity of NH_4^+—N was about 10 times higher than that of NO_3^-—N. Both forms of soil nitrogen were significantly affected by both nitrogen dressing and rice residues incorporation. Additionally, rice straw incorporated into soil may decrease both forms of available nitrogen.

Effects of Culture Treatments on Soil-Leachable Cations. It was thought that under various culture treatments the amount of available minerals in the soil might be affected and consequently influence rice growth. Under the same experimental treatments, the cations present in the aqueous leachates of soil, namely Na^+, K^+, Zn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+} were determined. Based on five sampling times, Table 1 shows that the concentration of Cu^{2+}, Zn^{2+}, and Fe^{2+} was lower than 0.1 ppm (1 g soil basis), that of K^+ and Mn^{2+} was lower than 6 ppm, and that of Ca^{2+}, Na^+, and Mg^{2+} was above 10 ppm. The Mg^{2+} content was much higher than 29 ppm. Comparing with the results of nitrogen dressing treatments, the amount of listed cations, except Fe^{2+} and K^+, was significantly higher in the NH_4^+—N dressing than in the NO_3^-—N dressing in the first crop of 1977. In addition, the amounts of all cations were higher in the former than in the latter in the second crop season of 1976. This indicated that different nitrogen fertilizer applications definitely influenced the availability of leachable cations in soil. Furthermore, comparing the data of cation contents in two crop seasons, the concentration of four cations, such as Cu^{2+}, Fe^{2+}, Mn^{2+}, and K^+, were shown to be higher in the first crop season, while that of other cations, namely, Zn^{2+}, Ca^{2+}, Mg^{2+}, and Na^+ were higher in the second. In some paddy fields of Taiwan, Zn^{2+} has been remarkably deficient in the second crop season, which in part agrees with the aforementioned findings.

To further understand the effect of rice straw incorporated into soil on the dynamics of aforementioned cations in pot soil, the results of analysis are shown in Table 2. With NH_4^+—N dressing, the amounts of cations Zn^{2+}, Fe^{2+}, Ca^{2+} and Na^+ were significantly higher in the S soil than that in the SR soil. However, regardless of fertilizer treatment, K^+ and Mg^{2+} were more concentrated in the SR soil. It is obvious that the introduction of rice straw into soil surely increases the amount of some cations and decreases others.

Effects of Culture Treatments on Growth and Yield of Rice Plants. Under the same treatments, the rice plants grown in the paddy field were examined at various growing stages. The results shown in Figure 4 indicate that the dry weight of roots and straw is significantly higher in the treatment of NH_4^+—N dressing than that of NO_3^-—N. With regard to the pot experiment, a similar pattern to Figure 4 was found in that the dry weight of rice roots and straw were significantly higher in the NH_4^+—N dressing than in the

TABLE 1. EFFECTS OF CULTURE TREATMENTS ON LEACHABLE SOIL CATIONS IN NANKANG PADDY FIELD[a]

Cation	Concentration (ppm)						Ratio of I/II[b]		
	I			II					
	A[c]	B	Means	A	B	Means	A	B	Means
K^+	1.72	2.01	1.87	2.32	1.36	1.84	0.71[d]	1.47[e]	1.01
Na^+	10.93	10.18	10.55	13.50	12.04	12.77	0.81	0.85	0.82
Ca^{2+}	10.92	10.74	10.83	14.71	10.22	12.46	0.72[d]	1.05	0.89
Cu^{2+}	0.08	0.05	0.07	0.06	0.06	0.06	1.33[e]	0.80	1.17
Fe^{2+}	0.02	0.06	0.04	0.04	0.03	0.03	0.50[e]	2.22[e]	1.35[d]
Mg^{2+}	38.33	29.14	33.74	42.99	32.50	37.59	0.89	0.90	0.90
Mn^{2+}	5.60	2.54	4.07	3.46	0.84	2.15	1.62[e]	3.02[e]	1.89[e]
Zn^{2+}	0.12	0.05	0.08	0.09	0.08	0.09	1.26	0.61[d]	0.96

[a]The data were obtained by means of five sampling times with four replications.
[b]I, II: The first crop season of 1977 and the second crop season of 1976, respectively.
[c]A, B: Top dressing of ammonium sulfate fertilizer (NH_4^+—N) and of potassium nitrate (NO_3^-—N), respectively.
[d,e]Statistical significance at 5% and 1% level, respectively.

TABLE 2. EFFECTS OF CULTURE TREATMENTS ON EXTRACTABLE SOIL CATIONS IN POT EXPERIMENT[a]

	Concentration (ppm)								
	S			SR			Ratio of S/SR[b]		
Cation	A[c]	B	Means	A	B	Means	A	B	Means
K^+	7.68	5.36	6.52	11.68	13.67	12.68	0.65[d]	0.40[d]	0.51[e]
Na^+	33.04	30.46	31.75	26.86	29.96	28.41	1.23	1.02	1.12
Ca^{2+}	52.20	50.86	51.53	34.82	37.44	36.13	1.50[e]	1.36[d]	1.43[e]
Cu^{2+}	0.05	0.04	0.05	0.07	0.02	0.05	0.71[d]	2.00[e]	1.00
Fe^{2+}	3.62	0.07	1.85	0.10	0.13	0.12	36.20[e]	0.54[e]	15.42[e]
Mg^{2+}	278.80	224.16	251.48	295.04	240.04	267.54	0.94	0.93	0.94
Mn^{2+}	11.78	16.19	13.99	14.47	7.88	11.18	0.81	2.05[e]	1.25[d]
Zn^{2+}	0.38	0.24	0.31	0.07	0.07	0.07	5.42[e]	3.43[e]	4.43[e]

[a]The data were obtained by means of five sampling times with four replications.
[b]S, SR: The pot was filled with 10 kg soil alone (S), and the soil was mixed with 100 g of chopped rice straw (SR).
[c]A, B: Top dressing of ammonium sulfate fertilizer (NH_4^+—N) and of potassium nitrate (NO_3^-—N), respectively.
[d,e]Statistical significance at 5% and 1% level, respectively.

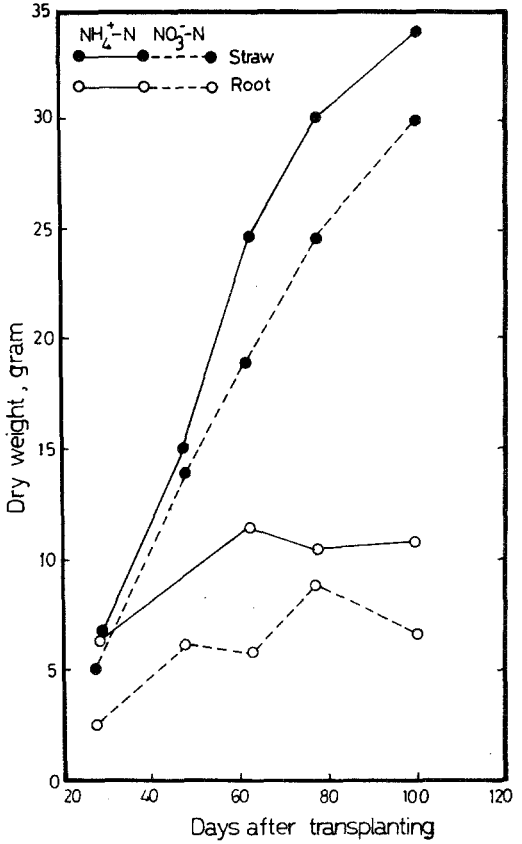

FIG. 4. The effects of nitrogen fertilizer treatments on the growth of rice plants growing in the paddy field during the second crop season of 1976.

NO_3^-—N dressing (Figures 5 and 6). In addition, the rice plants grew much more poorly and dry weight of both root and straw were lower in the SR soil than in the S soil.

The tiller number was significantly higher in the NH_4^+—N dressing treatment than that of NO_3^-—N treatment as observed by rice plants growing in the field in the second crop season of 1976 (Figure 7), and similar findings were obtained from the pot experiments. In the pot experiment, the tiller number was significantly lower in the SR soil than in the S soil (Figure 8).

Regarding the yield components of rice plants as affected by different culture treatments, the data of panicle number, total panicle weight, grain weight, and yield are shown in Table 3. These yield components were significantly different between the dressing treatments. In the NH_4^+—N dressing, these components were insignificantly different between the S and

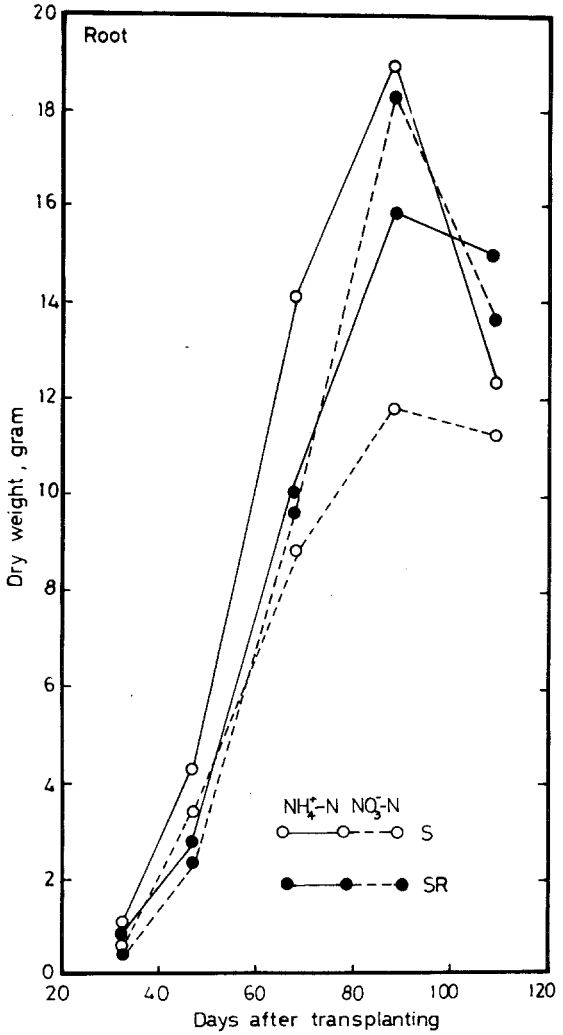

FIG. 5. The effects of culture treatments on the root growth of rice plants growing in the pots during the first crop season of 1977.

the SR soil, but were significantly different between them in the NO_3^-—N dressing treatment. This suggests that the suppression effect of rice productivity can be antagonized by NH_4^+—N fertilizer which agrees with our previous findings (Chou et al., 1977) and those of Chandrasekaran et al. (1973).

In conclusion, both the vegetative and reproductive growth of rice plants were significantly affected by culture treatments, showing that the NH_4^+—N dressing had a beneficial effect on rice growth and exhibited an antagonistic interaction on rice residues decomposed in soils.

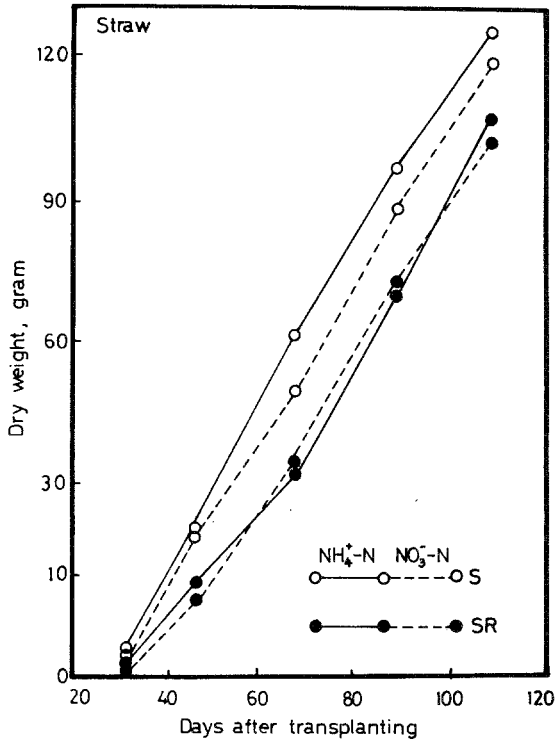

FIG. 6. The effects of culture treatments on the straw growth of rice plants growing in the pots during the first crop season of 1977.

Identity of Phytotoxins and Phytotoxicity Affected by Culture Treatments. We have previously reported seven phytotoxins, namely, vanillic, *p*-hydroxybenzoic, (*cis* and *trans*), *p*-coumaric, syringic, ferulic, and *o*-hydroxyphenylacetic acids, present in the paddy soil (Chou et al., 1977). However, the dynamics of these compounds as affected by culture treatments were not known. In the first crop season, only ferulic acid and syringic acids were found in lower amount in the NH_4^+—N dressing treatment than that in NO_3^-—N treatment, while in the second crop season other compounds were revealed in lower amount in the former treatment (Table 4). In addition, when the quantities of these compounds were compared between two crop seasons, it was found that the amount of four compounds, ferulic, *p*-hydroxybenzoic, *cis-p*-coumaric, and *o*-hydroxyphenylacetic acids, was significantly higher in the first crop season than that in the second crop season (Table 4). However, when the analysis was performed in pot conditions, higher concentrations of phytotoxic phenolics were found in the SR soil than the S soil control (Table 5). The ratio of S/SR in terms of the quantitative comparison of phytotoxins was generally lower than 1, which was more

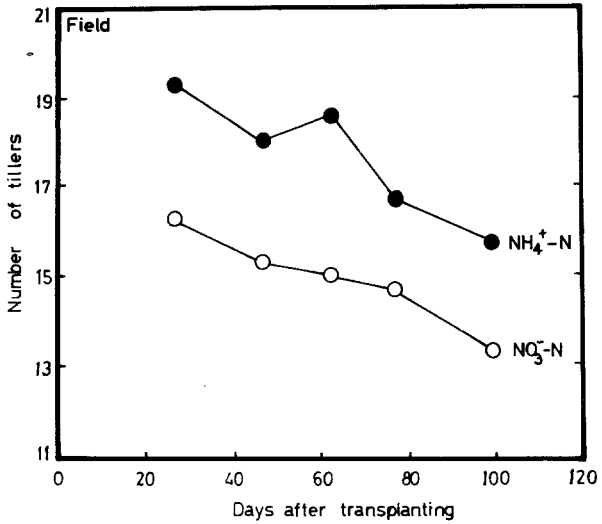

FIG. 7. The effects of nitrogen fertilizer treatments on the tillering number of rice plants growing in the field during the second crop season of 1976.

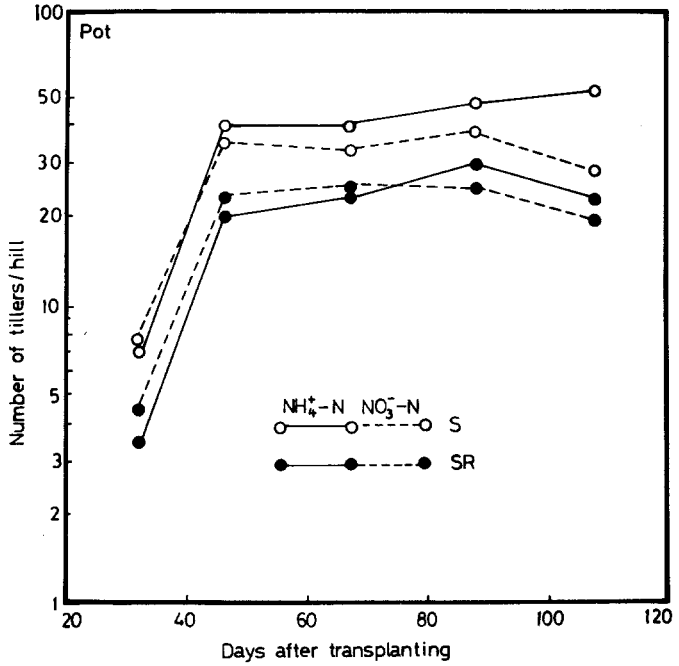

FIG. 8. The effects of culture treatments on the tillering number of rice plants growing in the pots during the first crop season of 1977.

TABLE 3. COMPARISON OF YIELD COMPONENTS OF RICE PLANTS GROWING IN POTS UNDER DIFFERENT SOIL TREATMENTS

Yield component (per hill)	A[a]			B			Ratio of A/B	
	S[b]	SR	% decrease	S	SR	% decrease	S	SR
Panicle number	38.75	32.25	17	26.25	19.50	26[c]	1.53[d]	1.65[d]
Total panicle weight (g)	64.33	58.24	10	52.25	42.26	20	1.23	1.37[c]
Ripening rate (%)	90.90	89.13	1	97.65	92.70	5	0.93	0.96
Testing weight (g/1000 seeds)	27.27	27.16	0.5	28.01	26.01	7	0.97	1.01
Grain weight (g)	61.85	55.24	11	50.26	40.14	21[c]	1.23[c]	1.37[c]
Yield (g/m^2)	1546.30	1380.90	11	1256.40	1003.57	20[c]	1.23[c]	1.37[c]

[a]A, B: Top dressing of nitrogen fertilizer (NH_4^+—N) and (NO_3^-—N), respectively.
[b]S, SR: The pot was filled with 10 kg soil alone (S), and the soil was mixed with 100 g chopped rice straw (SR).
[c,d]Statistical significance at 5% and 1% level, respectively.

TABLE 4. QUANTITATIVE COMPARISON OF PHYTOTOXINS PRESENT IN PADDY SOIL UNDER DIFFERENT CULTURE TREATMENTS IN 2 GROWING SEASONS[a]

Compound	Ratio			
	A/B[b]		I/II[c]	
	I	II	A	B
Ferulic acid	0.57[d]	0.75	1.33[d]	1.75[e]
trans-p-Coumaric acid	1.63[e]	1.27	0.94	0.73
Syringic acid	0.77[d]	2.16[e]	0.53[e]	1.50[e]
Vanillic acid	1.25	0.96	0.83	0.61[d]
p-Hydroxybenzoic acid	1.11	0.36[e]	2.50[e]	0.81
cis-p-Coumaric acid	1.10	0.40[e]	5.00[e]	1.80[e]
o-Hydroxyphenylacetic acid	1.00	0.50[d]	0.80	4.00[e]
Unknown	1.10	1.23	1.40[d]	1.60[e]

[a]The data were obtained by means of five sampling times with four replications.
[b]A, B: Top dressing of nitrogen fertilizer (NH_4^+—N) and (NO_3^-—N), respectively.
[c]I, II: The first crop season of 1977 (I) and the second crop season of 1976 (II).
[d,e]Statistical significance at 5% and 1% level, respectively.

significant in the NO_3^-—N dressing treatment. However, regarding the ratio of A/B, the data were more irregular. Phytotoxic phenolics were found in significantly high amounts in the SR soil, indicating that rice straw is a major source of these plant phenolics.

On the other hand, aqueous extracts obtained from the pot soils at five sampling times during the first crop season of 1977 were assayed for phytotoxicity. The results shown in Figure 9 indicate that the phytotoxicity revealed from the lettuce bioassay is more obvious, ranging from 30% to 91% inhibition. Before the 60th day of sampling, the aqueous extract of the SR soil exhibits significantly greater toxicity than that of the S soil, but the toxicity pattern is reversed thereafter. The toxicity revealed an irregular pattern and was not significant between the treatments of nitrogen top dressings. In the rice bioassay, the phytotoxicity varied irregularly and was as low as 30% against the distilled water control (Figure 9). The toxicity was not due to an osmotic effect of the extracts, which were below 17 mosmols.

DISCUSSION

Patrick and Mikkelsen (1971) indicated that the redox potential (Eh) in soil is brought about by the utilization of the available oxygen by soil microorganisms and by the production of reduced metabolites. The SR soil

with ammonium sulfate might reduce Eh (Figure 1B); however, the Eh was slightly oxidized in soil where the potassium nitrate was added. These findings are in agreement with those of Patrick et al. (1971) who found that nitrate and manganese dioxide are reduced at fairly high potential (above 200 mV). In addition, they also pointed out that most of the chemical changes which occurred in flooded soils were associated with microbial metabolism. Wu et al. (1976) concluded that, at the maximum tillering stage, the lower the redox potential in soil the more sulfate reducers and denitrifiers on the surface of the roots. Among those reducers *Pseudomonas putida* was found to be most common, but none of that was found normally growing rice roots. At this stage, the concentration of phytotoxic substances in the soil was also high, especially *p*-hydroxybenzoic acid. Our results agree with their findings, and we found more than seven substances potentially phytotoxic (Tables 4 and 5). These phytotoxins were predominant in the field with poor water drainage, particularly in the area where rice residues had been previously incorporated into soil. A large-scale experiment was conducted by improving water drainage in the paddy field of Kaohsiung Agricultural Improvement District in 1976. The results of this experiment show that the rice yield has been increased by at least 30% since that time (Wu et al., 1968 unpublished data).

TABLE 5. QUANTITATIVE COMPARISON OF PHYTOTOXINS PRESENT IN POT SOIL MIXED WITH OR WITHOUT RICE STRAW AS AFFECTED BY TWO DIFFERENT NITROGEN DRESSING TREATMENTS[a]

	Ratio			
	S/SR[b]		A/B[c]	
Compound	A	B	S	SR
Ferulic acid	0.33[e]	0.14[e]	2.00[e]	0.86[d]
trans-p-Coumaric acid	0.66[e]	0.55[e]	1.27	1.05
Syringic acid	0.54[e]	0.72[d]	0.85	1.00
Vanillic acid	0.85	0.63[e]	1.10	0.81[d]
p-Hydroxybenzoic acid	0.91	0.73[d]	1.10	1.00
cis-p-Coumaric acid	0.85	1.00	1.10	1.30
Unknown	0.85	1.00	1.10	1.30

[a]The data were obtained by means of 10 samplings with four replications both in the second crop season of 1976 and the first crop season of 1977.
[b]S, SR: The pot was filled with 10 kg soil alone (S), and the soil was mixed with 100 g chopped rice straw (SR).
[c]A, B: Top dressing of nitrogen fertilizer (NH_4^+—N) and (NO_3^-—N), respectively.
[d,e]Statistical significance at 5% and 1% level, respectively.

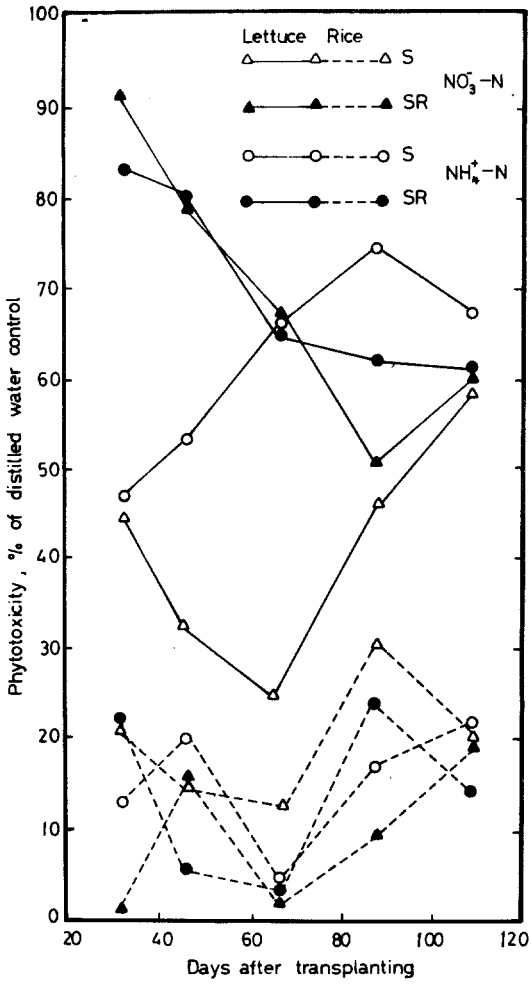

FIG. 9. Relative phytotoxicity of the aqueous extracts of pot soils as affected by culture treatments during the second crop season of 1977. The phytotoxicity was expressed as % inhibition on the radicle growth of lettuce and rice against the distilled water control.

Temperature is entirely different between the two crop seasons. During the early growing period of the second crop season the surface soil temperature will reach above 30°C, expediting the decomposition of rice residues left in soil, and resulting in release of greater quantities of organic substances. In this situation the phytotoxins may be accumulated and naturally would have a detrimental effect on rice growth. In addition, the phytotoxins may also affect the population of nitrogen-fixing microorganisms or nitrifying bacteria

in the soil of paddy fields. This effect of phytotoxins on microbes has not been evaluated in this study; however, such effects have been demonstrated by Rice and his coworkers (Rice, 1974).

Furthermore, the concentrations of cations, namely K^+,Ca^{2+}, and Mn^{2+} were higher in the first crop season, while those of Na^+, Ca^{2+}, Mg^{2+}, and Zn^{2+} were higher in the second crop season in Nankang paddy soil regardless of nitrogen fertilizer application (Table 1, last column). Most of our findings agree with those of Patrick et al. (1971), who found that the rapid decrease in Eh after flooding is a characteristic of soil low in reduceable iron and manganese. When the pot soil was mixed with rice straw, the amount of K^+ was significantly higher than that of soil alone, but that of Cu^{2+}, Fe^{2+}, Mn^{2+}, and Zn^{2+} was on average significantly lower in the soil in terms of the ratio of S/SR (see Table 2). It is interesting to note that in several poorly drained areas in Taiwan, such as Changhwa, Taitung, Pintung, the Zn deficiency is particularly noticeable during the second crop season.

Regarding the growth and yield of rice plants as affected by soil treatments, the root development was retarded by phytotoxins present in the SR soil (Chou, 1978 unpublished data), but this suppression could be overcome by application of NH_4^+—N fertilizer (Figures 5-7). Chandrasekaran et al. (1973) concluded that ammonium sulfate effectively eliminated the injury. Thus, the phytotoxic organic acids can be detoxified by ammonium ions leading to well-developed root systems. It is also possible that soil phytotoxicity can be reduced by incorporation of humic substances; Wang et al. (1977) found that protocatechuic acid, one of the phytotoxins related to *trans-p*-coumaric acid, can be polymerized into humic acids by using clay minerals as heterogenous catalysts. If this is the case, the incorporation of humic acid into soil may accelerate the detoxification of soil, and possibly increase its rice productivity. However, it is also possible that the polymerized phytotoxins fixed into a humic complex can be depolymerized under some conditions, resulting in free phenolic compounds which will produce an immediate phytotoxic effect on nearby susceptible plants.

Nevertheless, the autointoxication mechanism of rice is thought to be influenced by other environmental parameters, such as temperature, light, soil texture, nutrient availability, and the degree of water drainage. Some of them have been discussed previously; however, under an extreme environmental regime of poor water drainage, the quantity of phytotoxic substances may be significantly higher than that in the normal condition. For example, Koeppe et al. (1976) demonstrated that a higher concentration of chlorogenic acid and its isomers was found in the extracts of sunflower plants grown under phosphate deficiency as compared with phosphate-sufficient plants. This can be interpreted to mean that survival of plants growing in nutrient-poor soil may depend on the production of phytotoxic substances, thereby excluding

other plants without competing with them for the available nutrients. Thus, the autointoxication or allelopathy (Muller, 1970, 1974) may play a significant role in the initial important stages of plant development, such as seed germination, radicle growth, tillering, panicling, and other metabolic processes. This autointoxication or allelopathy aspect may be helpful for understanding the formation of a dominant species and the productivity of crop plants. Nevertheless, the relation of phytotoxins to rice productivity is only one aspect; important parameters as mentioned previously should be considered regarding the cause of lowering rice yield in the second crop season.

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CHEMICAL CONSTITUENTS OF THE CHEST GLAND SECRETION OF THE THICK-TAILED GALAGO (*Galago crassicaudatus*)

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Abstract—The naturally exuded chest gland secretions of adult male and female thick-tailed bush babies were collected directly in capillary tubes. The pure secretion was subjected to GC–MS analysis which revealed three major components. These were identified by comparison with the appropriate standard compounds: benzyl cyanide, *p*-hydroxybenzyl cyanide, and 2-(*p*-hydroxyphenyl)ethanol. Analysis of a male and a female secretion indicated that some sexual dimorphism in the relative concentrations of these components may exist.

Key Words—Thick-tailed bush baby, *Galago crassicaudatus*, chest gland secretion, benzyl cyanide, *p*-hydroxybenzyl cyanide, 2-(*p*-hydroxyphenyl)-ethanol.

INTRODUCTION

The results of numerous behavioral studies have indicated that chemical signals play a major role in prosimian communication (Epple, 1974; Doyle, 1974; Harrington, 1974; Clark, 1975; Schilling, 1978). However, in most of these studies, the chemical components of the scents have not been identified and hence the interpretation of what is being communicated by the chemical signals is uncertain (Schilling, 1978). In order to unravel the complexities of chemical communication in primates, we feel that the analysis of a communication channel within an "interactional perspective" as advocated by Smith (1977) should be undertaken. Smith suggests that the first level

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of analysis involves the specification of the signals used in the communication channel. In this case it would involve the identification of the major active components of the galago chest gland secretion.

Analysis of the *G. crassicaudatus* chest gland secretion was initiated by Wheeler, Blum, and Clark (1977). The material they identified was tested on laboratory animals and found to elicit a curious response in which the galago slowly placed its open mouth over the marked area (Clark, 1975). As the initial phase of our reinvestigation of the importance of chest gland secretions in the communication systems of these animals, we have found two additional major components whose identification we present.

METHODS AND MATERIALS

Collection of the Secretion. The males and females of *G. crassicaudatus* have well-developed chest glands as described by Clark (1975). The secretions exuded from this area of the skin were collected directly into capillary tubes (Figure 1). Collection of the secretion was facilitated by confining the animals to a special restrainer developed for this purpose (Katsir, 1979.) Secretions from two males were collected in 40 capillary tubes, each containing 0.2 μl of pure secretion. Similarly, 40 capillary tubes of secretion from three females were collected. Pooled samples from 3-5 capillary tubes gave sufficient quantities of the secretion for chemical analysis. Prior to analysis the secretions were stored in the capillary tubes at $-18°C$.

The animals were kept under a reversed day/night cycle (Doyle, 1974). The secretion was collected 1 hr before the red (night) light was turned on. At this time they were caught and put into the restrainer quickly. The laboratory animals used were accustomed to this treatment, and the amount of secretion produced did not seem to be affected by any stress to which the animals were subjected.

Analysis of the Secretion. Analytical gas chromatographic separations were carried out on a Carlo Erba 2150 gas chromatograph equipped with a flame ionization detector, using helium as the carrier gas. Quantitative determinations were achieved with the aid of a Hewlett Packard 3385A reporting integrator and by introducing samples into the gas chromatograph without the aid of a solvent or a syringe. This was accomplished by inserting a melting point capillary, sealed at one end and containing two or three collection capillary tips, open end first, through a hole in the septum which was otherwise closed with an empty, sealed melting point capillary. As the withdrawal of this stopper and introduction of the sample capillary resulted in pressure changes, this method could not be used for retention time determinations. Samples for the comparison of retention times of the constituents of the secretion with those of synthetic compounds were prepared by

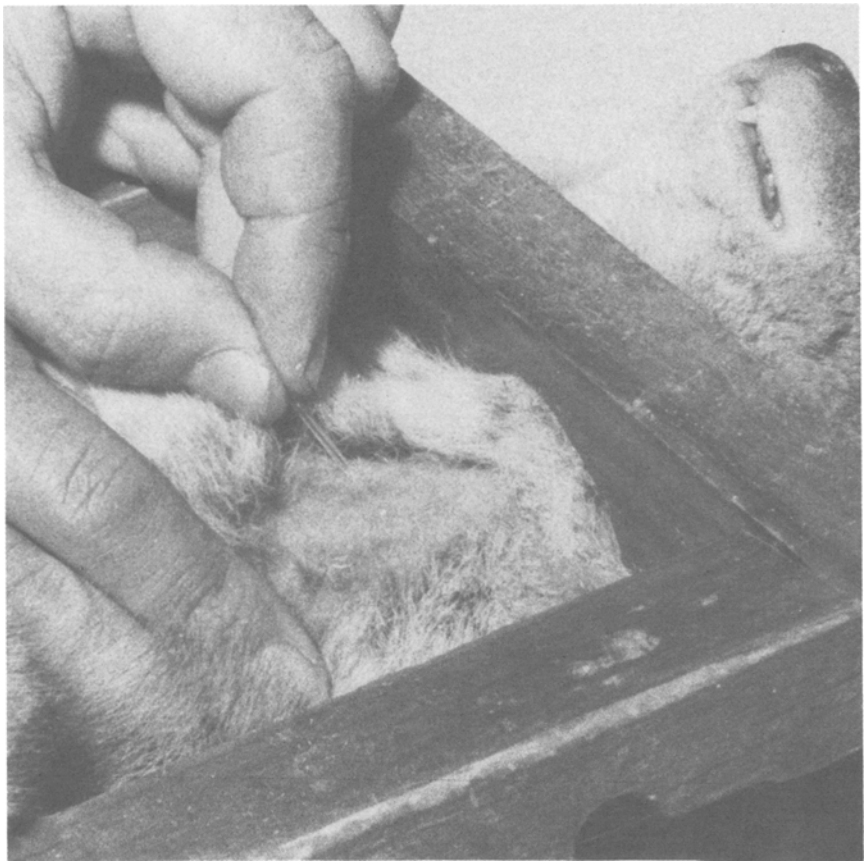


FIG. 1. A male *G. crassicaudatus* being held in the restrainer while the chest gland secretion is collected in a capillary tube.

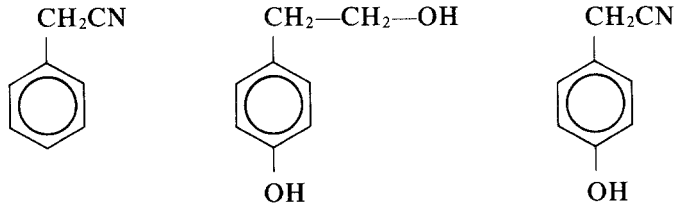
dissolving the contents of four or five collection capillaries in the absolute minimum of dichloromethane (Merck, residue analysis grade) in a glass capillary and injecting portions of this solution with a syringe in the normal manner.

All gas chromatographic fractionation (both analytical and GC-MS analysis) of the secretions was done with a glass capillary column coated with Carbowax 20M, 78 m × 0.3 mm, 3 ml He/min, temperature program 60–220°C (2°C/min).

The GC-MS analysis of the secretion was carried out with a Varian MAT 311A mass spectrometer coupled to a Varian 2700 gas chromatograph with a Schomburg interface (Henneberg, Hendrichs, Schomburg, 1975). Spectra were recorded on magnetic tape with a Varian SS 100 MS data system.

RESULTS

A satisfactory total ion current plot was obtained from the GC-MS analysis (Figure 2). The first major constituent of the secretion (scan No. 1149) had a molecular ion and base peak at *m/e* 117 and another large peak at *m/e* 90. Similarly the third major constituent (scan No. 2074) had a molecular ion and base peak at *m/e* 133 and major peaks at *m/e* 106 and 78. These compounds were identified as benzyl cyanide (I) and *p*-hydroxybenzyl cyanide (III), respectively, by comparison with their gas chromatographic retention times and mass spectrographic properties with those of authentic synthetic samples. The component at scan No. 1914 had a molecular ion at *m/e* 138, base peak at *m/e* 107, apparently did not contain nitrogen, and was aromatic. Its mass spectrum was similar to that described by Wheeler, et al. (1977) for 2-(*p*-hydroxyphenyl)ethanol (II). Comparison of its gas chromatographic retention time and mass spectrum with that of an authentic synthetic sample confirmed this assignment.



(I) (II) (III)

Quantitative GC analysis of whole male secretion revealed that the concentration of the three major components, when expressed as w/w of whole secretion collected was (I) = 8%, (II) = 17% and (III) = 56%. A single male scent mark was estimated to contain 0.5 μg of benzyl cyanide. Quantitative GC comparison of the secretions of male and female animals showed that the ratio of the two *p*-hydroxy compounds (II) and (III) is more or less the same for the two sexes, whereas benzyl cyanide (I) is present in a higher concentration in the secretion of the male than in that of the female. The results of this quantitative analysis are summarized in Table 1.

The relative concentrations of the components of pooled chest gland secretions varied by 1%. Because the secretions were pooled, data for individual variation and for the ontogeny of the secretion are not available.

DISCUSSION

Collection of the natural chest gland secretion directly into capillary tubes is advantageous in that it ensures that no impurities are introduced, as may happen when solvents are used to extract the secretions. In addition,

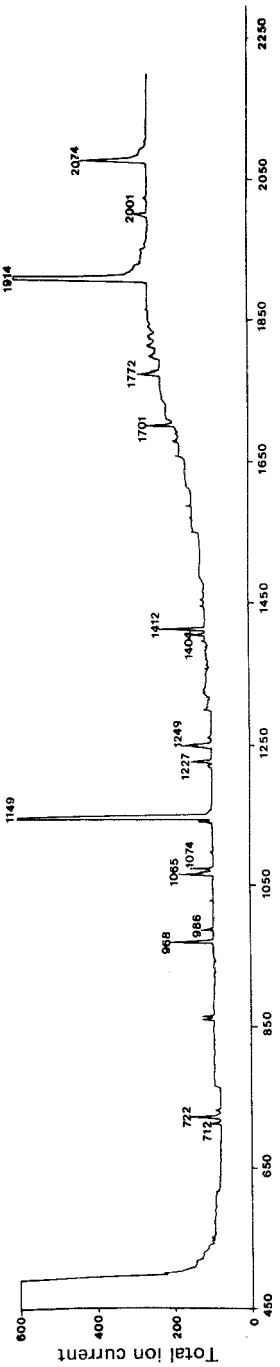


FIG. 2. Total ion current plot of the *G. crassicaudatus* chest gland secretion on a Carbowax 20M capillary column.

TABLE 1. RESULTS OF QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF SECRETIONS OF MALE AND FEMALE *G. crassicaudatus*

Major constituents	Relative concentrations (%)	
	Males	Females
Benzyl cyanide (I)	69	43
2-(*p*-Hydroxyphenyl)ethanol (II)	21	37
p-Hydroxybenzyl cyanide (III)	10	16

the method reduces the chances of contamination of the secretion from compounds which may be present in the fur of the animal.

Reexamination of *G. crassicaudatus* chest gland secretion has revealed that two additional volatile components are present in greater quantities than that of the component originally identified by Wheeler, et al. (1977). The gas chromatogram (Figure 2) indicates that there are other minor components in the secretion. The three major constituents of the secretion were tested under field conditions in comparison with natural chest gland secretion and found to elicit similar responses (Katsir and Crewe, 1979). Although there appears to be some sexual dimorphism in the quantities of volatile components present in the scent marks of the males and females we tested, this will have to be verified on a larger sample of animals. In addition, behavioral tests will have to be performed to indicate whether this difference is meaningful to the animals.

Bearder (1974) and Charles-Dominique (1978) have emphasized that galagoes, which are usually considered to be "solitary" prosimians, have a system of social relationships which is based on communication from a distance. This seems to be achieved mainly by means of a combination of auditory and olfactory signals and stems from the fact that the general activity and movements of the individuals involved are not synchronized. Bearder and Doyle (1974) have indicated that the *G. crassicaudatus* chest gland secretion together with a special vocalization might advertise the territory of individual animals. Clark (1975, 1978), in a more specific study of scent marking, has stated that the marks give an indication of sex, individual identity, and age of the animal producing the signal. She has said that "chest gland secretion is very long-lasting and is unlikely to reflect accurately the time of marking." Hence she feels that the timing cues are more likely to be obtained from urine washing.

However, in animals which organize their resource utilization temporally, the chest marking scent which is composed of at least three major components would give recipient individuals some indication of the time which has lapsed since the mark was deposited. Regnier and Goodwin (1977)

have given a very elegant indication of the way in which mammalian scent marks might produce a temporally patterned pheromone release. A similar situation exists in the case of the galago secretions (Katsir and Crewe, 1979). The persistent fluorescent components of the secretion such as ribitylflavin mentioned by Clark (1975) might be acting as modulators for the release of the three relatively volatile compounds we have discovered to be present in the secretion.

With a scent which has three components in differing proportions, the temporal pattern may be indicated more subtly than is the case in the example examined by Regnier and Goodwin (1977). The subtlety will stem from the fact that as the components volatilize, the quality of the odor of the scent mark will change with time. The analysis of this communication system is being pursued in order to verify whether the galagoes can indeed distinguish this change in odor quality and thus use it as a means for regulating social relationships which involve some spatial separation of the animals involved.

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RESPONSE OF CAPTIVE MALE RED FOXES (*Vulpes vulpes* L.) TO SOME CONSPECIFIC ODORS

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Abstract—Captive male red foxes were allowed access to urine and anal sac secretions collected from both familiar and unfamiliar foxes of both sexes. Conspecific odors commonly elicited higher visiting and marking frequencies than did their distilled water controls. Unfamiliar odors generally attracted a higher frequency of urinations than familiar odors, and unfamiliar male urine elicited a longer investigation time and was urine-marked more frequently than other stimulus odors.

Key Words—Foxes, scent marking, conspecific urine, anal sac secretion, familiar and unfamiliar secretions, frequency of visits, investigation time, odor discrimination apparatus.

INTRODUCTION

Members of the family Canidae display frequent urinations and defecations throughout their environment (Fox, 1971). Kleiman (1966) suggested that such scent marking be defined as urination, defecation and/or the rubbing of certain areas of the body which are (1) oriented towards a specific object, (2) elicited by familiar conspicuous landmarks and novel objects or odors, and (3) repeated frequently in response to the same stimulus.

Scent marking in red foxes has been discussed by several authors

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(Tinbergen, 1965; Burrows, 1968; Tembrock, 1968; Henry, 1977; Macdonald, 1977a). In common with other members of the Canidae, the predominant marking mode is urination. Typically, foxes deposit a small quantity of urine (<20 ml) onto prominent objects, inedible food, and previous scent marks (Henry, 1977). The marking postures are sexually dimorphic; males show a raised or elevated leg lifting posture to mark a vertical object, and either the lean, flex, lean-raise or flex-raise posture to mark an object at ground level. Females urinate from the squat or squat-raise position (Peters and Mech, 1975; Sprague and Anisko, 1973). Henry (1977) presents data which suggest that urine marking in scavenging wild foxes serves as a "book-keeping system," so that a urine mark on small inedible food remains indicates "no food present" and saves foxes from further fruitless investigations. In a series of experiments he showed that foxes spent less time investigating small food remnants when they were accompanied by fox urine odor. Urine marking was also repeatedly performed at regular sites where no food remnants were found and, although they accounted for only 12% of the observed urinations in Henry's study, it is possible that these scent posts may serve a social function (Tinbergen, 1965; Macdonald, 1977a). Burrows (1968) has also suggested that feces and anal sac secretion may serve as scent marks in the fox. As in dogs (Sprague and Anisko, 1973) both sexes adopt either the arch or the arch-raise posture for defecation and the tail is held in an arc, unlike typical urinations where the tail is held parallel to the ground. Occasionally the defecation posture is prolonged following deposition of feces and is accompanied by several contractions of the anus and jerking movements of the tail resulting in the expulsion of drops of anal sac secretion (personal observations). Ewer (1968) also suggested that the supracaudal gland may be used to mark the entrance of the fox's refuge.

The experiments reported in this paper investigated the reactions of foxes to urine and anal sac secretions from both familiar and unfamiliar foxes of both sexes. Seasonal variations in the reproductive cycle may be reflected in both the composition of the scent marks (Albone and Perry, 1976) and the frequency of marking behavior, which increases as the breeding season proceeds (Tembrock, 1954; Burrows, 1968). Male red foxes are fecund between October and April, and the weight of the testes, epididymides, and prostate increase during this period, reaching a peak in the latter part of January and early February. This peak coincides with the much shorter breeding period of the female, who is receptive on only three days of the year (Creed, 1972; Lloyd and Englund, 1973). For this reason, although collection of urine from fecund males was a relatively simple operation, for females this was not the case and it was necessary to use female urine collected some time before signs of estrus could be expected to have appeared.

EXPERIMENT 1

Method

Animals. Three mature males (George, Jack, and Hercules) were used in the experiment. Jack and Hercules sired litters during the course of the experiment and sperm was detected in the testes of the third as late as April. The foxes were housed in separate enclosures with different companions (Table 1). They were housed in concrete pens measuring at least 3 × 4 m and containing an open and covered section. A varied diet of offal, meatloaf, and small rodents together with water, was available *ad libitum.*

Odor Samples

Urine. Urine was routinely collected using a clean metabolism cage measuring 160 × 40 × 60 cm high. A fox was held in the cage overnight and urine collected in a clean, glass bottle beneath the cage. The following morning the urine was decanted into storage jars, labeled, and frozen until required. This regularly yielded quantities of urine in excess of 100 ml/night.

Anal Sac Secretion. The anal sac secretions were collected from restrained foxes by palpating the soft tissue on either side of the anus; under these conditions anal sac secretion was expelled and collected in a clean glass bottle, transferred to a clean storage bottle, and frozen until required. The quantities collected by this method varied from 0 to 800 μl. An odor sample was designated familiar if collected from a cage-mate of the experi-

TABLE 1. DETAILS OF FOXES USED IN STUDY

Fox	Age at beginning of expt.	Experiment	Caged with	Sexual experience	Distant origin
Hercules	5 yr	1,2	1 female, 1 castrate male, 1 ovariectomized female.	Yes	240 km to east
Robin	2 yr	2	2 females, 1 male	Yes	160 km to northwest
George	10 mo	1,2	1 male	No	160 km to northwest
Jack	10 mo	1,2	1 female	Yes	32 km to north
Peter	1 yr	2	1 male	No	160 km to northwest

mental male, and unfamiliar if collected from a fox caged separately. In only one case had the "unfamiliar" donor previously been in contact with the experimental animals, and in this case the contact was limited to 5 min duration, 11 months earlier. The quantities used in this experiment corresponded to the average volumes expelled by captive foxes in one ejection. One ml of urine and 50 μl of anal sac secretion were used.

Procedure

The foxes were observed in an outside enclosure, 4 × 9 m in area. They gained access to the enclosure through a small doorway 40 × 40 cm remotely controlled from a hidden observation point situated 10 m from the enclosure. Two clean tiles were placed 3 m apart in the arena and on one tile, selected at random, was placed either 1 ml urine for the urine condition or 50 μl anal sac secretion for the anal sac condition. One ml of distilled water, representing the control odor, was placed on the other tile. To initiate a trial, the door was opened and the fox allowed to enter the enclosure. A trial lasted for 5 min from the time the first tile was investigated. Failure to visit the second tile during this period resulted in a cancellation of the test. A visit was recorded when the nose of the fox came within about 5 cm of the tile. A marking response was recorded when the subject urinated or defecated within 30 cm of the stimulus tile after a visit. At the end of each trial the fox was withdrawn, the tiles removed, and the enclosure thoroughly cleaned. Each fox was given four trials under each odor condition and was not tested more than twice on any one day. A record was kept of the number of visits made to each tile and the marking responses during the trial period. The order of testing was randomized and tests carried out between December and March between the hours of 4 and 8 PM.

Results

The first tile visited was almost invariably that nearest the entrance to the arena, and for this reason no differences were observed between the control and odor tile for the initial visit. However, the tiles treated with conspecific scent were visited on average 2.92 ± 1.40 occasions per trial compared to 1.60 ± 0.71 for controls ($t_{71} = 10.00$; $P < 0.005$). On average, the treated tiles were marked on 57.6% of the visits and the control tiles on only 7.8% of the visits; a summary of the marking frequencies is included in Table 2. All types of marking showed a higher frequency of occurrence in the vicinity of a conspecific odor source than elsewhere, this being most obvious for urinations. The distribution of defecations was fairly even across the treatments and, owing to the small numbers observed, further analyses were limited to urination marking only.

TABLE 2. SUMMARY OF MARKING RESPONSES DURING ENCLOSURE TESTS

	Directed Urine/anal sac secretion	Directed Control or elsewhere
Urinations	105	13
Defecations	11	5
Prolonged defecations	5	3

Visits. Owing to the caging conditions, not all the experimental animals were tested under all the conditions (Table 1). For example, neither Hercules nor Jack had a familiar intact male cage-mate, and for this reason data for some of the cells were unavailable. Table 3 shows the means for the frequency of visits to the control and experimental odor sources. Comparisons between treatments and their controls and between different treatments were carried out by means of the sign test (Siegal, 1956) except where the number of trials was too small for an adequate test. In all other cases the treatment odor differed significantly from the control odor for the frequency of visits. No significant differences emerged between treatments. However, the results suggest that unfamiliar odors elicit more visits than the corresponding familiar odor.

TABLE 3. FREQUENCY OF VISITING AND URINE MARKING DURING ENCLOSURE TESTS

	Male urine	Control	Female urine	Control	Male anal sac	Control	Female anal sac	Control
Familiar odors								
Mean no. visits per trial	2.50	1.50	2.75	1.63	2.25	1.50	2.75	1.63
Urine marks per trial	0.75	0.0	1.13	0.0	0.75	0.0	1.13	0.0
Urine marks per visit	0.33	—	0.41	—	0.33	—	0.41	—
No. of trials	4		8		4		8	
Unfamiliar odors								
Mean No. visits per trial	3.58	1.50	3.08	1.33	2.67	1.66	2.92	1.50
Urine marks per trial	2.25	0.25	1.33	0.08	1.50	0.17	1.67	0.0
Urine marks per visit	0.63	0.17	0.40	0.06	0.40	0.10	0.57	—
No. of trials	12		12		12		12	

Marking. The figures for marking are similar to those for visits (Table 3). In this case all treatments differed from their controls except odors from familiar males where the number of trials was too small for an adequate test. Unfamiliar odors elicited significantly more urinations than did familiar odors ($P = 0.03$; Fisher's test). Unfamiliar male urine tended to elicit more urinations than any other odor type but only when compared with unfamiliar female urine did this difference approach a satisfactory level of significance ($P = 0.06$; Fisher's test).

Discussion

Both familiar and unfamiliar scent marks were investigated by male red foxes and, although no significant differences emerged between the odor types as measured by the frequency of visits, unfamiliar odors generally attracted greater attention than did the corresponding familiar odor. Unfamiliar odors elicited higher frequencies of marking by urination. Data obtained by Henry (1977, Table 3, p. 91) shows that wild foxes overmark food odors on approximately 70% of visits and on only 20% of visits when urine odor is also present. Clearly the present data, collected under very different conditions during a different season of the year, differ from the data of Henry. On occasions, urine marking was observed on 63% of visits (to unfamiliar male urine) and never did it fall below 33% (familiar male urine). These figures, however, are compatible with Macdonald's observations (1977a). Taken together, these findings support the hypothesis that the marking behavior of foxes performs some social function. The apparent differences between our data and Henry's (1977) may be due to several factors from which the effects of sex, season, captivity, distance from home range, past experience, and motivational state can neither be immediately ignored nor disentangled. Further experiments are needed to clarify these differences.

Studies of domestic dogs (*Canis familiaris*) have shown how odor may mediate social behavior (Le Boeuf, 1967a,b; Doty and Dunbar, 1974; Anisko, 1976; Dunbar, 1977). For example, Dunbar (1977) found that social investigation times between beagle dyads followed a similar pattern to their olfactory preferences. Males spend the longest time visiting estrous females, less time visiting nonestrous females, and even less time visiting males. These differences are paralleled by the findings that males showed a marked preference for estrous urine and vaginal secretions compared with nonestrous samples, and furthermore preferred female urine and feces compared with samples taken from males. Under conditions of artificial estrus, females spent more time investigating male dogs compared to females and similarly preferred male urine to female urine. Finally, both males and females urinated more frequently in the vicinity of the preferred animals and their odors. These findings suggest that urine marking behavior and olfactory preferences of

both sexes may interact to increase the probability of an encounter between a male and a receptive female.

Under different conditions and using different breeds, it has also been shown that olfactory mechanisms may also play a role in mediating intermale relationships. Graf and Meyer-Holzapfel (1974) showed how urine from a strange male or an estrous female elicits a greater reaction from a male than urine from itself or a nonestrous bitch. A female reacted no more strongly to the marks of a strange male than a male did to its own markings.

The following experiment was therefore designed to look closely at the investigation times of foxes to conspecific urine. Doty and Dunbar (1974) previously demonstrated in an enclosed space apparatus an association between odor preferences and social responses of beagles, and the nature of both the test material and target species suggested that a similar technique might be appropriate in the present situation.

EXPERIMENT 2

Animals (see Table 1).

Urine Samples. These were collected as before, from females out of season and fertile males.

Apparatus. The odor testing apparatus consisted of a wire cage measuring 140 × 75 × 75 cm high. One side of this cage was constructed from a galvanized steel sheet containing two inlet ports consisting of several small holes situated 75 cm apart and 30 cm from the base of the cage, equidistant from each end. The cage was situated inside a large Perspex box measuring 180 × 105 × 105 cm high. An extractor fan was positioned at the top of this box. Connected to the inlet ports of the metal cage were plastic tubes that passed through the Perspex box, leading to a simple odor delivery system situated in the adjoining room. The odor delivery apparatus was powered by an oil-free electric pump and allowed air to pass through gas dispersion bottles containing the experimental urine and arrive at the odor ports at a rate of 4 liters/min. A system of stopcocks controlled the air flow to both odor ports and allowed rapid changes in odor types.

Procedure

The fox was placed in the test cage. The experimenter then left the room and followed the reactions of the fox on a video monitor connected to a television camera positioned 2 m in front of the test cage. All animals used were familiar with the test apparatus and were allowed 2 min to adapt. Urine samples were presented simultaneously with a control of distilled water. Two ml of each were placed in dispersion bottles in the delivery apparatus. Urine

odor was delivered to one odor port, selected at random, and the control odor to the other. After 2 min the flow of odors was terminated and the extractor fan turned on; 2 min later the fan was turned off and a different urine odor and control odor were delivered to the odor ports. In this way each fox was tested against each odor type (maximum four types per fox). Each fox was tested on two separate days. The order of presentation was selected at random and balanced between the two tests, as was the position of presentation. A fox was said to visit the odor source when its nose was 5 cm from the odor port. A record was made of the latency and frequency of visiting during each 2-min period (a criterion of one response in at least two periods was adopted). Testing took place between 3 and 6 PM during the breeding season.

Results

The results of the experiment are recorded in Table 4. A large proportion of trials elicited zero approach to either the urine or control odors. This factor reduced the power of the analyses for the latency to first visit. No significant differences were observed for this latency either between urine and control or between urine types, although the latencies were clearly reduced when urine was visited. Unfamiliar male urine elicited the highest frequency of visits, almost double the score obtained for other urine types. This urine differed significantly from the control odor ($P < 0.02$ sign test) (Siegal, 1956), and tended to be visited more than unfamiliar female urine ($P < 0.09$ sign test). Unfamiliar female urine tended to elicit significantly more visits than the control odor ($P < 0.06$ sign test). Unfamiliar male urine, furthermore, elicited a significantly longer total investigation time than the control (Wilcoxon matched pairs, $P < 0.01$). Of the other comparisons, unfamiliar female urine differed significantly from the control (Wilcoxon matched pairs, $P < 0.01$) and elicited longer investigation than familiar female urine (Wilcoxon matched pairs, $P < 0.05$).

Discussion

Under different experimental conditions male foxes again distinguished between conspecific urine odors and spent longer periods investigating urine odors than distilled water controls. Urine from unfamiliar male foxes again elicited the greatest response. In the dog, at least, differences between latencies of investigation to conspecific odors have been shown to correspond well to differences observed in social interactions (Doty and Dunbar, 1974; Dunbar, 1977).

Whether such correspondence exists in foxes so far remains a matter for speculation. For example, it is well known that male foxes engage in increased agonistic behavior during the breeding season (Tembrock, 1954; Burrows,

TABLE 4. RESPONSES OF FOXES TO URINE ODORS DELIVERED BY AIR

	Unfamiliar male urine	Control	Unfamiliar female	Control	Familiar male	Control	Familiar female	Control
Number of trials	10	10	10	10	6	6	6	6
Number of zero visits	2	6	3	6	2	5	2	4
Latency to 1st visit (sec)								
$\overline{X}$	13.37	32.25	13.14	35.75	15.50	15.00	21.00	26.00
SD	9.21	24.47	9.38	36.84	7.51	0.00	18.31	4.24
Frequency of visits ($N = 5$)								
$\overline{X}$	2.30	0.40	1.20	0.40	1.25	1.00	1.25	1.00
SD	1.70	0.52	1.03	0.52	0.50	0.00	0.50	0.00
Total investigation time (secs)								
$\overline{X}$	9.73	0.61	4.71	0.42	3.03	4.40	2.27	1.20
SD	6.13	0.93	3.68	0.57	2.93	0.00	2.28	0.85

1968; Lloyd, 1975). Macdonald (1977b) has further suggested that the increase in agonistic encounters may be partly due to the intrusion of dispersing itinerant male foxes into established male home ranges. Our studies show that males express greatest interest in strange male odors, and this suggests one mechanism for the location and identification of dispersing or breeding rivals.

Further observations in our laboratory suggest that intermale rivalry may be mediated by male urine odor. One male fox, Hercules, consistently displayed aggressive behavior, including biting and tearing at the cage, scratching, tail lashing, vocalization, and often urination in response to stimulus male foxes held in close proximity to his home enclosure. Stimulus females, however, elicited only mild interest unless they had been previously sprayed with male urine when they, too, would elicit considerable aggressive responses. Females sprayed with female urine or anal sac secretion elicited only mild interest, and this corresponds well with some observations made by Preston (1975), who showed that females displayed lower levels of aggression in territorial encounters. Male foxes would elicit aggressive behavior whether they had been treated with female or male odors. Further studies are required to test the generalization of these responses to wild foxes. There is some evidence that among natural populations agonistic interactions between males can be subtly related to geographical distribution. Barash (1975) reported that trapped male foxes exhibited high levels of aggression when confronted with males from distant locations but when males from neighboring locations were presented, the aggressive interactions were much reduced, suggesting some degree of recognition and mutual tolerance, possibly mediated by an olfactory mechanism.

Tembrock (1968) reported that foxes urine mark their partners during the breeding season and females have also been observed rubbing their shoulders upon social scent marks (Macdonald, 1977a; Lloyd, 1978). Such behavior may have consequences for social exchanges between male and female foxes within and between established breeding groups.

Macdonald (1977a) has also presented evidence that suggests the urine marking of foxes is limited to a specific area, outside which marking is curtailed; he suggests this area corresponds to a home territory that is maintained in a similar way to wolf-pack territories (Peters and Mech, 1975). They found that raised leg urinations (RLUs) performed principally by the alpha male varied according to the season and the position of the pack within the territory. RLUs increased along the territorial boundaries where the scent marks of a neighboring group were also present. The implications for studies of captive animals are critical and may explain why several of our animals did not display urine marking under experimental conditions. It is possible that they failed to recognize the test arena as a home territory.

Chemical and bacteriological studies of fox secretions have made considerable advances in recent years (Albone and Perry, 1976; Albone and Flood, 1976; Gosden and Ware, 1976). Differences have been observed between urine samples collected in our laboratory from males and females over several months. Profiles obtained using gas-chromatography techniques showed male samples with consistently more peaks in some parts of the profile of volatile constituents. Jorgenson et al. (1978) analyzed urine samples, collected on snow during the breeding season, from free-ranging foxes and found consistent differences between male and female samples. In another comparison of urine samples from males, females, and castrates the results indicated that urine collected from a castrate male resembled more closely the female profile (Bailey et al., 1977). Further observations are needed to understand more closely how these chemical differences are related to the behavioral ecology of the red fox.

Henry (1977) showed that foxes used urine marks during scavenging behavior in order to exploit their available resources. The evidence from our results and also those of Macdonald (1977a) suggest that scent marking, predominantly by urination, also plays a role in the social interactions of foxes. The high frequency of social-oriented marking in our study was probably due to the fact that among captive foxes, group scavenging behavior is greatly reduced and social marking may assume an increased importance.

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SEASONAL VARIATION OF THREE KETONES IN THE INTERDIGITAL GLAND SECRETION OF REINDEER (*Rangifer tarandus* L.)

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Abstract—The seasonal variation of the three ketones, 6-methyl-2-heptanone, 7-methyl-3-octanone, and 7-methyl-1-octen-3-one, in the interdigital gland secretion of reindeer (*Rangifer tarandus* L.) has been investigated. A group of four adult animals, three females and one male, was used as a test group during a period of two years. Even though the three compounds are monitored in only a small number of test animals, it is evident that the ratios of the secretion's components vary over time. The variations are cyclic and are repeated from year to year.

Key Words—Reindeer, skin glands, interdigital gland, ketones, seasonal variation, *Rangifer tarandus*.

INTRODUCTION

The existence of annual cycles of mammal behavior has been known for a long time—for example, the rutting, calving, antler-growing, and antler-casting seasons in the case of ungulates. Intensive studies of behavior in reindeer and caribou reveal, for instance, the existence of cycles, such as harem-formation and migration and intragroup relations, such as the mother-calf relation (Müller-Schwarze et al., 1979; Kelsall, 1968). Only a few mammals have been investigated both for behavior and for constitution of the skin glands or the skin gland secretion (Brownlee et al., 1969; Müller-Schwarze et al., 1973; Mykytowycz et al., 1974; Quay, 1970). Attempts to classify mammals according to sex, group membership, and perhaps even individual identity, by comparing skin gland secretions are complicated by seasonal variations in the secretion composition.

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The chemical comparison of the secretions were prompted by our study of seasonally varying behavior in reindeer. We examined the possible covariation of behaviour and secretions (see Discussion).

Three dominant peaks were observed in the chromatogram of the volatile part of the secretion, and a preliminary investigation showed that these peaks were not constant with time as regards the relation between the peak size (see Figure 1) when measured according to the method described below. The three ketones were selected because, by this isolation method, they were found to be about 10 times more abundant than, for example, the short acids previously found in the interdigital gland secretion (Brundin et al., 1977).

METHODS AND MATERIALS

Secretion from the hindfoot interdigital gland pocket was taken from four reindeer of the forest variety—three females: ♀ C, ♀ H, and ♀ K; and one male: ♂ M; over a period of two years. Two other reindeer, one male and one female were used intermittently as controls (see Results). The animals were fed on lichens, commercial food, and water in the same proportions during the test period. All the animals were mature, and every female calved in 1976 as well as in 1977.

The secretion, taken with cotton-wool swabs, was immediately (i.e., within 15 min) taken to the laboratory in sealed glass tubes for evaluation. The samples were all taken at 10 AM the sampling day. Reference tests were also performed by immediate freezing of one part of the material in liquid nitrogen for comparison with unfrozen material. With our evaluation methods it was not possible to see any difference in the relative amounts of the three selected peaks from frozen and unfrozen material (see below).

The volatile compounds were transferred to methylene chloride, using the apparatus described by Andersson et al. (1975). In this method a gentle stream (30 ml/min) of nitrogen is passed over the sample heated to 80° C, after which the gas bubbles up through 5 ml methylene chloride. The solution is thereby also evaporated to 100 μl; of this, 10 μl is used in GLC. Evaporation losses were estimated to be the same in all experiments as the experimental conditions were unchanged during the test period.

In the last five months the method was changed to headspace analysis, which was found to be more convenient for the purpose. Cotton-wool swabs with interdigital gland secretion were taken in 3 ml glass bottles sealed with silicone septa. The bottles were kept at 80° C, and 0.5 ml of the atmosphere in each bottle was removed with a preheated (50° C) gas-tight syringe and injected in the column (see below). During the change-over to headspace analysis, both evaluation methods were used in parallel until analysis with the new method was reproducible. The volatile compounds were then inves-

tigated on a Pye Unicam GCD gas chromatograph equipped with flame ionization detectors. The glass column (1.8 m, ID 0.4 cm), filled with DMCS-treated Chromosorb W (100–120 mesh and coated with 5% Reoplex 400), was run with nitrogen as carrier gas (40 ml/min) under isothermal conditions (85° C). The detector temperature was 200° C and the injection temperature 130° C. Identification of the GC peaks was made in each experiment with a solution containing the three ketones (see below).

From the chromatograms in Figure 1, the three dominant peaks were selected for analysis over a longer period. They were denoted A, B, and C in order of increasing retention time (see below). A graphical description of the seasonal variation was made by calculating the ratios B/A and C/A, using the integrals of A, B, and C, and plotting them against time (method I). Another method consisted in summation of the peak areas of A, B, and C, followed by calculation of the integrals of each peak as percentage of the total sum of integrals (method II). Since there were only three peaks to be compared internally, method I was found to be a better mathematical description method.

The individual values of B/A and C/A have respectively been connected with straight lines, so as to simplify the interpretation of the seasonal variation. This method was used as there are too few points along the curves to afford a detailed picture of the variation. Some parts of the curves are dotted owing to lack of sample during periods exceeding two months. To check the reproducibility, some controls have been made with samples from several successive days.

RESULTS

The three peaks A, B, and C (see Figure 1) were analyzed by GC-MS, verified by synthesis, and found to correspond to 6-methyl-2-heptanone (A), 7-methyl-3-octanone (B), and 7-methyl-1-octen-3-one (C) (Andersson et al., 1979). Examination of the curves in Figure 2 shows drastic changes in the secretion composition over a yearly cycle. The B/A curves from ♀H and ♀C show obvious similarities when the above-mentioned amplitude differences are not emphasized. They both have a minimum in March surrounded by the two maxima around October–January and May–June. The former maximum is uncertain owing to lack of sample from both animals. The described minimum appears once more in March 1977, although with lower amplitudes of the surrounding maxima and some extra peaks at the end of March and the beginning of April. The B/A curve from ♀K has the same main fluctuations as those of ♀C and ♀H, but with some individual variations and gaps owing to lack of sample during some periods.

As there was only one male in the investigation, no sexual differences in

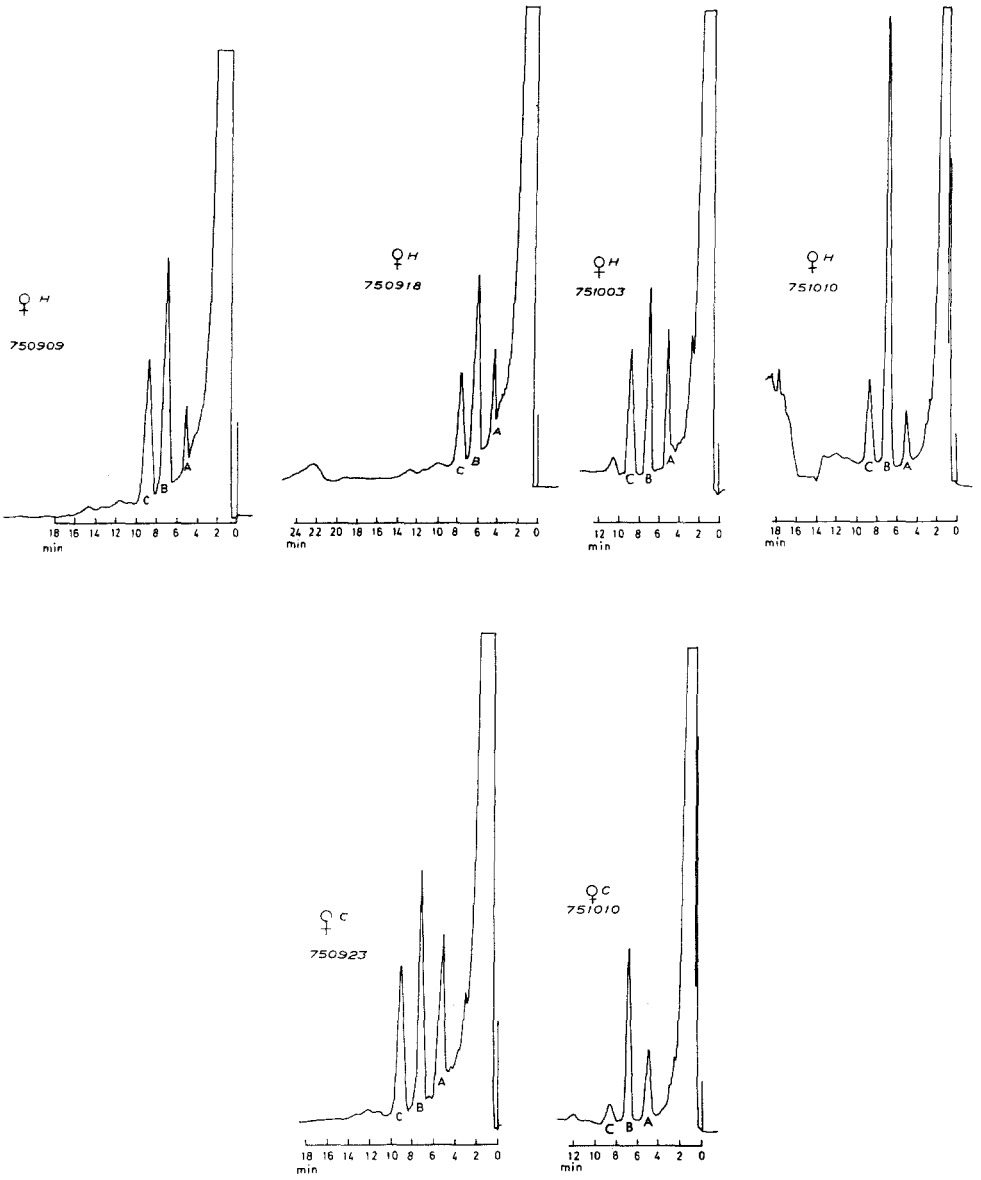


FIG. 1. Gas chromatograms of three ketones, A, B, and C in the interdigital gland secretion from reindeer. The chromatograms have been taken according to the marking in each figure [specimen and date (year, month, and day)].

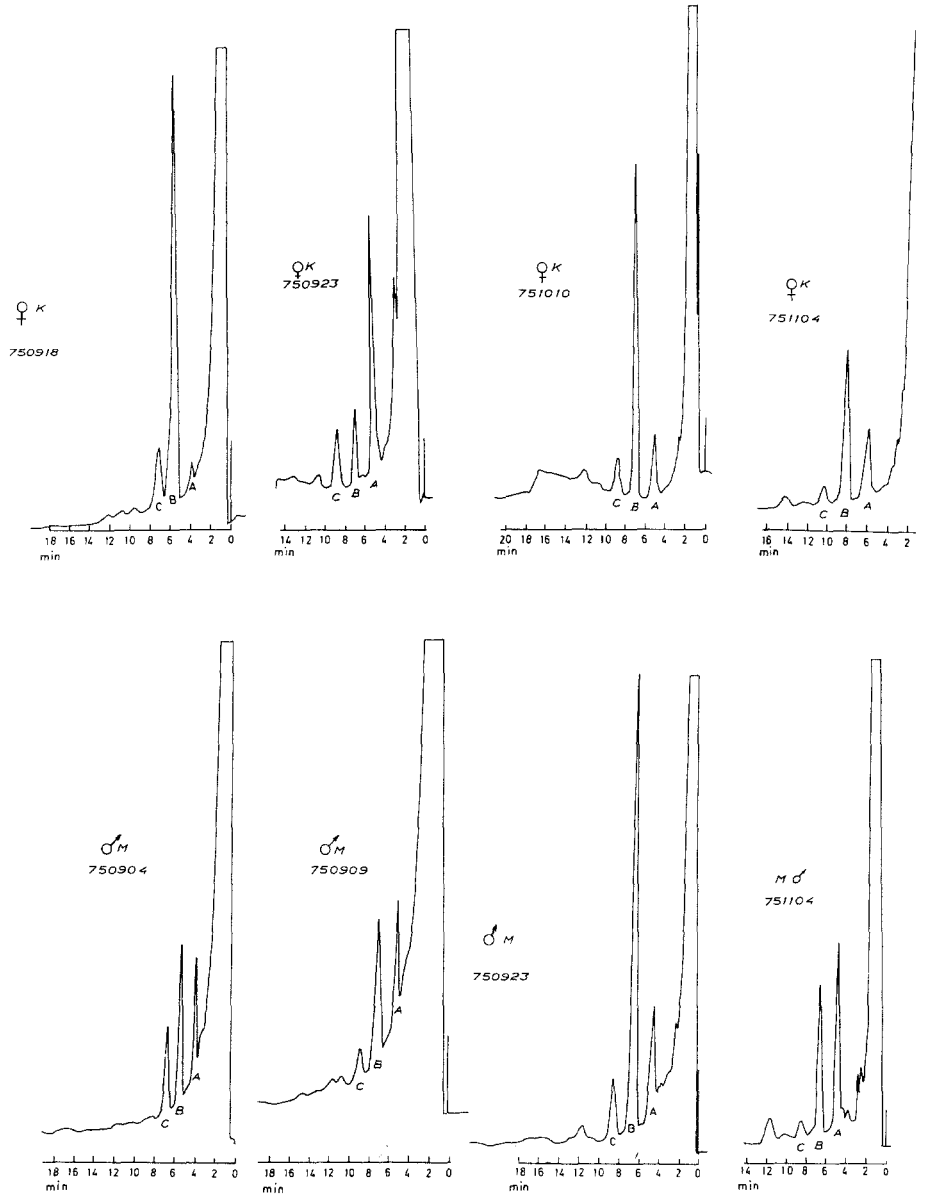
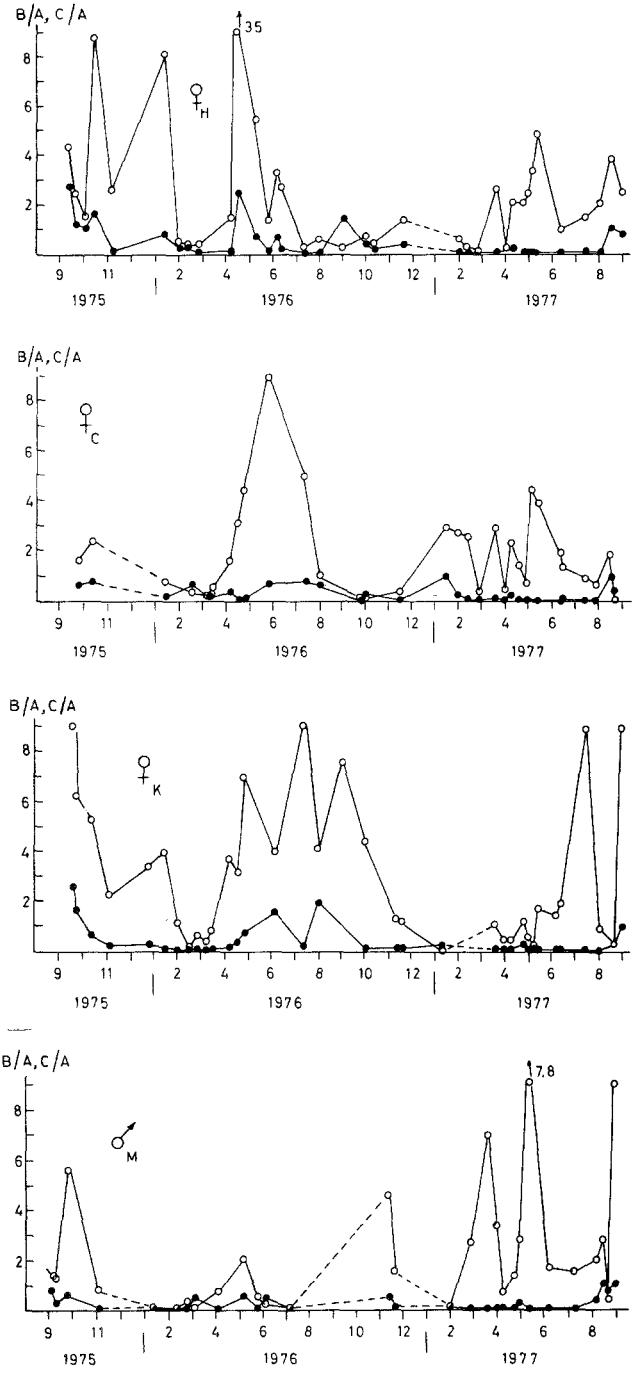


FIG. 1. Continued.


B/A, C/A
35
♀H
♀C
♀K
♂M
7.8
9 11 2 4 6 8 10 12 2 4 6 8
1975
1976
1977

the secretion will be postulated. However, in the secretion from ♂ M drastic changes in the composition during the test period were noted. As in the female material, the cyclic variations for B/A are found to recur from year to year.

In all animals the C/A curves show no clear consistency or similarity from one animal to another.

In order to obtain an idea of the variation in the total amount of secretion, the sum of integrals from A, B, and C was compared from time to time. This is an inexact method since it is impossible to remove equal amounts of secretion from the gland. However, this investigation showed, for the females at any rate, more secretion in May and September than at other times of the year. The given result also fits well with observations made in the field (Dietland Müller-Schwarze, personal communication).

The individual values of B/A and C/A from the control animals also agreed, with minor irregularities, with the results from the animals described above, i.e., when a minimum appeared for B/A (described above) for a female, control material from another female followed the same line.

DISCUSSION

Reindeer are semidomesticated ruminants. They show cyclic changes in certain types of behavior and physiology, such as sexual behavior, antler-growing, velvet-shedding, antler-casting, calving, and group formation. Their general behavior also follows a yearly cycle. This concerns the frequency of agonistic behavior, chemical communication, solitary sniffing and licking, hindleg-head contact, forehead rubbing, and thrashing (Müller-Schwarze et al., 1979).

It is known that reindeer and caribou adapted to widely fluctuating environments exhibit fluctuations in metabolic activities, such as mineral status (Helle et al., 1977) and energy intake (McEwan, 1968; McEwan and Whitehead, 1970), which is reflected in a yearly cycle of body weight. The seasonal variation of the amount of secretion in the interdigital gland of reindeer has been investigated by Kurt (1968). However, no specific correlation between the cycles described above and the variation of secretion composition is known so far. An analysis of the anal gland secretions of the red fox (*Vulpes vulpes*) has been reported by Albone and Perry (1976). There, individual differences and differences between left and right anal sac secretion has been presented, but no seasonal variation.

In our case left and right interdigital gland secretion has been investigated

FIG. 2. Relative abundance of three ketones A, B, and C from reindeer interdigital gland secretion, presented as the ratios B/A and C/A against time (o-o: B/A; •-•: C/A).

and found to be very similar, and because of that, material from the two glands was combined during the test. The generality of the results presented in this article is of course limited by the low number of test animals and by the fact that only three compounds were tested. However, the limitation consists mainly in the inability to give sexual and individual recognition to the secretions. The answers concerning sexual differences and individuality can only be provided by using, say, 20 animals and by testing approximately 15 peaks in the gas chromatograms from the volatile compounds over a period of 2–3 years.

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SEX PHEROMONE OF THE SAN JOSE SCALE[1,2]

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Abstract—The sex pheromone of the San Jose scale, *Quadraspidiotus perniciosus* (Comstock), was isolated from airborne collections on Porapak Q. Two components, present in approximately equal amounts, were identified as (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate and 3-methylene-7-methyl-7-octen-1-yl propanoate. Greenhouse bioassays and field tests have shown that the compounds are independently attractive to male San Jose scale. These structures are compared with those of other known scale pheromones.

Key Words—sex pheromone, San Jose scale, *Quadraspidiotus perniciosus*, Diaspididae, (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate, 3-methylene-7-methyl-7-octen-1-yl propanoate.

INTRODUCTION

The San Jose scale, *Quadraspidiotus perniciosus* (Comstock), is a major and widespread orchard pest that attacks all parts of host trees including the fruit. According to Downing and Logan (1977) good control is possible in light infestations with a dormant oil spray, while heavily encrusted infestations require additional applications of insecticides. The latter sprays are aimed at the crawlers since an early oil treatment may not reach below dead scales or

[1]*Quadraspidiotus perniciosus* (Comstock) (Homoptera: Diaspididae).

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under loose bark. The period of crawler emergence is predicted using heat accumulation (degree day) data timed from the male flight.

Rice (1974) demonstrated the presence of a sex pheromone for the San Jose scale by using traps baited with virgin females in orchard tests. Further studies over a 3-year period (Rice and Jones, 1977) showed that these traps could be used successfully to monitor the multiple male flights that occur throughout the host's growing season. As with other species of pests, synthetic pheromone could be used in place of virgin females to measure the extent and level of infestations. In addition, the pheromone would be extremely useful in timing the spring control sprays for the period during which both sexes of the scale are most susceptible; that is, when the males are emerging and the females have extended their pygidia. This paper describes research leading to the identification of the sex pheromone of the San Jose scale.

METHODS AND MATERIALS

The scales were reared on banana squash as described by Rice (1974). For pheromone collection, the males were mechanically removed from the squash with a brush when their scale covers became loose. This occurred during the male pupal stage, approximately 3 days before the females began releasing pheromone. [Early work found that 98% mortality of males would result by spraying the banana squash to run-off with a solution prepared by dissolving 3.3 ml of 2.0 EC dichlorvos (Vapona®, DDVP or 2,2-dichlorovinyl dimethyl phosphate) in 500 ml water. However, this treatment was discontinued as it also killed 50% of the females]. The squash were then placed in a plastic ice chest (ca. 100 liters) fitted with a Plexiglas cover (Figure 1). Ambient air, precleaned by activated charcoal, was pulled over the infested squash and then through a column of 40 g Porapak Q by a vacuum pump at a rate of 1-3 liters/min. Usually pheromone was collected for 2 weeks before the Porapak Q was rinsed with 250 ml pentane, which was then concentrated under vacuum to form a crude pheromone extract.

Bioassays were conducted in California in a greenhouse containing scale-infested banana squash. The traps, similar to those used to capture California red scale (Rice and Moreno, 1969), consisted of a sticky card (7.6 × 12.7 cm) held above a pint ice cream carton with a screen top. A check trap contained a wild gourd, *Cucurbita foetidissima* (H.B.K.), from which all scales had been mechanically removed except for a specific number of virgin females. To test a liquid solution, however, a hole (radius = 0.9 cm) was cut from the center of the coated card and a slit was made below the hole so that a semicircle of filter paper (radius =1.2 cm) could be positioned in the opening perpendicular to the card. Solutions of natural extracts and synthetic chemicals were applied to the filter paper dispenser. Ten, or fewer, of these traps were positioned about

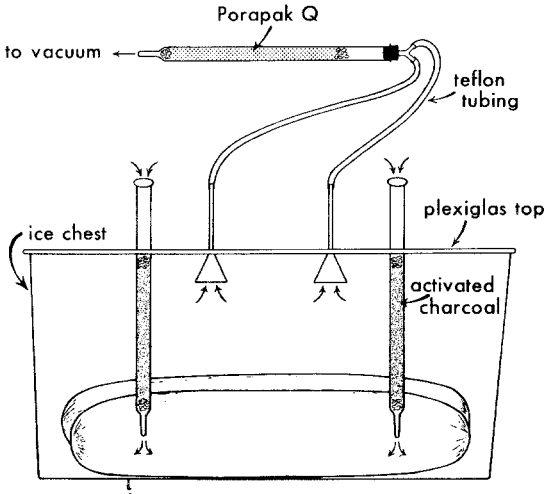

FIG. 1. Apparatus used to collect sex pheromone from female San Jose scale. The inside of the ice chest is shown containing charcoal-packed columns, for precleaning the air, and infested banana squash. Unlabeled arrows indicate ambient air flow.

the perimeter of a 1.22-m-diameter table which rotated at a rate of 0.083 rpm. The tests were begun in the evening and terminated after a few hours of flight activity.

The solution of the crude extract was applied first to a 1.5 × 50 cm column of Florisil (60-80 PR mesh, Floridin Co.). The column was eluted with 900 ml of a 0-50% gradient of diethyl ether in Skelly B. Eight fractions were collected and 5% of each was bioassayed.

Active fractions were combined and applied to both polar and nonpolar GLC columns (see below). Again fractions were collected and bioassayed until the material was considered pure as determined by observing a single peak on polar and nonpolar GLC columns.

After fractionation of the crude material on Florisil and either before or after GLC purification, the pheromone was injected on a 1 × 100-cm-high pressure column packed with 10% $AgNo_3$ on Bio-Sil A (20-44 μm, Bio-Rad Laboratories). The column was eluted with 500, 600, and 250 ml of 0, 2.5 and 20% diethyl ether in benzene, respectively.

Mass spectra were obtained at 20 eV on a Hitachi RMU-6E spectrometer interfaced with an OV-1 column.

Proton magnetic resonance (PMR) spectra were obtained with either a Varian HR 300 (Fourier transform) spectrometer (TNO, Delft, The Netherlands) or a Varian HA-100D spectrometer (Geneva, New York).

For the purposes of chemical identification microreactions on the natural

products included the following: saponification with dilute (ca. 1%) NaOH in 90–95% EtOH at 45° for 1 hr; esterification of alcohols with propanoyl chloride in CS_2; and hydrogenation at atmospheric pressure and room temperature with 5% Pd on $BaSO_4$ in 95% EtOH.

The GLC columns used were 3% OV-101 (methyl silicone on 100–120 mesh Gas-Chrom Q; 2 m × 4 mm glass column) and 10% XF-1150 (50% cyanoethyl methyl silicone on 100–120 mesh Chromosorb W-AW-DMCS; 2 m × 2 mm glass column). Retention values were obtained by coinjection with normal alkanes and are reported as Kovats retention indices (KRI) (Kovats, 1958).

RESULTS AND DISCUSSION

Table 1 shows bioassay data obtained during pheromone purification. After Florisil chromatography and GLC collections, all biological activity was found in a single peak on both OV-101 (KRI = 1418) and XF-1150 (KRI = 1860). Saponification of this material, however, produced two product peaks having KRI values of 1200 and 1218 on OV-101 and 1814 and 1844 on XF-1150. The relative amounts of the two alcohols were equal in one sample and in a 3:2 ratio, in favor of the earlier eluting compound, in

TABLE 1. SAN JOSE SCALE MALE CATCHES IN GREENHOUSE BIOASSAYS TO PHEROMONE PURIFICATION FRACTIONS

Fraction number	Males attracted to fractions from			
	Florisil[a]	OV-101[b]	XF-1150[c]	$AgNO_3$[d,e]
1	14	6	7	1
2	9	506	9	17
3	16	6	249	31
4	367	48	9	25
5	317	26	3	2
6	329	28	7	1
7	64	71	8	14
8	92	19	3	29
9				27
10				14
11				7
12				9

[a]Crude extract was applied to the Florisil column.
[b]Fractions 4–6 from the Florisil column was injected on OV-101.
[c]Fraction 2 from the OV-101 collection was injected on XF-1150.
[d]Fraction 3 from the XF-1150 collection was applied to the $AgNO_3$ column.
[e]Fractions 2–4 were combined for SJS-1 and fractions 7–10 were combined for SJS-2.

another. The difference in relative amounts could result from selective loss during collection or purification of one of the samples or could represent a variation in pheromone production by the scales. When the two alcohols were collected separately and treated with propanoyl chloride, GLC peaks having the same retention value on XF-1150 and nearly the same on OV-101 (KRI = 1414 and 1423) as the original material were produced. Each of these esters was bioassayed and found to be active and thus both compounds were shown to be propanoates.

Argentation chromatography also separated the two components. When the active material, either taken directly from the Florisil column or after GLC purification, was applied to the $AgNO_3$ column, two areas of biological activity were obtained. There were labeled SJS-1 and SJS-2 in order of their elution. It was found that SJS-1 had a slightly longer retention time on OV-101 than SJS-2, when injected separately, as did the saponification product of SJS-1 on both GLC columns relative to that of SJS-2.

Mass spectra of the individual components (Figure 2) were obtained and indicated that both compounds had branched 10-carbon skeletons and two degrees of unsaturation. When the compounds were hydrogenated separately, the mass spectra of the products were identical (Figure 3) and the same as 3,7-dimethyloctan-1-yl propanoate obtained by hydrogenation of geranyl propanoate. Also, GLC retention values of the hydrogenation products and of hydrogenated geranyl propanoate were identical (KRI = 1398 on OV-101 and 1707 on XF-1150), thus determining the hydrocarbon skeletons of both compounds.

A PMR spectrum (Figure 4) of the active material containing both components was obtained in CS_2. (The sample was prepared and sent to The Netherlands before it was realized that the GLC single peak was actually a mixture of two compounds. However, since there was no ambiguity in the interpretation of the resulting spectrum, PMR spectra of the individual components were not obtained.) Detailed analysis of this spectrum and of decoupled spectra by TNO allowed the assignments of chemical shifts and the 2-ene double bond configuration in one of the compounds. These data and those discussed above were consistent with structures (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate (40%) and 3-methylene-7-methyl-7-octen-1-yl propanoate (60%) (Figure 5) for SJS-1 and SJS-2, respectively.

As part of this project, scientists at Zoecon (Anderson et al., 1979) synthesized the two components of the San Jose scale sex pheromone. The synthetic and natural compounds were found to be identical by GLC retention times on polar and apolar columns, mass spectra, and PMR spectra, thus confirming the above structural assignments. In addition, a synthetic sample of the *E* isomer of SJS-1 was prepared and found to elute from the GLC considerably later than SJS-1 (KRI = 1451 and 1906 on OV-101 and XF-

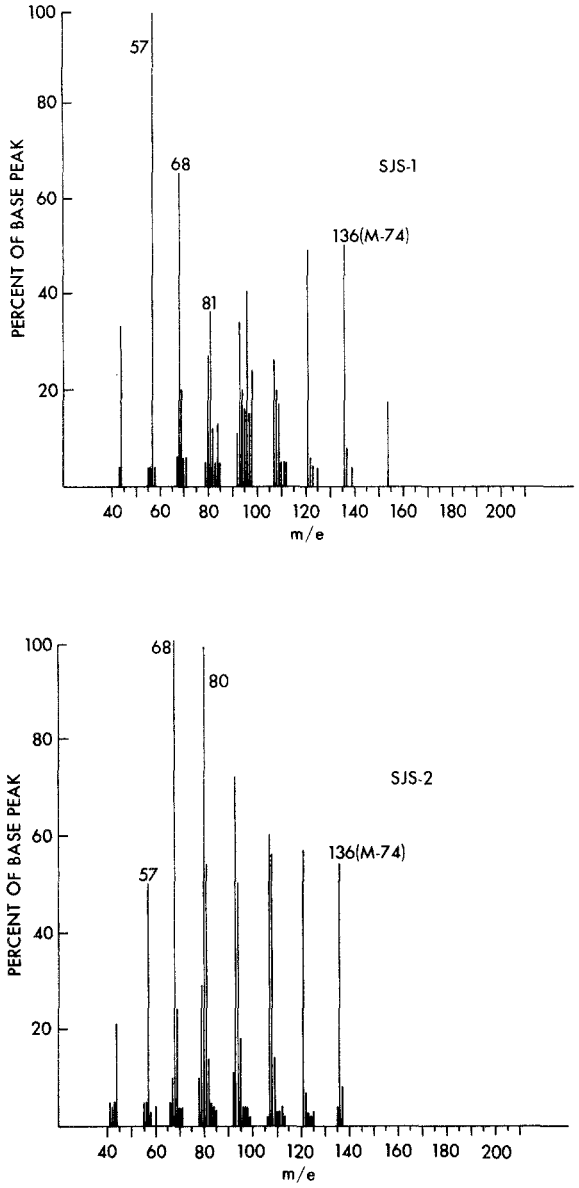

FIG. 2. Mass spectra (20 eV) of SJS-1 and SJS-2.

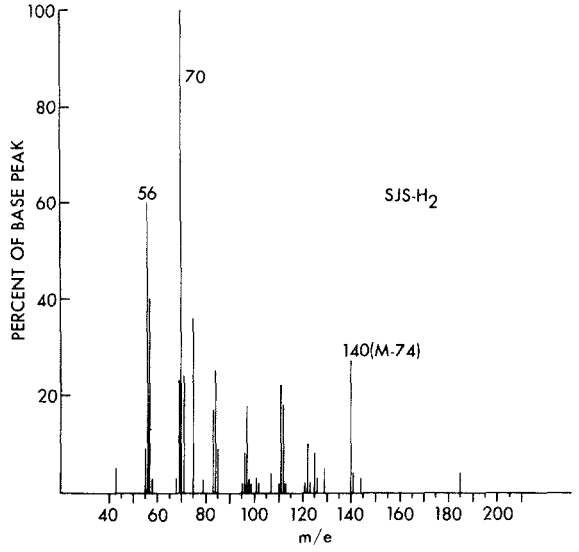

FIG. 3. Mass spectrum (20 eV) of a hydrogenated sample of either San Jose scale pheromone component (also 3,7-dimethyloctan-1-yl propanoate).

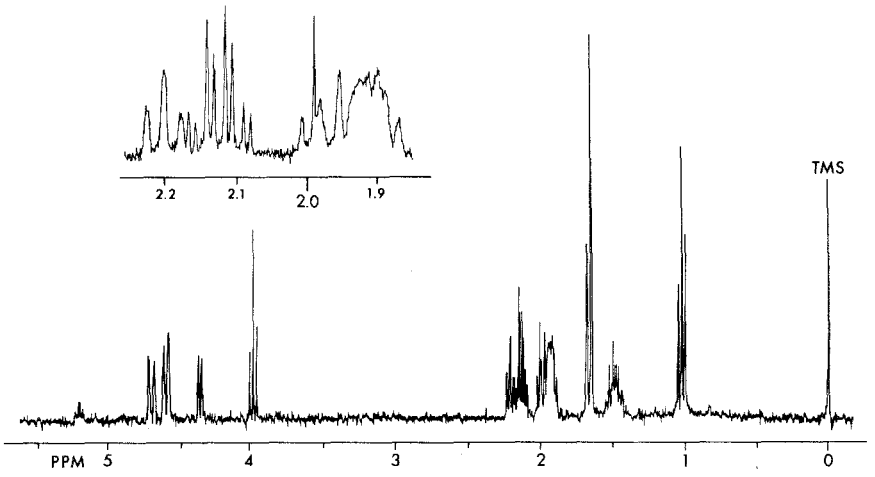

FIG. 4. PMR spectrum of San Jose scale active components taken in CS_2 at 300 MHz. Insert is enlargement of spectrum between δ 1.85 and 2.25 ppm.

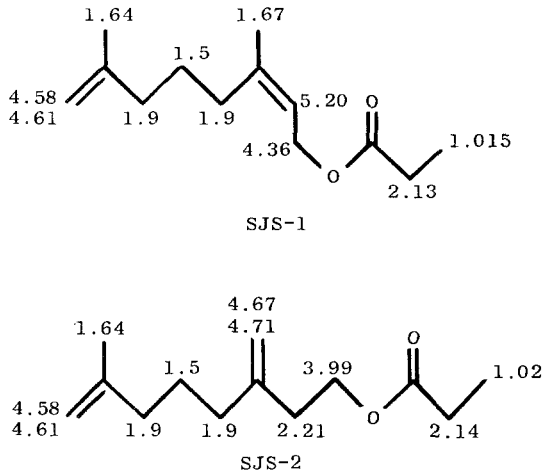

FIG. 5. Structure of San Jose scale sex pheromone components with PMR assignments in δ ppm.

1150, respectively, as compared with KRI = 1423 and 1860 on the same two columns for SJS-1).

Early results of greenhouse and field tests show both pheromone compounds to be active. In one field test, in a peach orchard in California, synthetic SJS-1 was tested at load rates of 100 and 300 μg on rubber septa. The total males trapped in four replicates for 3 nights were 273 and 325, respectively, compared to 197 for female-baited traps (ca. 250 females/trap) and 26 for blank traps. In another California field test, SJS-2 was tested at septa load rates of 33, 100, 300, and 900 μg. The total overnight catches of 4 replicates were 65, 50, 46, and 24 males, respectively, compared to 26 males in female traps and 2 males in blank traps. These data show that both compounds are independently attractive to male San Jose scale. A similar apparent redundancy of pheromone components was noted with the California red scale (Roelofs et al., 1978). (Preliminary field tests in New York apple orchards showed that traps baited with SJS-2 could become covered with thousands of San Jose scale males in several days in moderate infestations). More extensive field testing is being conducted with the pheromone compounds, both separate and together, as well as on isomers and analogues (Rice et al., unpublished).

The sex pheromone of three other scale species have been previously identified: the California red scale, *Aonidiella aurantii* (Maskell), (Roelofs et al., 1977); the yellow scale, *A. citrini* (Coquillet), (Gieselmann et al., 1979); and the white peach scale, *Pseudaulacaspis pentagona* (Targione-Tozzetti), (Heath et al., 1979). Some interesting comparisons can be made among these (shown in Figure 6) and the pheromone of the San Jose scale. All six com-

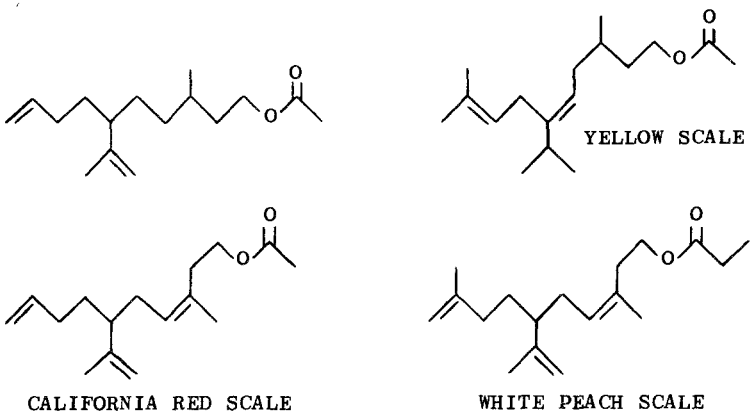

FIG. 6. Previously identified scale pheromones (see text for references).

pounds are branched, acyclic, unsaturated (isoprenoid) acetates or propanoates. However, the four compounds of previously identified scale pheromones have hydrocarbon skeletons of 14 or 15 carbons and lack the normal head-to-tail joining of isoprenoid units. The components of the pheromone of the San Jose scale, on the other hand, have 10-carbon skeletons and typical terpene structures. The California red scale, the yellow scale, and the San Jose scale belong to the same tribe, Aspidiotini, while the white peach scale is of the tribe Diaspidini. It will be interesting to note the correlation between pheromone structures and phylogenetic relationships as more scale pheromones are identified.

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HYDROGEN CYANIDE-PRODUCING GLANDS IN A SCOLOPENDER, *Asanada* N.SP.[1] (CHILOPODA, SCOLOPENDRIDAE)

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Abstract—A new type of defensive gland has been discovered in a scolopendromorph centipede, *Asanada* n.sp. The defensive secretion contains hydrogen cyanide and protein. It is produced in numerous unicellular glands, spread all over the body from the second to the next to the last body segment and over the legs. The morphology of the gland cells has been studied histologically. They contain two different secretions which are mixed during release.

Key words—*Asanada,* Scolopendridae, Chilopoda, unicellular defensive glands, hydrogen cyanide, protein.

INTRODUCTION

Scolopenders are known to be dangerous venomous animals, which inject the toxic secretion of their poison claw glands not only into their prey but also into their enemies. Therefore we were rather astonished in catching a small scolopender belonging to the genus *Asanada*, which was able to release a secretion with a strong almond-like odor over the entire length of its body. This publication reports first results on the chemical composition of this secretion. The glands which produce it have been localized and studied morphologically and histologically.

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METHODS AND MATERIALS

Asanada n.sp. was found in Anuradhapura (Sri Lanka). During a 5-month stay only 4 specimens of this rare species could be caught. They were living within wood infested by nasutitermitines. Therefore only spot tests (Feigl, 1960) could be performed for identification of the secretion compounds. For histology the animals were fixed in Bouin and embedded in methyl methacrylate (Rathmayer, 1962). The sections were stained with Azan (Haidenhain) (Romeis, 1968). The micrographs were taken with a Hitachi 5500 scanning electron microscope.[4]

RESULTS

When an *Asanada* n.sp. was caught, it fiercely beat and twisted its body around. After this our hands smelled strongly of bitter almond, but the secretion was not visible. When the animal was pressed against a smooth paper, the secretion could be seen as a thin shining colorless layer on the paper surface. Due to the smell of the secretion we supposed that *Asanada* produced hydrogen cyanide. This was tested with three different spot tests: copper sulfide, copper acetate-benzidine acetate, and thiocyanate. The positive reaction of the secretion in all three tests demonstrated the presence of hydrogen cyanide. The characteristic color of the secretion, stained with Ponceau S and ninhydrin, indicated the presence of a protein. Furthermore, the secretion turned to deep yellow when sprayed with 2,4-dinitrophenyl-hydrazine HCl, suggesting the presence of a carbonyl compound. Identification of this compound was not possible because of lack of material.

The mode of release of the secretion and its highly toxic hydrogen cyanide component prove that it has defensive functions. Its defensive effectiveness against enemies could not be tested because we had not enough animals.

After pressing an animal on a cellulose acetate membrane the release pattern of the secretion could be seen by staining it with Ponceau S. The fluid was released in small droplets sometimes converting to larger spots. It formed repeated segmental patterns along the anterior, posterior, and lateral margins of the sternum and along the legs (Figure 1). An examination of the body surface with a binocular and a scanning electron microscope showed a great number of pores from the second to the penultimate body segment. Pores were found on the sternites, pleurites, tergites, and legs (Figure 2). This explains the distribution of the secretion shown in Figure 1. About 300 pores per segment could be observed on an animal three centimeters in length. A smaller centipede had fewer pores. The pores were not regularly arranged

[4]We are indebted to Dr. R. Barkhausen, Frankfurt, for his help with the SEM photographs.

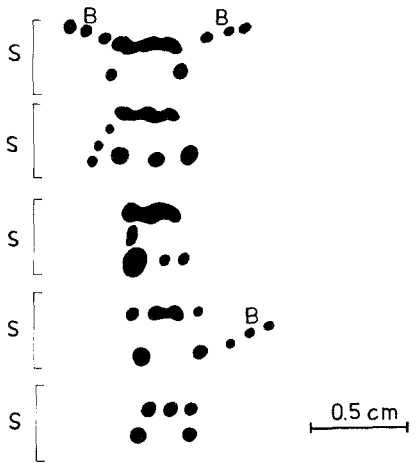

FIG. 1. Schematic drawing of the secretion released ventrally on a cellulose acetate membrane (staining: Ponceau S). S length of a segment, B print of a leg.

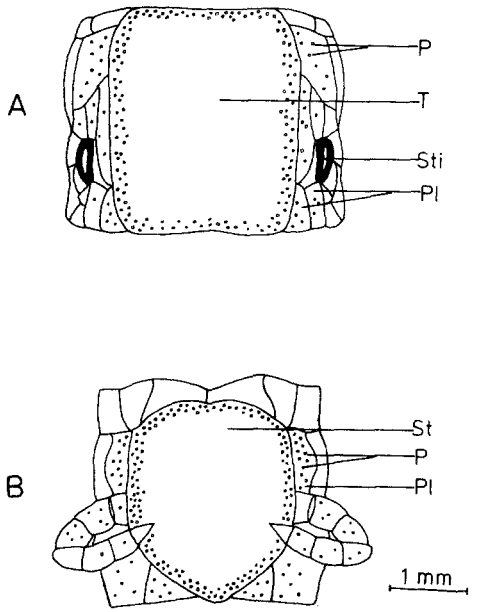

FIG. 2. Distribution of the gland pores on the surface of a segment (no. 5). P, pores; T, tergite; Sti, spiracle; Pl, pleurite; St, sternite.

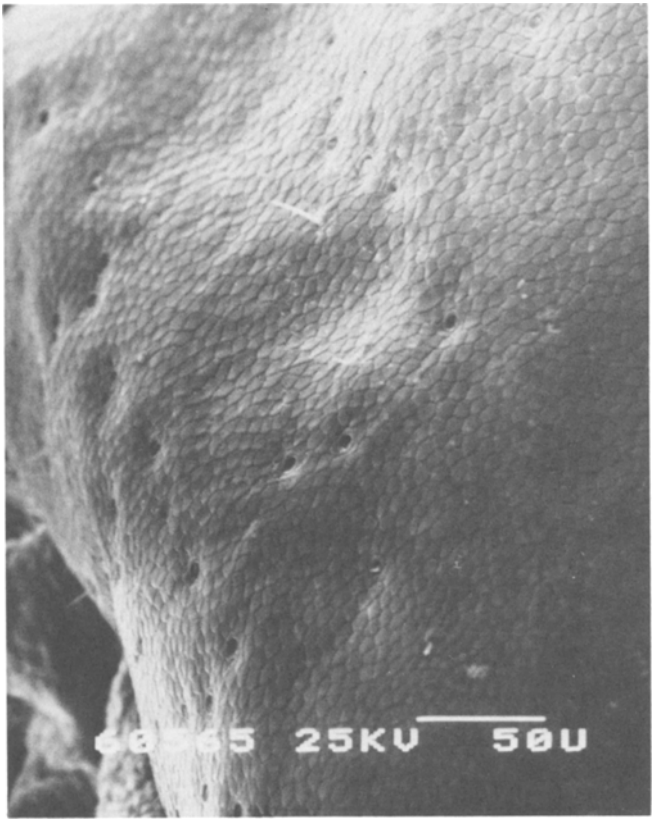

FIG. 3. Margin of a tergite with gland pores (SEM photograph).

(Figure 3). Some of them were scattered and some were arranged in groups of two to four. The pores were of the same size (about 5 μm ϕ) over the entire body. When fixed with Bouin, light spots which were apparently gland cells filled with secretion shone through the animal's cuticle. In histological sections the gland cells were found at the margins of the sternites, tergites, and pleurites, and in the legs.

Depending on the region from which the cross-section was taken, one to four gland cells or gland cell layers could be found filling the whole width of the sternite or tergite at its anterior or posterior margins (Figure 4). The gland cells were elongated tubes (diameter 30 μm) with constricted ends. The lens-shaped nucleus (ϕ 5 μm) with its clearly visible nucleolus was marginally situated in a thin plasmatic layer, which covered the whole wall of the gland cell. The orifice, a cuticular nozzle with a length of 7 μm, projected half way

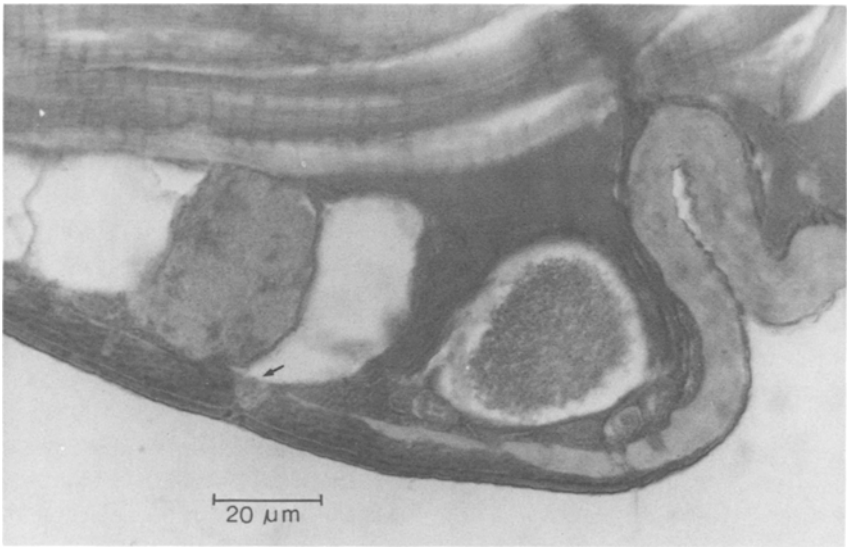

FIG. 4. Cross-section of a tergite (posterior margin) with a gland cell layer covered by muscles. Three of the five visible cells are emptied. At one emptied cell the orifice (↓) is visible penetrating the cuticle.

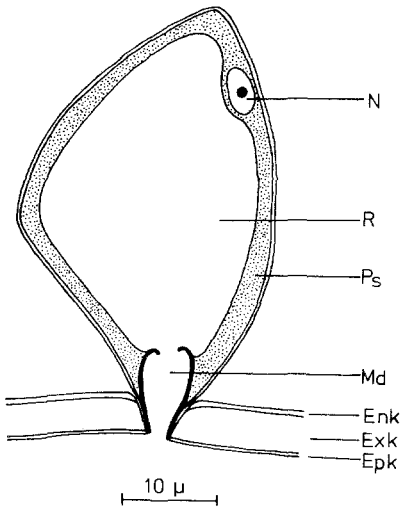

FIG. 5. Schematic drawing of a single gland cell (empty cell). Enk, endocuticle; Epk, epicuticle; Exk, exocuticle, Md, orifice structure; N, nucleus; Ps, cytoplasma; R, intracellular reservoir.

into the gland cell (Figure 5). It was constricted distally. A special structure for closure could not be observed.

The interior of the cells was largely filled with a finely granulated mass. Some cells appeared to be empty to different extents. Although no morphological differences were seen, with Azan staining two types of cells could be distinguished, one containing a brown and the other a red secretion. Both types of cells occurred with equal frequency, lying close together and regularly alternating. Different stages of the secretions could not be observed.

Gland cells or groups of gland cells always bordered on cross-striated muscles, attached at the cuticle. As we could not observe any special mechanism for emptying the cells, we presume the contraction of these muscles, together with increasing hemolymph pressure, to be responsible for the discharge of the secretion.

In addition to the newly discovered unicellular defensive glands, *Asanada* possess a typical poison claw gland like that of other Scolopendromorpha (Attems, 1926).

DISCUSSION

All Chilopoda possess poison claw glands, which were used for attacking prey and also partly for defense. Additional pugnatory glands, which are exclusively defensive in function, are known in the corhorts Lithobiomorpha and Geophilomorpha (Kaestner, 1963): unicellular sticky glands on the legs of *Lithobius* and unicellular sternal glands producing hydrogen cyanide in *Pachymerium, Geophilus,* and *Strigamia* (Schildknecht et al., 1968; Jones et al., 1976).

With the finding of the hydrogen cyanide-producing unicellular glands in *Asanada,* additional defensive glands are now known in a further cohort of the Chilopoda. In the Geophilidae, hydrogen cyanide is probably produced in a catalyzed cyanogenetic reaction. Mandelonitrile and benzoyl cyanide have been identified as precursors (Jones et al., 1976). The catalyst is probably an enzyme (Schildknecht et al., 1968). The sternal glands consist of two types of gland cells (Koch, 1927), which are presumed to produce on one hand the protein and on the other hand the precursor.

How the hydrogen cyanide is produced in *Asanada* is not yet known. The following facts support the hypothesis that it is generated in a cyanogenetic process: (1) the presence of a protein in the secretion; (2) a carbonyl compound in the secretion, possibly a product of the cyanogenetic reaction; and (3) two different stainable secretions which occur in alternately lying cells.

The unicellular glands of *Asanada* are not as regularly arranged as in many geophilomorph centipedes. They are located not only on the sternites, but also on the pleurites, tergites, and legs. In their morphological structure, however, the gland cells so strongly resemble those of the Geophilomorpha

that we presume a homologous origin of the both. Our knowledge concerning the distribution of such organs in Chilopoda, however, is too limited to allow any further evolutionary speculations.

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ATTRACTION OF NEMATODES TO METABOLITES OF YEASTS AND FUNGI

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Abstract—The free-living nematodes *Panagrellus redivivus* and *Rhabditis oxycerca* are strongly attracted to methyl, ethyl, propyl, butyl, and amyl acetate, to ethyl, propyl, and amyl formate and to ethyl propionate, but all the respective alcohols and acids are without effect. No loss of attraction is observed when the attractants are combined with lethal concentrations of the commercial nematicide sodium methyl dithiocarbamate.

Key Words—Attractants, nematodes, *Panagrellus redivivus, Rhabditis oxycerca, Saccharomyces cerevisiae,* predacious fungi, methyl acetate, ethyl acetate, propyl acetate, butyl acetate, amyl acetate, ethyl formate, propyl formate, amyl formate, ethyl propionate, sodium methyl dithiocarbamate.

INTRODUCTION

Predacious fungi are microorganisms which are able to capture and kill nematodes, protozoa, rotifera, crustacea, and miriapoda. The trapping of nematodes by fungi was first described by Zopf (1888) and more than 50 species of such fungi, of which most belong to the family of Hyphomycetes, are known. The organelles of capture are either sticky knobs, loops, or three-dimensional reticula in which the prey becomes entangled and soon dies. The dead prey is then used as a source of nutrients. The most sophisticated organelles of prey are constricting rings consisting of three cells. A nematode entering such a ring triggers the mechanism of trap closure and

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within a fraction of a second the three cells of the trap expand into spherical shapes and obliterate the lumen of the ring thus gripping the nematode so tightly that it cannot escape. Predacious fungi are not obligatory parasites and are able to live on media not containing nematodes. Trap formation in predacious fungi is a morphological response of the microorganism to the presence of substances produced by the potential prey (Pramer and Stoll, 1959). We have found that trap formation can also be induced by adverse cultivation conditions (Balan and Lechevalier, 1972) and other factors influencing trap formation have also been described (Nordbring-Hertz, 1978). Nematode-trapping fungi also produce attractants luring the prey to the traps (Balan and Gerber, 1972; Monoson and Ranieri, 1972; Monoson et al., 1973; Balan et al., 1974; Križková et al., 1976). The captured prey is "killed chemically" by substances produced by the fungus and not by some kind of "mechanical strangulation" (Olthof and Estey, 1963; Giuma and Cooke, 1971; Balan and Gerber, 1972; Balan et al., 1974; Križková et al., 1976). Problems connected with predacious fungi were studied and described in several reviews (Drechsler, 1941; Duddington, 1955; Pramer, 1964; Soprunov, 1966).

All cultures of predacious fungi which we have studied produce attractants and at least some of them produce several substances of this character (Balan et al., 1976), but our attempts to isolate and identify attractants directly from cultures of predacious fungi are as yet unsuccessful.

The nematodes *Panagrellus redivivus* and *Rhabditis oxycerca* which we use in our experiments feed on yeasts, on fungal mycelium and spores, and even on the mycelium and spores of predacious fungi. These facts and the difficulties we have met in isolating and identifying attractants directly from cultures of predacious fungi have led us to also test media from yeast cultures and various known metabolites of yeasts and fungi for their potential attraction for the two nematodes.

METHODS AND MATERIALS

Cultivation of Nematodes Panagrellus redivivus (Linn. 1767) Goodey 1945 and Rhabditis oxycerca De Man 1895. Both nematodes were cultivated on a cooked mixture of oatmeal (30 g) and tap water (100 ml). For *R. oxycerca* this medium is supplemented by sugar (5 g). The cooked cereal is distributed into Petri dishes to form a layer of about 5 mm and inoculated by a suspension of nematodes and *Saccharomyces cerevisiae* and incubated at 24° C in the dark. After 14–21 days the nematodes are scraped from the walls and lids of the Petri dishes and, using a separatory funnel, thoroughly washed five times in tap water.

Attraction Assay. A modification of the method described by us (Balan and Gerber, 1972) was used. One end of a metal cylinder, like that used in

antibiotic assays, is covered with a polyamide screen held in place by a segment of rubber tubing. The cylinder with the screen bottom is then filled with ignited, washed, screened sand of a grain size between 0.315 and 0.800 mm. To a layer of 50 g of sand in a 9-cm inner-diameter Petri dish bottom, 24 ml of a suspension containing 50,000 washed nematodes in a 1.7% glucose solution (or in sterile cultivation medium) is added. Five sand-filled cylinders are moistened to saturation with the test liquid (experiment) and five cylinders are moistened in the same way with the 1.7% glucose solution or cultivation medium (control). The five experimental and five control cylinders are transferred to two Petri dishes containing the sand slurry and nematodes and are arranged in such a way as to form circles in which the centers of the cylinders are exactly 12 mm from the wall of the Petri dish.

Nematodes from the suspension in the dish enter the moistened sand of the cylinders through the openings of the screen. If no attractants are present, the nematodes move at random and enter experimental and control cylinders in about equal numbers. Attractants cause the nematodes to enter experimental cylinders in significantly greater numbers than the controls. After one hour the control and experimental cylinders are transferred into two flasks with appropriate volumes of water to give a suspension containing 50–100 nematodes/ml. The number of nematodes in the two suspensions is counted in a Scot Counting Slide using five 1-ml sandless samples per suspension and averaging the results. The number of nematodes in the control represents 100% and attraction is expressed in percentages.

Cultivation of Saccharomyces cerevisiae. For testing the attraction of nematodes to filtered and centrifugated media from *S. cerevisiae* cultivations, a yeast extract-glucose medium (Y.D.) was used (yeast extract, 15 g; glucose, 15 g; water, 1000 ml; pH 6.8; sterilization, 15 min at 121° C). Cultivation is done on a rotatory shaker at 28° C in 500-ml flasks containing 100 ml of Y.D. medium.

Preparation of Solutions of Tested Attractants. All substances described in this paper as attractants for *P. redivivus* and *R. oxycerca* are liquids sparingly soluble in water. Stock solutions of attractants were prepared at 20° C by saturating a 1.7% glucose solution with the respective attractant for 24 hr. After saturation the lower layer of attractant-saturated glucose solution is withdrawn, filtered, and further diluted with 1.7% glucose to the concentrations needed. Solubilities of attractants were taken from tabulated data (Hodgman et al., 1955).

Computerized Estimation of Concentration Gradients of Active Compounds in Petri Dish Under and Around Testing Cylinders. The concentration of attractants in the cylinders is relatively high but rapidly decreases in the surrounding sand, i.e., in the area from which the nematodes are attracted to enter the cylinders. We therefore attempted to estimate the concentration

profiles of one of the active compounds, ethyl acetate, under and around the cylinders.

This calculation is based on the conception of an unsteady mass transfer in a homogenous isotropic environment described by Fick's second law of diffusion. Taking into account that in our case diffusion takes place in a liquid surrounding solid particles, the equation describing this process is

$$\frac{\partial c}{\partial t} = \epsilon D\left(\frac{\partial^2 c}{\partial x^2} + \frac{\partial^2 c}{\partial y^2} + \frac{\partial^2 c}{\partial z^2}\right) \tag{1}$$[3]

In computing this problem it is most advantageous to use cylindrical coordinates. Equation (1) is then changed to

$$\frac{\partial c}{\partial t} = \frac{\epsilon D}{r}\left[\frac{\partial}{\partial r}\left(r\frac{\partial c}{\partial r}\right) + \frac{\partial}{\partial \vartheta}\left(\frac{1}{r}\frac{\partial c}{\partial \vartheta}\right) + \frac{\partial}{\partial z}\left(r\frac{\partial c}{\partial z}\right)\right] \tag{2}$$

For the description of the diffusion in a Petri dish under the conditions of our experiment it is

$$\frac{\partial c}{\partial \vartheta} = 0 \tag{3}$$

and equation (2) is simplified to

$$\frac{\partial c}{\partial t} = \epsilon D\left(\frac{1}{r}\frac{\partial c}{\partial r} + \frac{\partial^2 c}{\partial r^2} + \frac{\partial^2 c}{\partial z^2}\right) \tag{4}$$

Under our experimental conditions diffusion in the small cylinder takes place in one direction only, and it can be characterized by equation (5)

$$\frac{\partial q}{\partial t} = \epsilon D\frac{\partial^2 q}{\partial z^2} \tag{5}$$

[3]Symbols: c = concentration of attractant in liquid phase in slurry bed of Petri dish (mol/cm^3); D = diffusivity of the attractant in the liquid phase (cm^2/sec); h = step in direction of axis z (cm); k = step in radial direction (cm); q = concentration of attractant in liquid phase of small cylinder (mol/cm^3); r = radial coordinate (cm); R_1 = inner radius of small cylinder (cm); R_2 = inner radius of Petri dish (cm); t = time (sec); x, y, z = rectangular coordinates; ϵ = void fraction (cm^3/cm^3); ϑ = cylindrical coordinate; z_1 = height of small cylinder (cm); z_2 = thickness of slurry bed (cm).

Before the experiment the sand-water-glucose slurry in the Petri dish does not contain any attractant, and in the small cylinder a constant chosen concentration of the attractant is present. At the beginning of the experiment the small cylinders are superimposed onto the slurry in the Petri dish, and the attractant then successively diffuses into the surrounding of the small cylinder forming a concentration gradient under and around it. For the mathematical simplification of this problem, one cylinder was situated in the center of the Petri dish and then the experimental conditions for equations (4) and (5) can be formulated by the following initial and boundary conditions:

$$\begin{aligned} q(0,z) &= q_0 \qquad \text{for} \quad 0 \leqslant z \leqslant z_1 \\ c(0,r,z) &= 0 \qquad \text{for} \quad -z_2 \leqslant z \leqslant 0 \quad \text{and} \quad 0 \leqslant r \leqslant R_2 \end{aligned} \tag{6}$$

$$\begin{aligned} &\frac{\partial q}{\partial z} = 0 \Big|_{z=z_1} \qquad \text{for} \quad t \geqslant 0 \\ &\frac{\partial q}{\partial z} = \frac{\partial c}{\partial z} \Big|_{z=0} \qquad \text{for} \quad t > 0 \qquad \text{and} \quad 0 \leqslant r \leqslant R_1 \\ &\frac{\partial c}{\partial z} = 0 \Big|_{z=0} \qquad \text{for} \quad t \geqslant 0 \qquad \text{and} \quad R_1 < r \leqslant R_2 \\ &\frac{\partial c}{\partial z} = 0 \Big|_{z=-z_2} \qquad \text{for} \quad t \geqslant 0 \\ &\frac{\partial c}{\partial r} = 0 \Big|_{r=R_2} \qquad \text{for} \quad t \geqslant 0 \end{aligned} \tag{7}$$

Using the SIEMENS 4004/150 digital computer, equations (4) and (5) for conditions (6) and (7) were solved by simulation. The following forms of equations (4) and (5) were used for the computation:

$$\frac{dq_i}{dt} = \frac{\epsilon D}{h^2} \left(q_{i+1} - 2q_i + q_{i-1} \right) \tag{8}$$

$$\begin{aligned} \frac{dc_{ij}}{dt} = \frac{\epsilon D}{k^2} \Bigg[&\left(1 + \frac{1}{2j} \right) c_{ij+1} - 2 \left(1 + \frac{k^2}{h^2} \right) c_{ij} \\ &+ \left(1 - \frac{1}{2j} \right) c_{ij-1} + \frac{k^2}{h^2} \left(c_{i+1j} + c_{i-1j} \right) \Bigg] \end{aligned} \tag{9}$$

In the direction of axis z, 1-mm steps were chosen; in the radial direction, 1.5-mm steps were used.

The void fraction of the slurry bed is $\epsilon = 0.41\ cm^3/cm^3$. The diffusivity of ethyl acetate in the liquid was calculated according to Wilke and Chang (1955) and found to be $D = 9.19 \times 10^{-6}\ cm^2/sec$.

The mathematical simulation of the diffusion process was done with the presumption that the presence of nematodes in the void fraction of the slurry does not significantly influence this process.

RESULTS AND DISCUSSION

The attraction of *P. redivivus* to Y.D. medium on which *S. cerevisiae* was cultivated is presented in Figure 1. The strong attraction of *P. redivivus* to Y.D. medium on which *S. cerevisiae* was cultivated has indicated that for this phenomenon yeast metabolites are responsible. This paper describes the attraction of nematodes to some esters of fatty acids which are known metabolites of yeasts and some fungi.

The attraction of *P. redivivus* and *R. oxycerca* to methyl acetate, ethyl acetate, propyl acetate, butyl acetate, and amyl acetate is presented in Figure 2. Acetic acid and the respective alcohols were found to be without any significant effect.

Figure 2 shows that nematodes accumulated in the attractant-containing cylinders in numbers several times higher than in the cylinders of the controls. For *P. redivivus* attraction increases and the optimal concentration in the cylinder decreases with the lengthening of the molecule of the attractant. At 20° C the solubility limit for amyl acetate is 0.18% and thus no higher concentrations of this compound could be tested.

For *R. oxycerca* similar relationships were found but highest attraction is observed already with propyl acetate and the effects of butyl and amyl acetate are successively weaker.

We have not yet tested combinations of attractants, but it is conceivable that by combining two or more of these compounds even higher attraction could be registered.

The small cylinders containing relatively high concentrations of attractant only supply the compound into the slurry where it is spread by diffusion and forms a concentration gradient under and around each cylinder. For an initial concentration of 0.1% of ethyl acetate in the cylinder, the computed concentration profiles after 30 and 60 min of the experiment are presented in Figure 3.

Sampling of the liquid from defined locations of the slurry in the Petri dish and assaying the concentration of the attractant in these samples without grossly disturbing the delicate concentration gradient seemed to be impossi-

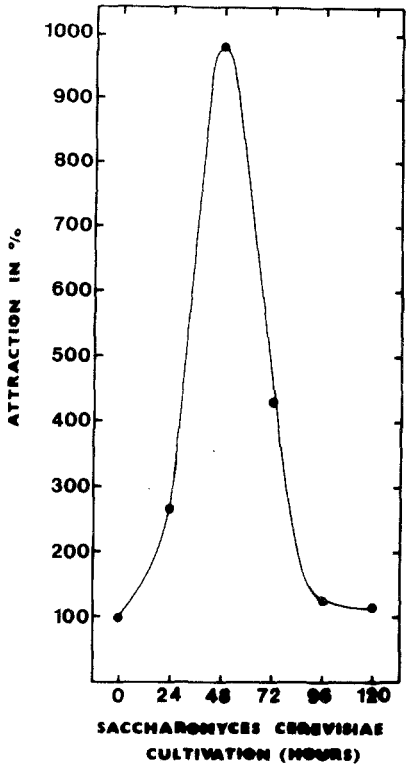

FIG. 1. Attraction of *Panagrellus redivivus* to centrifugated and filtered Y.D. medium on which *Saccharomyces cerevisiae* was cultivated.

ble. The simulated computation of concentration profiles give us at least a rough idea of the concentration gradients to which the nematodes in the Petri dish were subjected. The accumulation of nematodes in the small cylinders, i.e., at the location of highest attractant concentration, is a very complicated gradual process dependent on many factors. Our experimental data are unsufficient for an exact establishment of the concentration threshhold of attractant which *P. redivivus* is still able to detect and react to. Taking into account the number of nematodes accumulated in the cylinder within one hour and the total number of nematodes in the Petri dish, we may presume that the nematodes which have accumulated in a cylinder containing the optimal concentration of attractant have originated from the sand-medium-nematode slurry having at least a volume of 2 ml. If all the nematodes originally present in this volume had entered the cylinders within one hour, the highest concentration of attractant to which the nematodes would have been subjected on the border of this volume would be in the region between

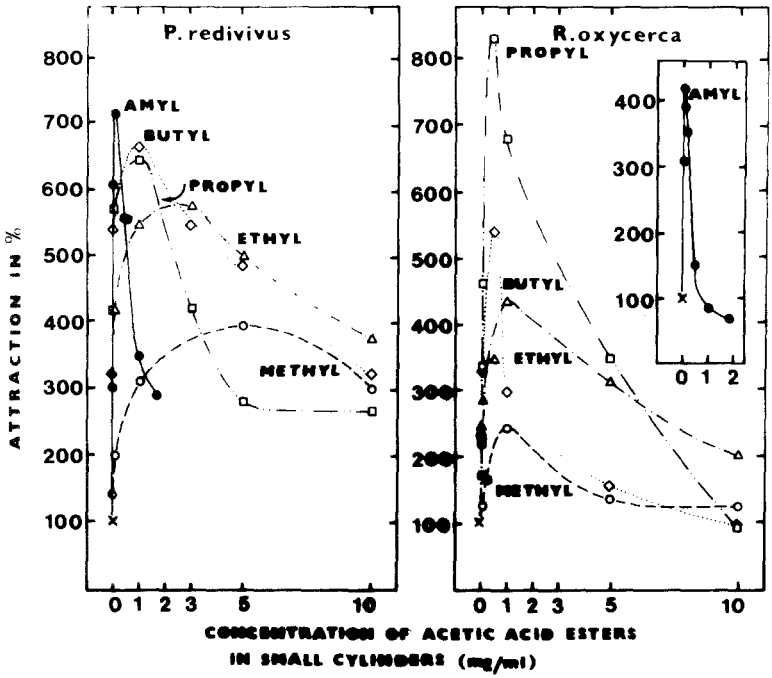

FIG. 2. Attraction of *Panagrellus redivivus* and *Rhabditis oxycerca* to some esters of acetic acid. Data on axis x are concentrations of attractants in small cylinders from which the compounds diffuse into the nematode-containing slurry of the Petri dish. The nematodes in the slurry are attracted by concentrations which are by several orders lower (cf. Figure 3). × = controls.

1×10^{-7} and 1×10^{-10} mol/liter (depending on the attractant used, its concentration in the small cylinder, and its diffusion coefficient).

Some other esters of fatty acids were also found to attract *P. redivivus*; for the ethyl, propyl, and amyl esters of formic acid attractions of between 300 and 400% were measured, and the ethyl ester of propionic acid had an attraction of 700%.

It is obvious that combining attractants and nematicidal compounds would be of greatest practical value. We have therefore tested combinations of our attractants with such concentrations of nematicides which kill *P. redivivus*. For instance the addition of from 0.30 to 0.1% of the commercial nematicide sodium methyl dithiocarbamate (Na *N*-methyl dithiocarbamate, tradenames Vapam and Nematin) to 0.01% of amyl acetate (optimal concentration in small cylinder) did not interfere with attraction but these combinations of compounds killed *P. redivivus* within 24 hr. In the concentrations used amyl acetate alone did not harm and sodium methyl dithiocarbamate did not attract the nematodes.

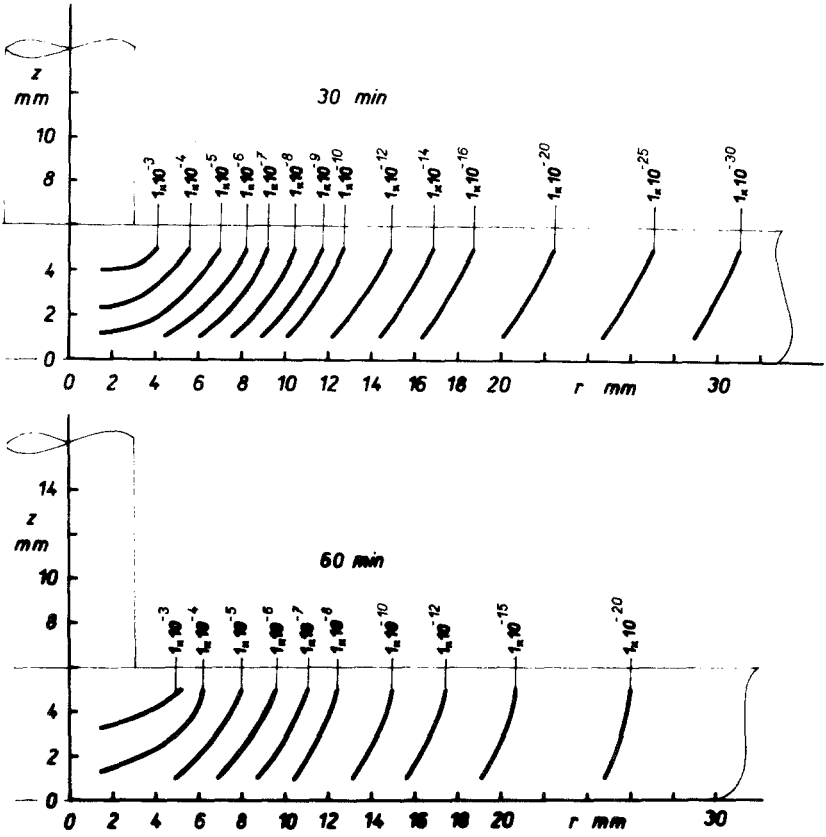

FIG. 3. Concentration profiles of ethyl acetate (in moles/liter) in slurry of Petri dish after 30 and 60 min of experiment. Initial concentration in small cylinder was 0.1% (i.e., 1.1×10^{-2} mol/liter).

Although *P. redivivus* is not a plant-pathogenic nematode, this result is rather encouraging for the potential use of attractant-nematicide combinations in the field. It goes without saying that plant-pathogenic nematodes could be attracted by other attractants as we have found for the free-living nematodes *P. redivivus* and *R. oxycerca.*

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SYNTHESIS OF 7-METHYL-3-METHYLENE-7-OCTEN-1-YL PROPANOATE AND (*Z*)-3,7-DIMETHYL-2,7-OCTADIEN-1-YL PROPANOATE, COMPONENTS OF THE SEX PHEROMONE OF THE SAN JOSE SCALE[1]

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Abstract—The two components of the sex pheromone of the San Jose scale, *Quadraspidiotus perniciosus* (Comstock) were synthesized. The first component, 7-methyl-3-methylene-7-octen-1-yl propanoate, was obtained by the addition of a 4-methyl-4-penten-1-yl copper complex to 3-butyn-1-yl propanoate (or 3-butyn-1-yl trimethylsilyl ether). Reaction of the same copper complex with propyne followed by treatment with carbon dioxide gave a key intermediate in the stereoselective synthesis of (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate, the second component of the pheromone.

Key Words—Synthesis, sex pheromone, San Jose scale, *Quadraspidiotus perniciosus*, 7-methyl-3-methylene-7-octen-1-yl propanoate, (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate.

INTRODUCTION

The San Jose scale, *Quadraspidiotus perniciosus* (Comstock), is a serious worldwide pest of fruit, shade, and ornamental trees. Its widespread and serious infestations particularly of deciduous fruit trees make it a pest of economic importance. Rice (1974) demonstrated the existence of a sex pheromone produced by the female San Jose scale which attracted males. This pheromone has now been identified (Gieselmann et al., 1979) as a two-component mixture of 7-methyl-3-methylene-7-octen-1-yl propanoate (I) and (*Z*)-3,7-dimethyl-2,7-octadien-1-yl propanoate (II). We have confirmed these

[1] *Quadraspidiotus perniciosus* (Comstock) (Homoptera: Diaspididae).
[2] Contribution No. 73 from the Research Laboratory of Zoecon Corporation.

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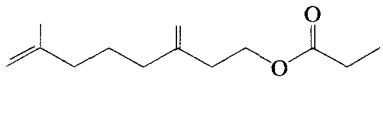

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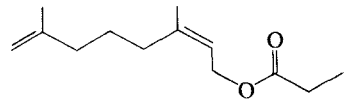

(II)

structural assignments by synthesizing these two compounds, and now report the details of their preparation. The synthetic routes reported here are short and economically attractive and should allow the ready preparation of samples for the field applications of these two pheromone components.

METHODS AND MATERIALS

Preparative thin-layer chromatography was, in general, carried out on 1-m × 20-cm glass plates coated with 1.3 mm of Merck (Darmstadt) silica gel PF-254. NMR spectra were determined on a Varian T-60 spectrometer. Infrared spectra were measured on a Unicam SP-200G or a Perkin-Elmer 281 spectrophotometer. Mass spectra were measured on a Hewlett-Packard model 5985 GC-MS-DS with an all glass jet separator at 70 eV ionization potential. Gas-liquid chromatographic analyses were performed on model 402 Hewlett-Packard instruments equipped with hydrogen flame ionization detectors. All solvents were dried over activated molecular sieves.

Ethyl 4-methyl-4-pentenoate (*V*). A mixture of 1100 ml (6.0 mol) of triethyl orthoacetate, 72.3 g (1.0 mol) of 2-methyl-2-propen-1-ol (IV), and 7.4 ml (0.1 mol) of propanoic acid was heated under reflux for 1 hr. The reaction was cooled and poured onto ice and 2 N HCl, and the mixture was extracted with ether. The organic layer was washed with 5% aqueous NaOH and brine and was dried ($MgSO_4$). Removal of the solvent in vacuo followed by distillation of the residue gave 96.0 g (0.68 mol, 68% yield) of ester V. Bp 64–66° (20 mm); NMR ($CDCl_3$, δ) 4.70 (br s, 2H), 4.12 (q, 2H, J = 7 Hz), 2.40 (br s, 4H), 1.76 (br s, 3H) and 1.23 ppm (t, 3H, J = 7 Hz).

4-Methyl-4-penten-1-ol (*VI*). To a suspension of 6.08 g (0.16 mol) of lithium aluminum hydride in 400 ml of dry tetrahydrofuran (THF) under a N_2 atmosphere and cooled to 0° was added dropwise 30.0 g (0.21 mol) of ester V in 75 ml of THF over 40 min. After 2 hr at room temperature, the reaction was quenched by the successive dropwise addition of 6.5 ml of water, 6.5 ml of 15% NaOH, and 19.5 ml of water. The granular precipitate was removed by filtration, and ether was added to the filtrate followed by washes with water and brine. The organic layer was dried ($MgSO_4$) and solvent was removed in vacuo. The residue was distilled (bp 64–65°; 15 mm) to give 18.8 g (0.19 mol, 90% yield) of alcohol VI. IR (neat) 3350 (OH) and 885 cm^{-1} ($C{=}CH_2$); NMR ($CDCl_3$, δ) 4.72 (br s, 2H), 3.63 (t, 2H, J = 6 Hz) and 1.75 ppm (br s, 3H).

1-Bromo-4-methyl-4-pentene (III). A solution of 53.4 g (0.204 mol) of triphenylphosphine in 600 ml of acetonitrile under a N_2 atmosphere was cooled in an ice bath, and then 32.4 g (0.203 mol) of bromine in 200 ml of acetonitrile was added, followed by 16.0 g of pyridine and then by 20.0 g (0.200 mol) of 4-methyl-4-penten-1-ol (VI) in 35 ml of acetonitrile. After stirring overnight, the reaction was worked up by addition of water and extraction with pentane. The organic layer was washed with 2 N HCl, water and brine, and was dried (Na_2SO_4). Solvent was removed by distillation, and the residue was then distilled under reduced pressure (bp 90–95°; 140 mm) to give 22.8 g (0.14 mol, 70% yield) of bromide III. NMR ($CDCl_3$,δ) 4.73 (br s, 2H), 3.38 (t, 2H, J = 6.5 Hz), and 1.72 ppm (br s, 3H).

7-Methyl-3-methylene-7-octen-1-yl propanoate (I). To 0.8 g (33.0 mmol) of magnesium turnings suspended in 10 ml of dry ether under a N_2 atmosphere was added about 0.5 g of bromide III. After the reaction had begun, 10 ml of ether was added followed by the remaining bromide (4.9 g total, 30.0 mmol) in 40 ml of ether. The reaction was heated under reflux overnight to give 4-methyl-4-pentenylmagnesium bromide (VII). Titration of the Grignard reagent (Watson and Eastham, 1967) gave a molarity of 0.4 M (80% conversion).

To a solution of 0.61 g (3 mmol) of cuprous bromide–dimethyl sulfide complex (House et al., 1975) in 5 ml of ether and 17 ml of dimethyl sulfide cooled to −50° under a N_2 atmosphere was added 6.8 ml (2.7 mmol) of 0.4 M 4-methyl-4-pentenylmagnesium bromide (VII) in ether while the temperature was maintained below −45°. The mixture was warmed to −40°, and 0.37 g (2.9 mmol) of 3-butyn-1-yl propanoate (VIII) in 10 ml of ether was added. After the mixture had been stirred at −25° for 4 hr, it was recooled to −40°, and 10 ml of saturated aqueous NH_4Cl was added. After the reaction had been warmed to room temperature overnight, it was poured into ether and saturated NH_4Cl solution. The organic phase was separated and was washed with saturated NH_4Cl, water, and brine, and dried ($MgSO_4$). Removal of solvent in vacuo gave crude ester which was purified by preparative thin-layer chromatography (silica impregnated with Rhodamine 6G; developed in 5% ether in hexane) to give 0.24 g (1.14 mmol, 42% yield) of propanoate I. By GLC analysis (2 m 3% OV-17/0.4% Carbowax), the chemical purity of the propanoate was shown to be 94.2%. IR (neat) 1735 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 4.82 (br s, 2H), 4.70 (br s, 2H), 4.18 (t, 2H, J = 7 Hz), 1.72 (br s, 3H) and 1.17 ppm (t, 3H, J = 7.5 Hz). MS (CI, CH_4) m/e (relative intensity) 211 (5, $M^+ + H$), 137 (100). Analysis: Calcd for $C_{13}H_{22}O_2$: C, 74.24; H, 10.54. Found: C, 74.42; H, 10.67.

Trimethylsilyl 3-butyn-1-yl ether (IX). To a solution of 3.50 g (50.0 mmol) of 3-butyn-1-ol in 50 ml of pentane and 11 ml of ether cooled in an ice bath under a N_2 atmosphere was added 9.2 ml (66.0 mmol) of triethylamine. Then 7.6 ml (59.9 mmol) of trimethylsilyl chloride was slowly added, and the mixture was stirred at 10° for 2 hr. About 30 ml of ice-cold water was added,

and the phases were separated. The organic layer was quickly washed with ice-cold 1 N H_2SO_4, saturated $NaHCO_3$ and brine, and was dried (Na_2SO_4). Solvent was removed by distillation to give 7.0 g (49.2 mmol) of ether IX in 98% yield. NMR (CCl_4,δ) 3.64 (t, 2H, J = 7 Hz), 2.34 (d of t, 2H, J = 3 Hz, J = 7 Hz), 1.85 (t, 1H, J = 3 Hz), and 0.14 ppm (s, 9H).

7-Methyl-3-methylene-7-octen-1-yl propanoate (I). A solution of 2.1 g (10.0 mmol) of cuprous bromide-dimethyl sulfide complex (House et al., 1975) in 15 ml of dry ether and 12 ml of dimethyl sulfide under a N_2 atmosphere was cooled to −60°. After the addition of another 14 ml of dimethyl sulfide to redissolve the complex, 24.0 ml (9.6 mmol) of 0.4 M 4-methyl-4-pentenylmagnesium bromide in ether was added over 5 min and the reaction was then stirred at −45° for 2 hr. Trimethylsilyl 3-butyn-1-yl ether (IX, 1.4 g, 9.8 mmol) in 35 ml of ether-pentane (1 : 6) was added at −45°, and the mixture was allowed to warm to −25° for several hours. The reaction was then poured into a precooled (−10°) solution of HCl-saturated NH_4Cl, and this mixture was warmed to room temperature and stirred overnight. Water was added and the organic product was extracted into ether. The ether fraction was washed with saturated $(NH_4)_2SO_4$, water, and brine, and was dried (Na_2SO_4). Removal of solvent in vacuo gave 1.05 g of crude alcohol X. Purification of a small sample by preparative tlc gave pure alcohol X. IR (neat) 3310 cm^{-1} (OH); NMR ($CDCl_3$, δ) 4.75 (br s, 2H), 4.58 (br s, 2H), 3.60 (t, 2H, J = 6 Hz), and 1.63 ppm (br s, 3H). MS (70 eV) m/e (relative intensity) 154 (0.4, M^+), 68 (100). Analysis: Calcd for $C_{10}H_{18}O$: C, 77.87; H, 11.76. Found: C, 77.68; H, 11.51.

A mixture of this crude alcohol, 4.0 ml of propanoic anhydride, and 4.0 ml of pyridine under a N_2 atmosphere was heated to 65° for 1 hr. Ice was added to the cooled mixture, and after 30 min the reaction was poured into ether and water. The ether fraction was washed with 2 N HCl, 2 M Na_2CO_3, and brine and was dried ($MgSO_4$). After removal of solvent in vacuo, the residue was applied to three 1-m × 20-cm preparative silica plates (impregnated with Rhodamine 6G) which were developed in 5% ether in hexane. The purified ester was removed and microdistilled [bp (bath) 70°, 1.0 mm], to give 0.91 g (4.33 mmol, 45% yield from Grignard reagent VII) of the propanoate I.

(Z)-3,7-Dimethyl-2,7-octadienoic acid (XI). A solution of 4.7 g (22.9 mmol) of cuprous bromide-dimethyl sulfide complex (House et al., 1975) in 20 ml of dry ether and 42 ml of dimethyl sulfide under a N_2 atmosphere was cooled to −50°, and 51 ml (20.4 mmol) of 0.4 M 4-methyl-4-pentenylmagnesium bromide was added over 1 hr. The reaction was stirred for 1.5 hr, and then 4 ml of propyne was added and the mixture was warmed to −20°. After 1 hr, 25 ml of hexamethylphosphoramide (HMPA) and 0.5 ml of triethylphosphite were added followed by excess carbon dioxide. The mixture was warmed to −15°, placed in a CCl_4-dry ice bath, and carbon dioxide was bubbled into the reaction overnight. The reaction was quenched with satu-

rated $(NH_4)_2SO_4$, and the solid was removed by filtration. The solid was washed several more times with ether. Combined filtrates (organic and aqueous phases) were saturated with NaCl, and the aqueous phase was separated and reextracted with ether. The combined ether phases were extracted with 10% aqueous NaOH. The basic layer was then separated, reacidified with 10% H_2SO_4, and the organic acid was extracted with ether. The ether layer was washed with brine and was dried (Na_2SO_4). Removal of solvent in vacuo gave 3.00 g of crude acid XI. NMR ($CDCl_3$, δ) 5.67 (br s, 1H), 4.69 (br s, 2H), 1.97 (d, 3H, $J = 2$ Hz), and 1.74 ppm (br s, 3H).

Methyl (Z)-3,7-Dimethyl-2,7-octadienoate (XII). To a mixture of 16 g (0.285 mol) of potassium hydroxide in 24 ml of water and 75 ml of ether cooled in an ice bath was added, in portions, 7.5 g (0.073 mol) of *N*-nitrosomethylurea. The yellow ether layer was removed by decantation and was dried over KOH pellets.

To 2.0 g of the crude acid XI in 15 ml of dry ether was added about 30 ml of the above diazomethane in ether solution. After overnight stirring, the reaction was poured into water. The organic phase was separated and washed with brine and was dried (Na_2SO_4). Removal of solvent in vacuo gave 2.25 g of crude ester which was purified by application to six 1-m × 20-cm preparative silica plates (developed in 4% Et_2O in hexane) to give 1.16 g (6.4 mmol, 47% yield from Grignard reagent VII) of ester XII. IR (CCl_4) 1715 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 5.70 (br s, 1H), 4.72 (br s, 2H), 3.70 (s, 3H), 1.88 (d, 3H, $J = 2$ Hz), and 1.73 ppm (br s, 3H). MS (70 eV) *m/e* (relative intensity) 182 (0.8, M^+), 114 (100). Analysis: Calcd for $C_{11}H_{18}O_2$: C, 72.49; H, 9.95. Found: C, 72.15; H, 9.89.

(Z)-3,7-Dimethyl-2,7-octadien-1-ol (XIII). A solution of 1.13 g (6.2 mmol) of the ester XII in 10 ml of benzene cooled to 0° under a N_2 atmosphere was treated with 11.4 ml (ca. 20 mmol) of 27.8% diisobutylaluminum hydride (DIBAH) in heptane. After 2 hr, excess hydride was quenched with saturated aqueous NH_4Cl. Ether was added to the mixture and the pH of the aqueous phase was adjusted to pH 3 with 1 M HCl. The layers were shaken, separated, and the organic phase was washed with saturated $NaHCO_3$ and brine and was dried (Na_2SO_4). Removal of solvent in vacuo gave 0.96 g (6.2 mmol, 100% yield) of alcohol XIII. IR (CCl_4) 3620 (OH) and 3075 cm^{-1} (C=CH_2); NMR ($CDCl_3$, δ) 5.45 (t, 1H, $J = 7$ Hz), 4.72 (br s, 2H), 4.17 (d, 2H, $J = 7$ Hz) and 1.73 ppm (br s, 6H). MS (70 eV) *m/e* (relative intensity) 154 (0.3, M^+), 69 (100). Analysis: Calcd for $C_{10}H_{18}O$: C, 77.87; H, 11.76. Found: C, 77.63; H, 11.71.

(Z)-3,7-Dimethyl-2,7-octadien-1-yl propanoate (II). A mixture of 0.96 g (6.2 mmol) of alcohol XIII, 1.5 ml (12 mmol) of propanoic anhydride, and 1.5 ml of pyridine was stirred overnight under a N_2 atmosphere. Ice was added to the mixture, and after 30 min the reaction was poured into a mixture of pentane and 5% aqueous HCl. The pentane layer was separated and washed with 2 M Na_2CO_3 and brine, and was dried (Na_2SO_4). Removal of solvent in

vacuo gave a residue which was applied to four 1-m × 20-cm preparative silica plates (impregnated with Rhodamine 6G) which were developed twice in 2% ether in hexane. Removal of the ester and microdistillation [bp 60° (bath), 0.25 mm] gave 0.81 g (3.85 mmol, 62% yield) of pheromone component II. GLC analysis (2 m 3% OV-17/0.4% Carbowax) of this component indicated a chemical purity of 95.2%. IR (CCl_4) 3075 (C=CH_2) and 1740 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 5.38 (t, 1H, J = 7 Hz), 4.70 (br s, 2H), 4.57 (d, 2H, J =7 Hz), 1.73 (br s, 6H), and 1.12 ppm (t, 3H, J = 7.5 Hz). MS (70 eV) m/e (relative intensity) 57 (100). Analysis: Calcd for $C_{13}H_{22}O_2$: C, 74.24; H, 10.54. Found: C, 74.29; H, 10.69.

RESULTS AND DISCUSSION

We have applied the well-known procedure of the addition of organocopper reagents to alkynes, developed by Normant and coworkers (1974), to the synthesis of both pheromone components. A retrosynthetic analysis of each component suggested that a common alkylcopper reagent, derived from 1-bromo-4-methyl-4-pentene (III), could be used in the addition to an alkyne substrate. The synthesis of 7-methyl-3-methylene-7-octen-1-yl propanoate (I), the first component of the San Jose scale pheromone, is outlined in Figure 1. Reaction of 2-methyl-2-propen-1-ol with triethyl orthoacetate gave via the orthoester Claisen rearrangement (Johnson et al., 1970) ethyl 4-methyl-4-pentenoate (V). Lithium aluminum hydride reduction of the ester gave the corresponding alcohol VI which on treatment with bromine and triphenylphosphine (Horner et al., 1959; Breitholle and Fallis, 1978) gave 1-bromo-4-methyl-4-pentene (III). The bromide was then converted to the corresponding Grignard reagent VII. In a manner analagous to that reported by the French workers (Alexakis et al., 1975), we reacted this Grignard reagent first with the cuprous bromide–dimethyl sulfide complex (House et al., 1975; Marfat et al., 1977) and then treated this intermediate with 3-butyn-1-yl propanoate (VIII) to give pheromone component I directly in 42% yield. We also obtained the same pheromone component by reacting the organocopper reagent, derived from Grignard reagent VII and the cuprous bromide–dimethyl sulfide complex, with the trimethylsilyl ether of 3-butyn-1-ol (IX) followed by an acidic workup and subsequent esterification with propanoic anhydride and pyridine. The regioselectivities of the organocopper addition to alkynes VIII and IX were nearly identical, with less than 2% organocopper attack occurring at the terminal acetylenic carbon in each case. This result is different from the results reported by the French workers (Alexakis et al., 1975), who found higher regioselectivity in the addition of organocopper reagents to IX than to 3-butyn-1-yl acetate.

As we had anticipated, the Grignard reagent VII, used in the synthesis of

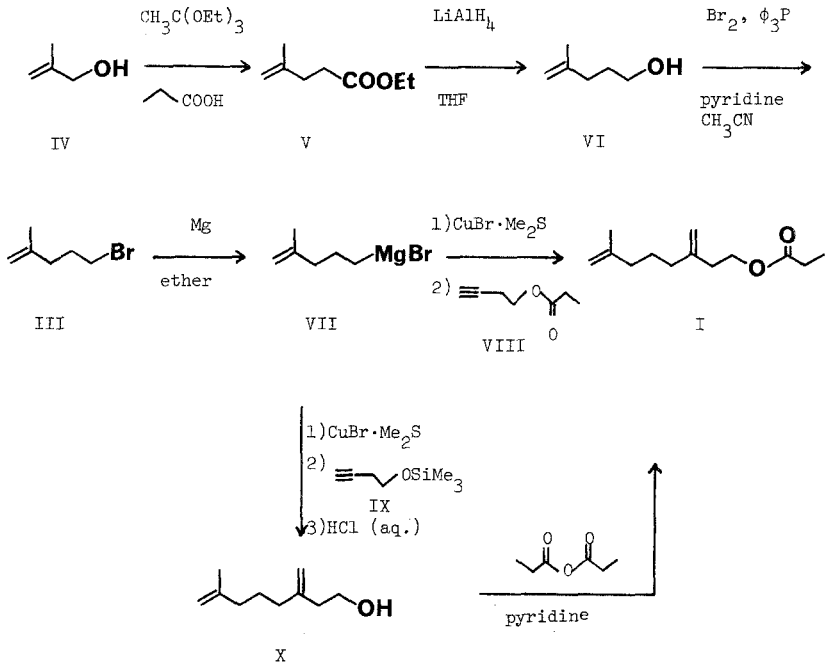

FIG. 1. Synthesis of component I of the San Jose scale pheromone.

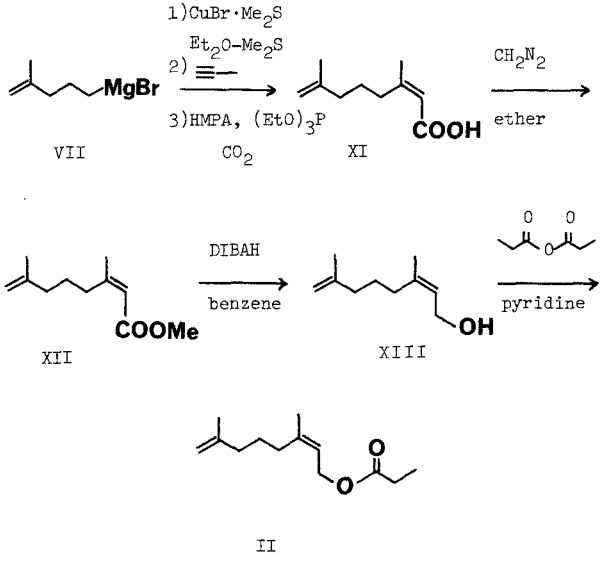

FIG. 2. Synthesis of component II of the San Jose scale pheromone.

the first pheromone component I, could also be used to prepare a key intermediate in the synthesis of the second component II. Thus, as outlined in Figure 2, reaction of the organocopper reagent derived from the Grignard reagent VII and the cuprous bromide-dimethyl sulfide complex with propyne followed by treatment of the intermediate with carbon dioxide gave (*Z*)-3,7-dimethyl-2,7-octadienoic acid (XI) in a manner analogous to earlier work (Normant et al., 1974). Esterification of the *Z* acid XI with diazomethane in ether gave the corresponding *Z* ester XII (47% yield from VII) which was reduced to the *Z* allylic alcohol XIII with diisobutylaluminum hydride (DIBAH). Finally, esterification with propanoic anhydride and pyridine gave the second component of the San Jose scale pheromone, II, in a 63% yield from ester XII.

The spectroscopic and chromatographic properties of the synthetic propanoates I and II were found to be identical to those of the two pheromone components isolated from the San Jose scale. In greenhouse and field bioassays, each synthetic component was independently attractive to male San Jose scale (Gieselmann et al., 1979).

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DEFENSIVE SECRETION OF CHRYSOMELID LARVAE[1] *Linaeidea aenea* LINNÉ and *Plagiodera versicolora distincta* BALY

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Abstract—The larval defensive secretions of *Linaeidea aenea* Linné and *Plagiodera versicolora distincta* Baly were identified as plagiolactone and epiplagiolactone. In addition to these compounds, chrysomelidial and the acetates of hexadecanol, octadecanol and (*Z*)-11-eicosenol from the former insect, and plagiodial and epichrysomelidial from latter insect were identified.

Key Words—Defensive secretion, *Linaeidea aenea* Linné, *Plagiodera versicolora distincta* Baly, chrysomelidial, plagiolactone, plagiodial.

INTRODUCTION

Recently chrysomelidial (I) and its epimer II, the novel cyclopentanoid monoterpenes, were identified in the defensive secretion of *Plagiodera versicolora* (Meinwald et al., 1977) and *Gastrophysa cyanea* Melsheimer (Blum et al., 1978). Plagiolactone (III) was also isolated from *P. versicolora* (Meinwald et al., 1977). In previous studies (Sugawara et al., 1978; 1979), chrysomelidial (I) and the acetates of octadecanol and (*Z*)-11-eicosenol were identified as defensive substances from *Gastrophysa atrocyanea* Motschulsky and *Phaedon brassicae* Baly.

[1]This is report No. 3 of the Defensive Secretion of Chrysomelid Beetles. Report No. 2 is Sugawara et al., 1979.

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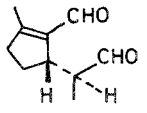

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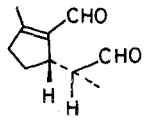

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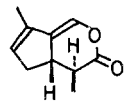

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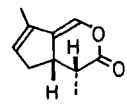
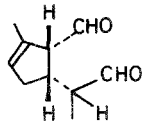


IV



V

In this paper, the defensive secretions of the larvae of two Japanese leaf beetles were investigated. *Linaeidea aenea* Linné, which feeds on alder leaves, secretes I, III, IV; and the acetates of hexadecanol, (*Z*)-9-octadecenol, octadecanol, and (*Z*)-11-eicosenol. Willow feeding *Plagiodera versicolora distincta* Baly also secretes cyclopentanoid monoterpenes II, III, IV, and a new isomer of I, named plagiodial (V) [5-(1-formylethyl)-2-methyl-2-cyclopentene-1-carbaldehyde] as a major component in the secretion. This report also describes the optimum conditions for the separation of these compounds (I or II, III, IV, and V) by gas chromatography.

METHODS AND MATERIALS

L. aenea were selected from our laboratory culture maintained on *Alunus hirsuta* Turcz leaves.

P. versicolora distincta were reared on *Salix bobylonica* L. leaves. The secretion from 350 *Linaeidea* larvae was collected by the previously described method (Sugawara et al., 1978) and weighed (45.4 mg). It was extracted with pentane, and dried over Na_2SO_4 to give 5.3 mg of the extracts. In the same way, 4.6 mg of extract was obtained from 50.2 mg of the secretions of 2700 *Plagiodera* larvae. R_f values on TLC were calculated for 5-cm × 20-cm glass plate (silica gel) developed with hexane-ether (1 : 1). A Hitachi gas chromatograph 163 equipped with FID was employed with the following columns: column I—30-m × 0.25-mm glass capillary column (coated with OV-101) at 150° C with a flow rate of 2 ml/min; column II—2-m × 2.5-mm glass column (packed with 5% DEGS) at 200° C with a flow rate of 20 ml/min; column III—column I was used under programed temperature from 200 to 220° C at the rate of 1° C/min. Quantitative ratios were calculated from their peak areas relative to that of chrysomelidial (retention time = 9.3 min, column II) and are expressed in parentheses after each retention time. PMR spectra were recorded on a JEOL JNM PS-100 (100 MHz) spectrometer using $CDCl_3$ as

solvent with 1% TMS as an internal standard. An electron impact mass spectrometer Hitachi M-52G was used (20 eV).

RESULTS

L. aenea. The extracts showed five spots (R_f0.88, 0.75, 0.61, 0.57, 0.35) on TLC, in which the spot of R_f 0.88 was identical to that of (*Z*)-11-eicosenyl acetate. GLC analyses were carried out after purifying the fraction (R_f 0.88, 0.75) with preparative TLC (PF_{254}). Retention times of 8.0 (0.1), 13.3 (0.2), 14.3 (0.4), 21.6 (1.5), and 28.0 min (0.5) were measured by using column III, and they were identified as the acetates of hexadecanol, (*Z*)-9-octadecenol, octadecanol, and (*Z*)-11-eicosenol, respectively, by comparison with authentic samples except the fraction at 28.0 min. Products of hydrogenation catalyzed by Pd/$BaSO_4$ in ethanol gave retention times of 8.0, 14.3, 23.0, and 29.8 min and were identified as the acetates of hexadecanol, octadecanol, and eicosanol, respectively, except the fraction of 29.8 min. The unknown peak of 28.0 min was thought to be shifted to 29.8 min after hydrogenation, and the presence of an unsaturated bond was suggested. However, the structure could not be established.

The fraction of R_f 0.35 and retention time of 9.3 min (column II, C in Figure 1) was identical to chrysomelidial isolated from *P. brassicae* in its R_f

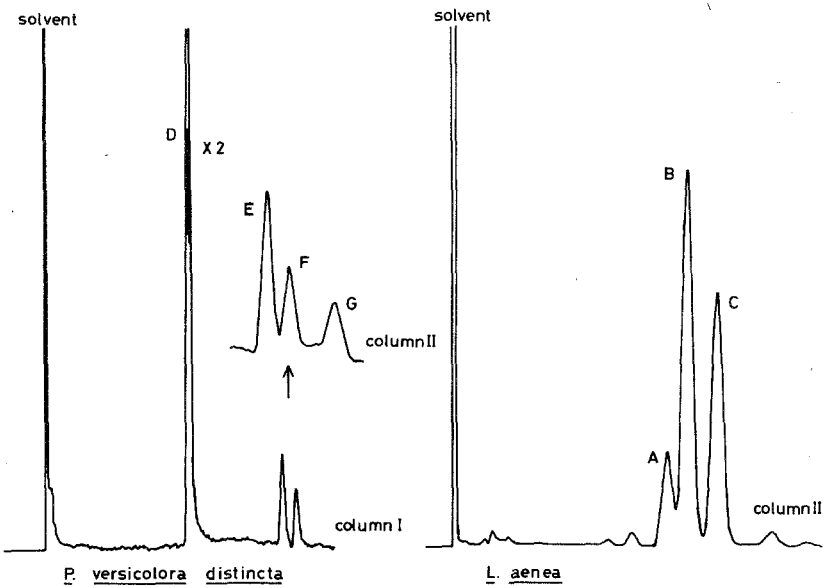


FIG. 1. Gas chromatogram of extracts of *P. versicolora distincta* or *L. aenea.* A,E: plagiolactone (III); B,F: epiplagiolactone (IV); C: chrysomelidial (I); G: epichrysomelidial (II); D: plagiodial (V).

and retention time, and its GC-MS spectrum also supported this identification (*m/e*: M^+ 166, $C_{10}H_{14}O_2$).

Two fractions with retention times of 7.6 (0.5, A in Figure 1) and 8.3 (2.0, B in Figure 1) corresponding to R_f 0.61 and 0.57, respectively, gave the same fragments and intensities of mass spectra, A: 164 (M^+; $C_{10}H_{14}O_2$, 56%); 136 (17), 121 (47), 108 (58), 107 (43), 93 (8), 91 (15), 80 (100), 79 (73), 77 (14), B: 164 (M^+; $C_{10}H_{14}O_2$, 89), 136 (30), 121 (66), 108 (92), 107 (72), 93 (76), 91 (34), 80 (100), 79 (86), 77 (11). After hydrogenation with Pd/$BaSO_4$ catalyst, fractions A and B showed a single major peak (9.6 min) on GLC (column I) and its mass spectrum was identical to that of iridomyrmecin (M^+; 168, $C_{10}H_{18}O_2$). In the PMR spectrum of mixture A, B, and C, three methyl doublets were observed at high field. The signal at 1.10 decoupled with 2.90 was due to IV, 1.28 decoupled with 2.52 to III, and 0.90 decoupled with 3.14 to I (Meinwald and Jones, 1978). These peak intensities approximated the ratio calculated from GLC. Therefore, A was identified as plagiolactone (III), B as epiplagiolactone (IV), and C as chrysomelidial (I). This is the first instance of the presence of epiplagiolactone in nature.

P. versicolora distincta: The extracts of secretions showed four spots on TLC (R_f 0.61, 0.57, 0.36, and 0.34) and four peaks (D, E, F, and G) on GLC (column II). The investigations of TLC, GLC, and GC-MS clearly showed that peaks E and F were due to plagiolactone (III) and epiplagiolactone (IV), respectively, and suggested that a peak G was due to a chrysomelidial-like substance. A major component in the secretions (D, 85% by GLC) gave quite similar mass fragmentations to chrysomelidial, but the relative peak intensities were completely different. Predominant ions from component D were *m/e* 166 (M^+, 2%, $C_{10}H_{14}O_2$), 138 (8), 119 (8), 110, (9), 109 (100), 108, (78), 107 (18), 95 (11), 93 (12), 91 (17), 82 (6), 81 (54), 80 (78), 79 (51), 77 (9), 67 (54), 55 (30), 53 (11), 43 (45), and 41 (20). The mass spectrum gave us the most information: in this, *m/e* 109 (C_7H_9O) was a fragment ion arising from the loss of a propanal radical from the molecular ion, and *m/e* 108 (C_7H_8O) was an ion caused by McLafferty rearrangement in a similar manner to chrysomelidial (Meinwald et al., 1977). Very intense IR absorptions (2730, 1730, 910 cm^{-1}) of the extracts showed the presence of saturated aldehyde and olefinic unsaturation. It was clear that fraction D was an olefinic isomer of chrysomelidial and was named as plagiodial.

We failed to obtain plagiodial as a single substance by preparative TLC (PF_{254}) and GLC (DEGS, GE, SE-30), because it was more unstable than chrysomelidial and easily changed to unidentified compounds during purifications. However, PMR spectra of the mixture (D, E, F, and G on GLC) were enough to elucidate the structure of plagiodial as 5-(1-formylethyl)-2-methyl-2-cyclopentene-1-carbaldehyde. There were two doublet aldehyde protons at 9.58 (J = 3.5) coupled with 3.03 (m) and at 9.64 (J = 1.8) coupled with 2.53 (q,

d, $J = 7.2, 6.5$), an olefinic proton at 5.55 (m), an olefinic methyl singlet at 1.74, and a most intense methyl doublet at 1.13 (d, $J = 7.2$) coupled with 2.53. Possible assignments of signals are indicated in Figure 2.

Moreover, chemical derivations confirmed the structure of plagiodial. The $NaBH_4$ reduction of plagiodial followed by acetylation was performed as follows: the extracts (2 mg) in ethanol were reduced with $NaBH_4$ in the presence of $NaHCO_3$ for 20 min at room temperature, and poured into dilute HCl. After extraction with ether, the organic layer was washed with brine, concentrated, and acetylated with Ac_2O–pyridine at 0° C. It was extracted with ether and washed with dilute HCl, $NaHCO_3$, and brine, successively. Concentration of the extract afforded the plagiodiol diacetate. A major single peak of plagiodiol diacetate was observed at 11.3 min (column II). The peak in the mass spectrum at m/e 134 ($M^+ - 120$) indicated that two molecules of acetic acid were lost from the molecular ion ($C_{14}H_{22}O_4$, 254). After hydrogenation catalyzed by $Pd/BaSO_4$ in ethanol, the retention time of the product was 10.4 min. Although M^+ ($C_{14}H_{24}O_4$, 256) was not detected, the significant ion peak at m/e 136 ($M^+ - 120$) proved that the hydrogenated product was iridodiol diacetate.

It is known that the aldehyde substituent on cyclopentane ring of iridodial was *cis* against 1-formylethyl substituent, and the absolute configuration of C-5 in chrysomelidial is the *S* form (Meinwald and Jones, 1978). Therefore, if plagiodial maintained the same configuration as chrysomelidial at C-5, the absolute configuration seems to be *S* at C-5 and *R* at C-1 about two asymmetric carbons in the cyclopentene ring.

The presence of epichrysomelidial (II) was also suggested from the PMR spectra of the mixture. Three weak methyl doublet signals at 1.07, 1.08, and 1.34 ppm were observed at the side of a methyl doublet signal of plagiodial, in which the weakest signal at 1.07 seemed to be epichrysomelidial (Meinwald et al., 1977) and its intensity corresponded to that of peak G on GLC. The

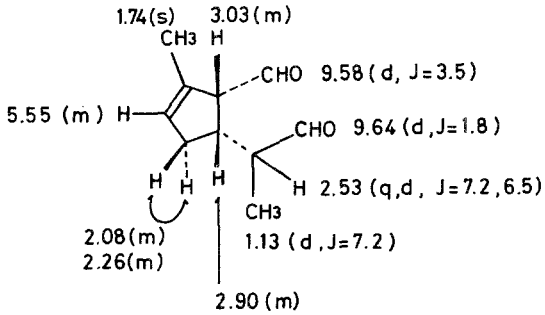


FIG. 2. Possible assignments of plagiodial (V) (JEOL JNM PS-100, $CDCl_3$).

identification of peak G and C as either I or II was impossible by GLC because of their identical retention times (9.3 min) under these gas chromatographic conditions.

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VARIATION IN ATTRACTION OF INDIVIDUAL MALE GYPSY MOTHS TO (+)- AND (±)-DISPARLURE[1]

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Abstract—Gyspy moth (*Lymantria dispar* L.) males atttracted to either (+)-*cis*-7,8-epoxy-2-methyloctadecane [(+)-disparlure] or its racemate [(±)-disparlure] do not represent distinct behavioral classes of individuals. Males attracted to within 2 cm of either (+)- or (±)-disparlure dispensers on a tree trunk were caught, marked as to the lure visited, and then released into a 5 × 5 grid at ca. 20-m spacing of traps baited alternately with (+)- or (±)-disparlure. Males then reattracted to these lures showed no evident preference for the lures at which they were first captured, indicating that males attracted to either of these lures are not behavioral phenotypes.

Key Words—*Lymantria dispar*, gypsy moth, pheromone, polymorphism, disparlure, enantiomer, *cis*-7,8-epoxy-2-methyloctadecane, attractant.

INTRODUCTION

Field tests of pheromone blends (or pheromone in combination with analogs) using trap catch as a criterion of response usually demonstrate that some individuals are captured at ratios of components that differ appreciably from the optimal lure. The gypsy moth, *Lymantria dispar* L., is lured to the (+)-enantiomer of *cis*-7,8-epoxy-2-methyloctadecane (disparlure) in 5- to 10-fold greater numbers than to its racemate (Cardé et al., 1977a, 1978a; Miller et al., 1977; Plimmer et al., 1977). The lowered trap catch at the racemate is evidently caused by the antagonistic effects of the (−)-enantiomer upon many behaviors that are reflected ultimately in trap catch: likelihood of upwind flight and landing and the persistence of wing fanning while walking (Cardé et

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al., 1977a). In this paper we report on a field study designed to assess the degree of individual variation in attraction to (+)- and (±)-disparlure by use of an attraction-mark-release-recapture technique.

METHODS AND MATERIALS

Males from a dense (10–100% defoliation) natural population near Stormstown, Centre County, Pennsylvania, were collected at cotton wicks 1 cm in diam. × 1 cm in length charged with 100 μl petroleum ether containing either 100 μg (+)-disparlure prepared by the procedure of Farnum et al. (1977) or 200 μg (±)-disparlure (Farchan Chemicals). One wick of each type was placed on an insect pin on 50-cm-diam. trees 20 m apart. Males that landed on the tree trunk and walked while wing fanning to within 2 cm of the source were captured, marked with Dayglo® fluorescent powder (color-coded to the lure) and released within a few (5–10) minutes in the recapture grid. A total of 425 males at the (+) station and 205 at the (±) station were caught, marked, and released. Several-fold as much time was spent collecting males at the (±)-baited as at the (+)-baited lure so that sufficient males attracted to racemate would be available for release. We did not evaluate the comparative "attractiveness" of these lures on tree trunks by estimation of the number of males lured to each dispenser type per unit of time.

Males were released 100 m away from the capture site in the center area of a 5 × 5 grid of recapture traps. Traps alternated with (+) and (±) lures 18–22 m apart. The four corner (outside) traps were baited with (±)-disparlure. The traps were of the high-capacity, no-exit design (Granett, 1973; Cardé et al., 1977b) with a 2 × 2-cm plastic strip containing 19% 2,2-dichlorovinyl dimethylphosphate as a killing agent.

Marking was conducted from July 16 to 20 and recaptures were tallied from July 16 to 22, 1978. Marked males were removed daily and were readily distinguished from unmarked individuals with the aid of a UV light. The overall recapture rate of marked males was 19.5%.

RESULTS

If the variation in differential male attraction to (+)- and (±)-disparlure represented in part distinct classes of responders, then males originally captured at (+)- or (±)-disparlure should tend to be recaptured at the same lure. Comparison of the actual recaptures of marked males (Table 1) indicates no obvious level of association with previous capture. In this test males marked at either (+)- or (±)-disparlure were recaptured in the same relative proportions at (+)- or (±)-disparlure baits. Thus, the differential levels of attraction to these lures is not obviously related to the existence of distinct

TABLE 1. CAPTURES OF MARKED AND UNMARKED MALES AT (+)- AND (±)-DISPARLURE STATIONS AND SIMILARLY BAITED TRAPS (TEST CONDUCTED JULY 16 TO 22, 1978)

	Marking blend (no. expected with null hypothesis)[a]			$\bar{X}$ unmarked males[b]
	(+)	(±)	Total	(trap/sample)
Capture blend				
(+)	58(61)	32(29)	90	171.6
(±)	23(22)	10(11)	33	50.7
Total	81	42	123	

[a] $\chi^2 = 0.448$, 1 df, with $0.50 < P < 0.75$ and $N = 123$.
[b] The two means are significantly different at $P < 0.01$ according to Student's t test. Means exclude marked males.

behavioral types. Overall capture rates of unmarked males indicate a 3.5:1 preference of (+)-disparlure over the racemate. This ratio is not unexpected because the four outside corner traps were baited with (±)-disparlure. These four traps outcaught the other four (±)-perimeter traps by a 2:1 ratio, and they accounted for 49% of the total (±) catch, although they represented only 31% of the number of (±) traps.

DISCUSSION

The basis of attraction within a species to different blend ratios has been accorded little attention, although such response variation is a common feature of most field tests. In the gypsy moth this differential response to (+)- and (±)-disparlure could be due in part to behavioral differences in the responders. Differences in attraction of male *Ostrinia nubilalis* (Hübner), the European corn borer, to blends of (*Z*)- and (*E*)-tetradecenyl acetates have been viewed as having a phenotypic (and likely genotypic) basis (Klun, 1975). The existence of two pheromone strains of *O. nubilalis* in North America (Kochansky et al., 1975) undoubtedly is related in part to separate introductions from Europe. In one area of full sympatry in central Pennsylvania (Cardé et al., 1975), gene flow between these strains as measured by isozyme frequency appears to be restricted (Harrison and Vawter, 1977; Cardé et al., 1978b), suggesting that factors in addition to pheromone polymorphism may be involved. In field studies of *Grapholitha molesta* (Busck) (Cardé et al., 1976), using an attraction-mark-release-recapture technique (similar to the procedure used in this study) and (*Z*) and (*E*) blends of the acetate components of the pheromone (Cardé et al., 1979), variation in attraction to blend

alterations could not be correlated with detectable levels of behavioral classes. The *G. molesta* findings verify that catch in traps where the responder is ensnared upon the first visit cannot be readily used to delineate the presence of behavioral types, for the trapping technique does not allow an individual responder to seek the most "attractive" lure if it first becomes ensnared at a less than optimal lure.

The natural pheromone of the gypsy moth is *cis*-7,8-epoxy-2-methyl-octadecane (Bierl et al., 1970); however, its optical configuration is as yet unknown. In view of the antagonistic effect of the (−)-enantiomer on attraction, it would appear unlikely that (−)-disparlure is a component of the natural communication system of this species. Thus, attraction to (±)-disparlure is dissimilar to the trap catch recorded at *O. nublilalis* or *G. molesta* blends, where the attraction response is altered by modifications of the ratio of components in the natural bouquet.

The (−)-enantiomer, when emitted with (+)-disparlure in the field, lowers the likelihood that males will fly upwind, land near the chemical source, or engage in prolonged walking while wing fanning (Cardé et al, 1977a); these mechanisms explain in part the lower trap catch at (±)-disparlure. The current test indicates that when trap catch is used as an indirect measure of behaviors, (−)-disparlure acts in a similar fashion upon all males. The use of a trap-mark-release-retrap procedure did not reveal "behavioral phenotypes" that were significantly more affected by the (−)-enantiomer. Such males could be present in a low proportion and thus not detectable by our procedure unless substantially more males were released and recaptured. On the other hand, detection of "phenotypic" variation in behavioral reactions may require a more discriminating assay than has been used. Considerable variation in the "ground" speed of male gypsy moths in relation to movement of a floor pattern (optomotor anemotaxis) in a wind tunnel (Cardé and Hagaman, 1979) does appear to have a phenotypic basis. Before the degree and the basis of individual variation in pheromone responses can be described for any species, the nature of the response mechanisms and the effects of internal and environmental cues on their expression will have to be quantified with considerable precision.

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IDENTIFICATION OF THE WHITE PEACH SCALE SEX PHEROMONE
An Illustration of Micro Techniques[1]

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Abstract—Micro techniques were used to obtain spectroscopic and degradative information from less than 5 μg of the sex attractant of female white peach scale, *Pseudaulascaspis pentagona* (Targioni-Tozzetti) isolated from airborne collections. The pheromone was identified as (*Z*)-3,9-dimethyl-6-isopropenyl-3,9-decadien-1-ol propionate. Both enantiomers of the *Z* isomer and also the enantiomers of the *E* isomer were prepared from (*R*)- or (*S*)-limonene. Bioassays of material with minimum enantiomeric purity of 95% showed that at extreme dilution only the *R,Z* isomer attracted male white peach scale; however activity of the *S,Z* enantiomer could not be completely excluded.

Key Words—White peach scale, *Pseudaulacaspis pentagona,* pheromone, attractant, microtechniques, enantiomer, isomers, (*R,Z*)-3,9-dimethyl-6-isopropenyl-3,9-decadien-1-ol propionate.

INTRODUCTION

The white peach scale, *Pseudaulacaspis pentagona* (Targioni-Tozzetti), is one of the principal armored scale pests of the world (Beardsley and Gonzalez, 1975). It is an introduced economic pest of peaches in the eastern United States south of Washington, D.C. (Smith, 1969).

[1]Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the USDA.

Scale insects are often inconspicuous and their detection at subeconomic levels can be difficult. Thus, a sex attractant would be useful for pest management (Rice and Jones, 1977). This realization spurred an extensive study of mating communication in the California red scale, *Aonidiella aurantii* (Maskell), and resulted in the identification of a female-produced sex pheromone (Roelofs et al., 1978, and references therein).

A project was therefore begun in 1974 at this laboratory, in association with studies of chemical and acoustical communication in other peach insects, to isolate and identify the sex pheromone produced by the female white peach scale. This project resulted in the identification and synthesis of a compound that attracts males and releases copulatory behavior, and in the development and refinement of chemical identification techniques for extremely small quantities of material.

METHODS AND MATERIALS

Pheromone Collection and Bioassay. The laboratory colony was established in March 1973 from crawlers collected locally from peach, *Prunus persica* (L.). The insect was reared on potato tubers at 28° C, 60% relative humidity, 13 hr light: 11 hr dark, using methods similar to those of Duston (1953). The life history on potato is documented, and the scale is strictly bisexual (Bennett and Brown, 1958, and references therein). The species exhibits sexual dichronism: females produce all their female offspring first and then all their male offspring. Exploitation of this characteristic and the mechanical removal of the pupating males with water spray were used to produce colonies of virgin females on tubers for pheromone collection.

Initial studies were conducted by cryogenic trapping of the air volatiles with liquefied N_2 (Browne et al., 1974). This method was limited to collections over a short time period because of the necessity for constant maintenance. Trapping of the volatiles on a Porapak-Q® (Waters Assoc., Inc.) solid adsorption trap (Byrne et al., 1975) resulted in a loss of ca. 10-20% in activity (according to bioassays of the crude extract) when compared with the cryogenic trapping. However, the ease of operation and the ability to collect over long periods warranted the use of the adsorption trap method. Collections were made by drawing air over scale-infested potatoes contained in a 45-liter glass jar and through two 1.5 × 6-cm glass traps coupled in series, each containing 3.7 g of Porapak Q as the adsorbent. Typically, 30-40 potatoes containing 8-90 thousand sexually mature females were used for each collection. Volatiles were collected continuously, and the traps were changed every 48 hr over a 2-week period. Based on an estimated 8 hr/day pheromone production period for females, 100 million female-hour equivalents (Feh) of

pheromone were collected on 208 Porapak-Q traps and ca. 4–5 μg of the pure pheromone was isolated.

Initially, the volatiles were removed from the adsorbent by Soxhlet extraction for 18 hr with 100 ml of hexane. This became too time consuming and expensive, so an alternative method of removing the pheromone from the Porapak was adopted. All the active material could be eluted from the traps with 2 ml of 20% ether-hexane by using a liquid chromatography pump. The traps were eluted with a solvent flow of 1 ml/min in the opposite direction of the air flow that was used during collection. Crude material obtained in this manner was equal in activity to the material obtained by Soxhlet extractions.

Bioassays were conducted during the afternoon hours of peak male emergence (ca. 1-4 PM), (McLaughlin and Ashley, 1977) in the laboratory under daylight-simulating (Vitalite®) fluorescent lighting. A sample in 5 μl hexane was placed on an 8 × 12-mm stainless-steel planchet at the center of a 90-mm-diam. arena (inverted plastic petri dish lid lined with filter paper). After the solvent disappeared, males (ca. 10, range 5–18) were introduced ca. 25–30 mm from the treated end of the planchet, the cover (dish bottom) was placed on the arena, and the males were observed for 2 min. The number of males on or touching the planchet was recorded. Each sample was tested in triplicate, and all assays were controlled by comparison with the response to a 0.1 Feh sample of a batch of material collected cryogenically in 1974 and reserved throughout the study for this purpose. Response to blank or solvent-treated planchets was virtually nil.

Field bioassays of the synthesized pheromone were conducted in a peach orchard during a major flight of males. Traps were constructed by coating one side of a 10-cm-diam. cardboard cup lid with Stikem Special®, and these were suspended 1.5 m above the ground (ca. mid-tree height) in a line with 30 cm between traps. The trap line was ca. 6 m from a group of scale-infested peach trees. Each trap was baited with 25 ng of one of the isomers of the synthetic pheromone applied in 25 μl of hexane to a 12.7-mm-diam. antibacterial assay disk. This disk was affixed with a straight pin to a 6.4-mm-thick cork platform at the center of the sticky surface. Four replicates of each treatment were applied at random to the traps; four untreated traps served as controls.

Isolation. The concentrated residue from the airborne collections was first chromatographed on a 6.4-mm × 50-cm silica column (10 μm LiChrosorb®, EM Laboratories, Inc.). The column was eluted with 3% ether in hexane at 3 ml/min. Thirty fractions (2 ml each) were collected. An additional 100 ml of 10% ether-hexane was used to remove the inactive polar material from the column. The active fractions were combined, concentrated, and injected onto a 6.4-mm × 25-cm, high-efficiency silica column (5 μm, LiChrosorb) prepared as described by Heath et al. (1977). By eluting with 3% ether in hexane at 1 ml/min, 20 fractions (1 ml each) were collected.

All micropreparative and analytical gas-liquid chromatography (GLC) was performed with a Varian model 1400 gas chromatograph equipped with a flame ionization detector. Stainless-steel columns were used, and the effluent from the packed columns was split 98:2 with 2% of the effluent routed to the detector, and 98% of the effluent collected in a cooled glass capillary tube 30 cm long (Brownlee and Silverstein, 1968).

The active fractions from the second silica column were concentrated and further purified by GLC on a 2-m × 2.3-mm (ID) column packed with 5% OV-101 on 80–100 mesh Chromosorb G-HP; the column temperature was held at 190° for 15 min and then programed at 260° at 10°/min with a He flow of 20 ml/min.

The active fraction collected from the OV-101 column was chromatographed on a 2-m × 2.3-mm (ID) column packed with 4% Carbowax 20M on 100–120 mesh Chromosorb G-HP operated isothermally at 180°, with a He flow of 20 ml/min. The active component collected from the Carbowax column was at least 99% pure when analyzed on a Dexsil stainless-steel capillary column (Dexsil 300 GC, 60 m × 0.76 mm ID, column temp. 190° C, He carrier velocity 20 cm/sec), OV-101 glass capillary column (OV-101, 35 m × 0.25 mm ID, column temp 190° C, He carrier velocity 20 cm/sec), and a SP-1000 glass capillary column (45 cm × 0.25 mm ID, column temp. 200° C, He carrier velocity 18 cm/sec).

Identification. The amount of pure pheromone available for spectral and microchemical degradation made it necessary to use improved micro techniques for structure elucidation. 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 column packed with 3% OV-101 of 100–120 Chromosorb W was introduced directly into the ionization source. Methane was the GLC carrier gas and the reagent gas. Data acquisition and reduction were accomplished with a Systems Industries Disc System 150 computer interfaced to the mass spectrometer.

The proton magnetic resonance spectra were obtained with a Bruker HFX-90 (equipped with a 2-mm micro insert, 5-μl sample volume) interfaced with a Nicolet 1080 computer. Approximately 2 μg of the active component was collected from the GC into a glass capillary tube in the same manner used in the GLC purification. The cooled collection tube was removed from the collector, and ca. 6 μl of deuterated benzene was used to concentrate the compound toward one end of the capillary. The tube was shortened to 7 cm in length and sealed at both ends. The residual amount of undeuterated benzene was used for chemical shift determinations, and the deuterated benzene provided the spectrometer stabilization signal for the long-term data acquisitions. Approximately 32,000 scans were accumulated over a 24-hr period.

The infrared spectra were obtained with a Nicolet 7199 Fourier Trans-

form Interferometer equipped with a 4× beam condensor and a mercury cadmium telluride liquid N_2 cooled detector. The active component (ca. 200 ng) that had been collected in a capillary tube was removed via a 2-μl slug of hexane. The capillary tube was drawn out to a fine point at one end, which allowed the hexane slug containing the sample to be deposited on a micro KBr pellet (1.5 mm OD). After the solvent evaporated (less than 1 min) and using a resolution of 4 cm^{-1}, approximately 1000 scans were obtained over a 15-min period and ratioed to the KBr blank which was also obtained with 1000 scans.

The pheromone (ca. 200 ng) was reduced in the inlet of the gas chromatograph with hydrogen and neutral palladium catalyst (Beroza and Sarimiento, 1966), equipped with a 2-m × 2.3-mm Carbowax 20M column. Microozonolysis of the pheromone (200 ng) was carried out in CS_2 at −70° C, and the ozonide was reduced with triphenyl-phosphine (Beroza and Bierl, 1967). The ozonolysis and hydrogenation products were collected and analyzed by chemical ionization mass spectrometry. When possible, synthetic material was used to confirm the identity of microdegradative products and also to verify spectral data.

Synthesis. The four isomers of 3,9-dimethyl-6-isopropenyl-3,9-decadien-1-ol propionate were synthesized by the same general procedure (Figure 1). The *R,Z* and *R,E* isomers of compounds VI were prepared from (*R*)-(+)-limonene. The *S,Z* and *S,E* isomers were obtained from (*S*)-(−)-limonene in an analogous manner. The *Z* and *E* isomers were separated on a preparative silica column (see later).

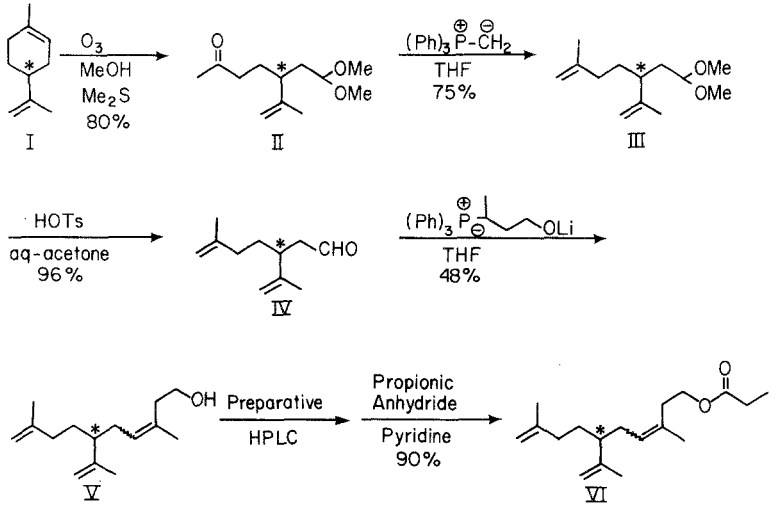

FIG. 1. Synthesis of the *Z* and *E* isomers of 3,9-dimethyl-6-isopropenyl-3,9-decadien-1-yl propionate.

(*R*)-(+)-limonene (I) (SCM Chemical Co.) was ozonized with 1 mole equivalent of ozone at −65° C in methanol. Reduction of the limonene ozonide with dimethyl sulfide was done in the presence of a trace amount of acid. The yield of the keto-acetal (II) was found to be greater than 80% with less than 5% of the keto-aldehyde being formed. The keto-acetal was then allowed to react with (methyl)-triphenylphosphonium bromide (Aldrich Chemical Co.) in THF to give the diene-acetal (III) (Maercker 1965). Hydrolysis of the acetal produced the diene aldehyde (IV). This diene aldehyde was added to the yilde generated from the corresponding 3-hydroxy-1-methylpropyltriphenylphosphonium salt to give the triene alcohols (V) according to the procedure of Roelofs et al. (1978). GLC analysis indicated that the *Z* and *E* isomers were formed in the Witting reaction in a ratio of 52:48, respectively, with ca. 50% yield. About 30% of the compound, 9-methyl-6-isopropenyl-3-9-decadien-1-ol, was also produced. The Wittig products were purified by liquid chromatography (LC) on a 12.7-mm × 25-cm high-efficiency silica column containing 5 μm LiChrosorb by using ether-hexane (1:1) at a flow rate of 3 ml/min. Elution order was *Z* isomer, the *E* isomer of the desired triene alcohol product (V), and then the unresolved *Z*-*E* isomers of the 3 de methyl compound (k' values of 1.9, 2.3, and 2.5, respectively, where the capacity ratio k' is equal to the elution time of the sample (T_s) minus the elution time of a unretained solute (T_0) divided by the elution time of the unretained solute, i.e., $k' = (T_S - T_0)$ (Ettre 1963). The *Z* and *E* isomers of V were at least 98% pure when checked on polar and nonpolar capillary GLC columns. Propionation of the pure isomers of V with propionic anhydride gave the corresponding triene propionates (VI), which were again purified by LC. By using a 6.4-mm × 25-m column of 5 μm silica eluted with 5% ether-hexane at 1 ml/min, *Z* and *E* isomers of VI with k' of 1.60 and 1.9, respectively, were purified. Analysis on several different capillary GLC columns indicated a purity of 99% or better. Optical rotations were obtained with a Randolph model 85 polarimeter using a sodium vapor lamp. Samples were either neat or dissolved in chloroform placed in a 1-dm cell (vol = 1 ml).

RESULTS AND DISCUSSION

As indicated, material obtained in 1974 by cryogenic trapping of the air volatiles from *P. pentagona* females was used as a standard at 0.1 Feh for the bioassays. When males were occasionally poorly responsive to the standard, bioassay data for these days were not used.

The active fractions (13-16) obtained from the 6.4-mm × 50-cm silica column (Table 1) were combined. This combined fraction was as attractive as the crude material when bioassayed at 0.1 Feh. Activity was not increased by recombination of all the fractions. The combined active fractions (13-17)

from the first silica column were further purified on a high-efficiency 6.4-mm × 25-cm silica column (Table 1). GLC of the combined active fractions (9–11) from the second silica purification on the packed OV-101 column yielded a single peak with a carbon equivalent of 17.76 (Kovats, 1965). No loss nor enhancement of activity was obtained when the material was recombined with other GLC fractions. Further GLC purification of the OV-101 purified compound gave on Carbowax 20M a single peak with a Kovats index of 21.30. This fraction was as active as the standard crude extract when bioassayed at 0.1 Feh. This material was determined to be greater than 99% pure when analyzed on the three capillary columns.

The amount of pure pheromone available for identification never exceeded 2–3 μg at any given time, although an estimated 4–5 μg of the pure pheromone was collected during the course of this investigation. However, previous work done in developing microstructure elucidation techniques (Tumlinson and Heath, 1976) suggested that this amount of material would be adequate for obtaining the usual spectral information and conducting the microchemical degradations necessary for structure elucidation if some improvements could be made in the existing techniques. Primary concern in the handling of micro samples is the typically large losses encountered in the

TABLE 1. RESPONSE[a] OF MALE WHITE PEACH SCALE IN LABORATORY BIOASSAY TO PURIFICATION FRACTIONS OF VOLATILES

6.4 mm × 50 cm silica[b]		6.4 mm × 25 cm silica[c]		OV-101[d]	
Fraction (3 ml)	% male response	Fraction (1 ml)	% male response	Fraction (min)	% male response
1-10	0	1-5	0	1-5	0
11	0	6	0	5-10	0
12	3.3	7	0	10-15	0
13	13.3	8	0	16	0
14	48.3	9	69.0	17	2.2
15	71.8	10	63.3	18	74.6
16	22.5	11	16.6	19	32.2
17	3.2	12	4.2	20	18.0
18	0	13	0	21	4.0
19-30	0	14-20	0	22	2.0
30-100	0			23-40	0

[a]Attraction from a distance of ca. 25 mm onto a metal planchet treated with the fraction. Assay conducted in 90-mm diam. petri dish arena.
[b]Crude pheromone obtained from airborne collections.
[c]Active fractions (13-17) from first silica.
[d]Active fractions (9-11) from second silica.

transfers of such samples and the removal of any interfering background that would limit the utility of the information obtained.

A chemical ionization (CH_4) mass spectrum was obtained on ca. 50 ng of the pure compound (Figure 2). A molecular weight of 278 was established by peaks at *m/e* 279 (P+1) and 277 (P−1). The peak at *m/e* 205 (P+1-74), along with the peak at *m/e* 75 ($C_3H_7O_2^+$) suggested a propionated compound. The intensities of the ions at *m/e* 203-207 were exactly the same as those seen in the spectrum of farnesyl-propionate run on the mass spectrometer. Hydrogenation of ca. 150 ng of the pheromone indicated three units of unsaturation since both the P+1 and P+1-74 were found at 285 and 211, respectively (6 mass units higher than the unsaturated pheromone). Ions at 313 (P+29) and 325 (P+41) found in the mass spectra of the hydrogenated pheromone confirmed the molecular weight of 284 for the saturated pheromone. Ozonolysis of ca. 150 ng of the pheromone produced one major product that was identified as 3-oxobutan-1-yl propionate by comparison of its retention time on Carbowax 20M and its mass spectrum with those of a synthetic standard. On the bases of the mass spectral data obtained for the pheromone and its degradative products, the partial structure was determined to consist of a propionate ester of 3-alkylidine-1-butanol; this left undefined an 11-carbon hydrocarbon moiety having two points of unsaturation.

The collection of microgram and submicrogram amounts of material is readily accomplished with the cooled glass capillary collector described by Brownlee and Silverstein (1968), and collection efficiencies are 80% (often 90%). However, the transfer of these small amounts to infrared and nuclear magnetic resonance (NMR) spectrometers has typically resulted in the loss of a very high percentage of the material. Also the transfer process may introduce impurities (H_2O for example), which limits the degree of information obtained to those spectral regions not obscured by background signals. The overall signal-to-noise improvement on multiscan instruments too may be limited, depending on the amount of impurity, because of dynamic range considerations.

We found that we could limit losses by using a small slug of solvent to transferred pure pheromone from the collection tube to the micro KBr pellet; as a result an infrared spectrum was obtained with only 200 ng of material. The infrared spectrum showed a strong carbonyl adsorption at 1740 cm^{-1}, and of particular interest a rather strong adsorption at 880 cm^{-1}, which strongly suggested the presence of two terminal olefins.

By using the collection tube as the NMR cell and eliminating the transfer process, a proton magnetic resonance spectrum (Figure 3) was obtained on ca. 2 μg of the pure pheromone. The spectrum is consistent with structure VI (Figure 1), with δ values of 5.55 (1H, t); 4.98 (4H, s) ($R_2C{=}CH_2$); 4.28 (2H, t)

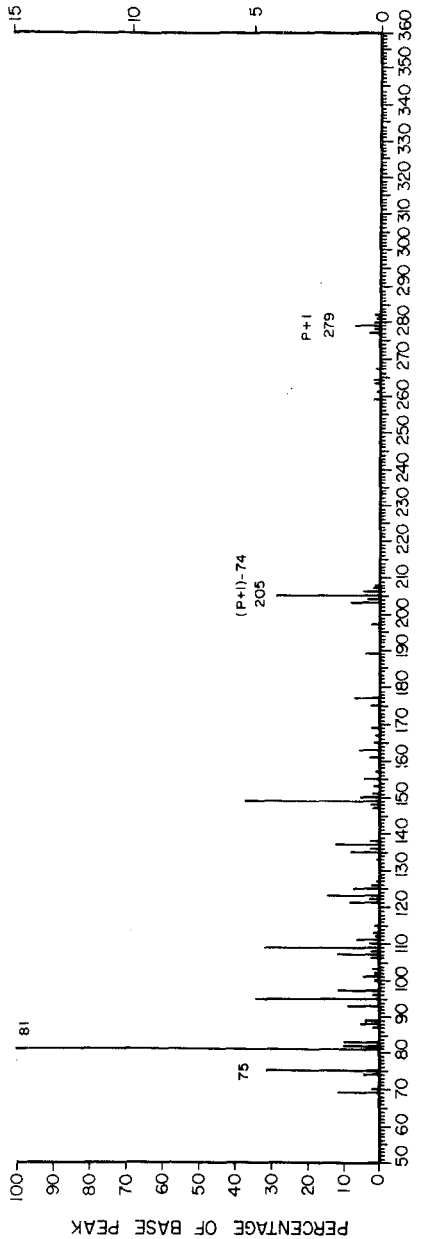

FIG. 2. Methane ionization mass spectrum of ca. 50 ng of white peach scale pheromone.

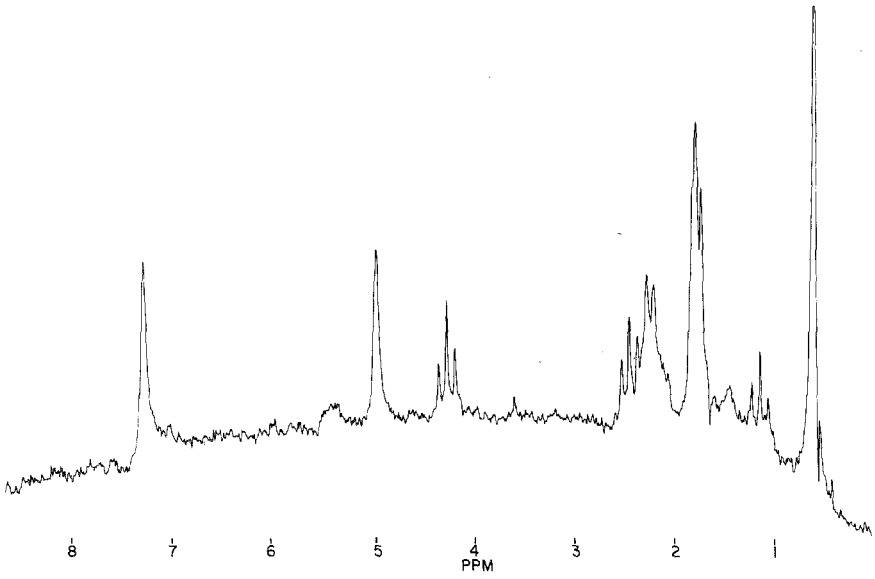

FIG. 3. 90 MHz proton NMR spectrum of ca. 2 μg of white peach scale pheromone. 32,000 transcents obtained, benzene d_6 used as solvent, and spectrometer lock signal.

$(CH_2—\underline{CH_2}—O)$; 2.46 (2H, q) and 1.16 (3H, t) $(—\overset{O}{\overset{\|}{C}}—CH_2—CH_3)$; 2.35 – 2.21 2H $C(R)=C—CH_2$ and 5 H allylic); 1.82 (3H, s) 1.80 (3H, s) 1.74 (3H, s) (three $CH_3—C(R)=C$); 1.4 ppm (2H, m). Thus the NMR spectra obtained on this amount of material did provide confirmation of the propionate group, the trisubstituted double bond and the two terminal methylenes, but there was no conclusive way to determine that the isopropenyl branch was located in the sixth position. Therefore the existence and position of the branch were hypothesized from previous work in pheromone chemistry and particularly from conversations with the team who had identified the California red scale pheromone (Roelofs et al., 1978).

Since neither the enantiomers nor the stereochemistry of the pheromone was known, it was necessary to design a synthetic method that produced the four possible isomers of the pheromone. By starting with the respective enantiomer of limonene, we were able to use ozonolysis to produce the key intermediate for the rest of the synthesis. The synthesized *Z* pheromone was identical in its mass, infrared, and PMR spectra and coeluted on the various capillary columns with the natural material.

Infrared and mass spectra of the *E* isomer were identical to those for the *Z*

isomer. However, in the NMR spectrum the 3 methyl group of the *E* isomer showed a slight upfield shift from 1.80 to 1.74 compared to the *Z* isomer. The *Z* and *E* isomers of this pheromone have subsequently been synthesized stereospecifically, and the NMR and chromatographic data confirm the original assignments of the geometry of the trisubstituted bond (Heath et al., in preparation). The geometrical purity of the synthetic pheromone was determined on an OV-101 glass capillary column (35 m × 0.2 mm ID) with ca. 100,000 theoretical plates. When analyses were carried out at 145° C with a linear flow velocity of 18 cm/sec, the *Z* isomer had a k' of 1.76 and the *E* isomer a k' of 2.07, with $\alpha = 1.18$ (k' of *E* isomer/k' of *Z* isomer) and an isomer resolution of 4.1 (calculated by multiplying the distance between the two isomer peaks by 2 and dividing by the product of the two peak widths at the base, i.e., $2DT/(W_1 + W_2)$ (Ettre 1963).

Optical rotations were obtained on two of the intermediates that were available during the synthesis that had a known purity of 97% or higher when checked on the three capillary columns. The *R* and *S* ketoacetal (II) had rotations of +1.167 and −1.125 (α_D^{25}, $C = 9.3$), respectively; and *R* and *S* diene-aldehyde (IV) had rotations of +1.588 and −1.565 (α_D^{25}, $C = 10.1$), respectively. On the basis of these values minimum enantiomeric purity is ca. 95%. The rotations measured on the products of the subsequent steps and on the final products were very small and accurate measurements were not possible. As further material becomes available enantiomeric purifications and determinations will be undertaken by using the method of Bergot et al. (1978).

Bioassays. Laboratory bioassays were conducted on 10 pg, 1 pg, and 100 fg dilutions of the four stereoisomers of *R,Z*; *S,Z*; *R,E*; and *S,E* (Table 2, test 1). The activity of the *R,Z* isomer was neither enhanced nor decreased by the addition of the other isomers (Table 2, test 2). In a field bioassay conducted July 12, 1979, 25 ng of the *R,Z* isomer captured (mean per trap ± SE) 240 ± 23.2 males, the *S,Z* isomer 91.0 ± 7.0, the *R,E* isomer 37.5 ± 6.0, and the *S,E* isomer 34.0 ± 10.9, and the 4 blank traps captured an average 24.0 ± 7.1 males. The small amount of activity elicited by enantiomer/isomers other than *R,Z* (particularly *S,Z*) was probably the result of the extremely high degree of sensitivity of this bioassay and the response to small amounts of *R,Z* not discernible by the analytical techniques used. We cannot totally exclude *S,Z* as a pheromone component, because the minimum enantiomeric purity of our test material was only ca. 95%.

Males responding to either female extracts or synthetic pheromone in the laboratory bioassay exhibited sexual behavior that appeared the same as that of males exposed to pheromone-releasing females. For example, either material released copulatory behavior in males at inappropriate locations and in the absence of any other female-like cues. Also, the sexual behavior of males

TABLE 2. MEAN (± SE) PERCENT RESPONSE OF MALE WHITE PEACH SCALE TO ISOMERS AND ENANTIOMERS OF BASIC PHEROMONE MOLECULE (LABORATORY BIOASSAY)[a]

	10 pg	1 pg	100 fg
Test 1			
R,Z	75 ± 6	48 ± 4	36 ± 6
R,E	3 ± 3	0 ± 0	0 ± 0
S,Z	30 ± 12	12 ± 7	0 ± 0
S,E	3 ± 3	0 ± 0	7 ± 4
Test 2[b]			
R,Z	84 ± 3	50 ± 7	35 ± 8
R,Z + *R,E*	84 ± 4	56 ± 3	40 ± 7
R,Z + *S,Z*	84 ± 11	56 ± 3	40 ± 7
R,Z +*S,E*	84 ± 5	52 ± 3	37 ± 9
S,Z + *S,E*	46 ± 0	31 ± 3	3 ± 3
S,E + *R,E*	15 ± 4	19 ± 7	2 ± 2

[a]Isomeric purity >99%, enantiomeric purity ca. 95%. Analysis techniques in text.
[b]Mixtures contained the indicated amount of each compound.

toward a locus of evaporating pheromone was the same as that toward a female. This response may be concentration dependent, at least at some upper limit, because males exposed to very high concentrations of the pheromone did not approach the locus of evaporation, but stopped a few millimeters away and began copulatory thrusts. At times, very low concentrations of female extracts appeared to release flight behavior from males in the petri dish bioassay chambers, but usually the males walked to the treated metal planchets.

In the field, and during some greenhouse assays, males were observed in flight toward synthetic and natural pheromone sources. When near a source, they frequently exhibited the corkscrew pattern of flight described for male red pine scale males (Coccidae), *Matsucoccus resenosae* (Bean and Bedwine), by Doane (1966). Males did not land directly at a pheromone-treated spot, but came to rest several centimeters away and walked over to the treated locus.

Thus, from chemical and behavioral evidence we submit that (*R,Z*)-3,9-dimethyl-6-isopropenyl-3,9-decadien-1-ol propionate is the principal if not the only component of the sex pheromone produced by white peach scale females. The pheromone is quite similar to that of the California red scale (Roelofs et al., 1978), which suggests that the diaspidid scales may utilize this general class of chemical for sexual communication.

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ISOLATION, IDENTIFICATION, AND SYNTHESIS OF A FEMALE SEX PHEROMONE OF THE NAVEL ORANGEWORM, *Amyelois transitella* (LEPIDOPTERA: PYRALIDAE)[1]

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Abstract—A sex pheromone of the navel orangeworm, *Amyelois transitella* (Walker), was obtained from ether rinses of the sex pheromone gland of calling females. The pheromone was isolated by means of liquid and gas chromatography and was identified as one of four possible geometrical isomers of 11,13-hexadecadienal by means of spectroscopic and microchemical methods. Synthesis and laboratory bioassay of all four isomers revealed that only the (*Z,Z*) isomer was biologically active. (*Z,Z*)-11,13-hexadecadienal elicited quantitatively similar activation and attraction responses by *A. transitella* males as did the natural product.

Key Words—Sex pheromone, navel orangeworm, *Amyelois transitella*, (*Z,Z*)-11,13-hexadecadienal, Pyralidae.

INTRODUCTION

The navel orangeworm, *Amyelois transitella* (Walker), is a well-established primary pest of almonds in California for which no completely satisfactory method has been developed for monitoring adult populations. The principal methods that have been used to monitor adult activity for the purpose of timing chemical control measures include light traps (Madsen and Sanborn, 1962; Summers and Price, 1964) and traps baited with laboratory rearing

[1]Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the USDA. Accepted for publication February 28, 1979.

medium. The latter were reported by Rice (1976) and Rice et al. (1976) to be attractive to *A. transitella* females.

Proshold (1967) and Asoka Srinivasan (1969) studied the sex pheromone of *A. transitella* females and reported that traps baited with live females were attractive to males in commercial almond and walnut plantings. The lack of a synthetic sex pheromone has, however, limited the usefulness of population monitoring by this means. The availability of a synthetic sex pheromone for this species would allow more extensive use of this survey method than is presently possible and would be a useful adjunct to extant methods (light traps, oviposition attractants) of monitoring. In addition, the feasibility of using the synthetic pheromone or related compounds as disruptants of mating communication (Mitchell, 1975) could be explored.

This paper reports the isolation, identification, and synthesis of a sex pheromone of *A. transitella* that was obtained from diethyl ether rinses of the sex pheromone glands of laboratory-reared females.

METHODS AND MATERIALS

Moths used in this study were reared in this laboratory at 26.7 ± 1° C and ca. 60% relative humidity under a reversed 14:10 light:dark photoperiod. Photophase and scotophase light intensities were >250 and ca. 0.3 lux, respectively. Details of the insect diet and rearing procedure are described elsewhere (Coffelt et al., 1978). Periodic introduction of new stock during the course of this study was made from eggs and larvae that were obtained from the Stored-Product Insects Laboratory, Fresno, California.

Details of the bioassay apparatus and procedures are described elsewhere (Coffelt et al., 1979). Briefly, two kinds of bioassays were conducted: In the first, males were placed individually in glass vials (22 × 53 mm) and the test sample placed within 2 cm of them; onset of locomotion and "wing-buzzing" within 15 sec of sample introduction was considered evidence of pheromone response. In the second bioassay, upwind movement (ca. 0.4 m) in Plexiglas tubes (apparatus described by Sower et al., 1973b) to within ca. 3 cm of the pheromone locus within 30 sec after sample introduction was taken as evidence of pheromone response. Male response to applicators treated with solvent only was ca. 10% in either bioassay.

Initial trials to obtain the pheromone from either solvent rinses of excised female abdominal tips, whole female bodies, or filter papers upon which females had been held yielded nonreproducible quantities as determined by bioassay. Subsequently, superficial diethyl ether rinses of excised pheromone glands of calling females (7-10th hr of scotophase) were made by the method described by Sower et al. (1973a). Quantitative bioassays of pheromone obtained by this method were reproducible, and the extracts were clean

enough for gas-liquid chromatography (GLC) without further purification.

All analytical and micropreparative GLC during the identification phase of the project were done on Varian model 2100 instruments that were equipped with H_2 flame ionization detectors. Instrument operating parameters were the same as those described by Coffelt et al. (1978).

RESULTS

Isolation. A superficial pheromone gland rinse (30 females) was concentrated to ca. 4 μl and injected into a 1.8-m × 2-mm (ID) glass column packed with 3% OV-1 on 100/120 mesh Gas Chrom Q® column; the temperature was programed (6°C/min) from 100°C at injection to 220°C. The resulting chromatogram, shown in Figure 1A, revealed two detectable peaks in the

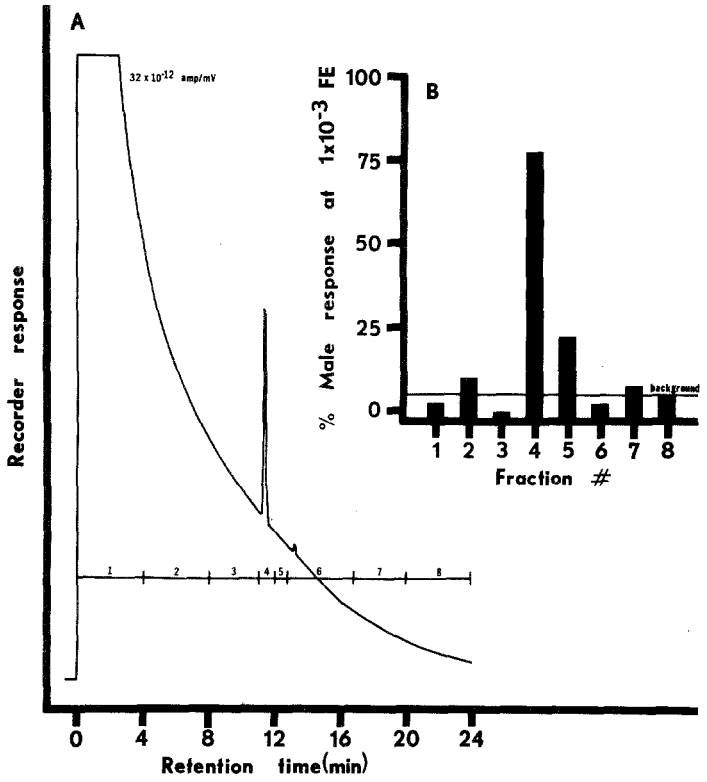

FIG. 1. (A) Gas chromatogram of superficial diethyl ether rinse of terminal abdominal tips of 30 calling *A. transitella* females. Instrument parameters as described in text. (B) Response of male *A. transitella* to micro-preparative GLC fractions as indicated in (A).

elution range of most previously identified lepidopteran sex pheromones. The major component, ca. 1.0–1.5 ng/female, had a retention time of 11.6 min (170° C) and a carbon number of 18.1 relative to normal paraffins. The minor component of the gland rinse eluted at 13.0 min (178° C) (carbon number 19.0 relative to normal paraffins) and was present in concentration of ca. 0.1 ng/female. A similar preparation (25 females) was injected as above, and the column effluent was collected in 1.0 ml of hexane at 0–4, 4–8, 8–11, 11–12, 12–13, 13–16.7, 16.7–20, and 20–24 min after injection. Each fraction was diluted to yield 1×10^{-3} female equivalents (FE) / 5 μl and bioassayed (activation test). The results of this series of tests (5 replications) are shown in Figure 1B. More than 95% of the biological activity (based on additional bioassays of further dilutions) was contained in the 11 to 12-min GLC fraction, which corresponded to the retention time of the major component indicated in Figure 1A. Male response to the 11 to 12-min fraction was not significantly different from that recorded for an aliquot of the unchromatographed gland rinse over a range of concentrations from 1×10^{-2} to 1×10^{-4} FE. The 13.0 to 16.7-min fraction (fraction 6, Figure 1A) containing the minor component of the gland rinse was inactive.

Subsequently, similar gland rinses were collected from 5% Carbowax 20M on 100/120 mesh Chromosorb W®, column temperature 170° C, and 5% HiEff-1BP on 60/80 mesh Gas Chrom Q®, column temperature 140° C. In each case, one area of biological activity was found (Carbowax), 10.5–12.0 min; HiEff-1BP, 13.6–15.5 min). When the active fraction from either polar GLC column was recollected from the OV-1 column, the biological activity eluted only in the 11 to 12-min fraction shown in Figure 1A. No significant loss of biological activity (activation or orientation bioassays) was noted following GLC on any of the three columns used.

Identification. Pheromone was purified for mass spectral analysis as follows: Diethyl ether gland rinses from ca. 5000 calling females were stored in lots of 100 at −10° C until used. These extracts were combined, carefully concentrated under dry N_2 to near dryness, and taken back up in 0.5 ml of hexane. The extract was injected onto a 1.0-cm (ID) glass high-pressure liquid chromatographic (HPLC) column packed to a height of 50.0 cm with Poragel® 60A, 37–75 μm (Waters Associates). Forty 2.5-ml fractions were collected with hexane as the eluting solvent at a flow rate of 120 ml/hr. Biological activity (activation and orientation bioassays) appeared as a single band with an elution volume of 60–70 ml (fractions 24–28). Analytical GLC (OV-1) of each fraction showed one detectable peak with a retention time identical to that of the major component of the crude extract shown in Figure 1A. Analytical GLC runs of inactive Poragel 60A fractions did not contain this peak. Quantitative bioassays of pooled active fractions indicated no loss of biological activity relative to dilutions of an unchromatographed aliquot of the crude extract.

The combined active fractions from the Poragel 60A column were further purified by GLC on OV-1 (140° C isothermal). The biologically active fraction contained ca. 2-3 μg of a compound whose retention time (programed temperature run on OV-1) was identical to that of the major component shown in Figure 1A. Collection of this active fraction from the Carbowax 20M (170° C) column yielded a single apparently symmetrical peak (11.7 min) on both columns and had the same biological activity as the starting material.

A mass spectrum of the purified (>98%) compound was obtained with a Finnigan model 105C chemical ionization mass spectrometer that was equipped with a chromatographic inlet (Varian model 1400). The effluent of a 2.0 m × 2.3 mm (ID) column packed with 3% SE-30 on 100/120 Varaport® 30 was introduced directly into the ionization source. Methane served as the carrier and reagent gas. The resulting spectrum had a base peak at m/e 95 and a molecular weight of 236 established by peaks at m/e 237 (P+1), 265 (P+29), and 277 (P+41). A peak at m/e 219 (P+1-18) and the character of the remaining portion of the spectrum suggested that the compound was an unbranched aldehyde or alcohol. Thus, the pheromone appeared to be either a 16-carbon triunsaturated alcohol or a diunsaturated aldehyde.

Hydrogenation (Pd on charcoal) of ca. 300 ng of the purified compound resulted in the loss of biological activity and the disappearance of the original peak (GLC on 3% OV-1 and 5% Carbowax 20M) and the appearance of a new peak with a retention time on both polar and nonpolar stationary phases identical to that of hexadecanal. This result, along with mass spectral data, suggested that the compound was a diunsaturated aldehyde.

Supportive evidence for an aldehyde (as opposed to alcohol) functionality was obtained from each of three independent tests. First, the pheromone eluted from silicic acid columns (Sower et al., 1973b) with standard aldehydes. Second, treatment of the pheromone with acetic anhydride in pyridine did not significantly reduce biological activity relative to untreated pyridine controls. Third, treatment of the pheromone with $NaBH_4$ resulted in a significant reduction in biological activity.

The retention times of the pheromone relative to hexadecanal on both polar and nonpolar GLC were much longer than expected for a hexadecadienal with two isolated double bonds. On OV-1, for example, the purified pheromone eluted after hexadecanal. This observation suggested that the two centers of unsaturation were conjugated, i.e., that the pheromone was a conjugated diene or an α,β-unsaturated aldehyde. To confirm this, we obtained the UV spectrum of ca. 750 ng of GLC purified (~96%) pheromone in 200 μl of ethanol. Maximum absorbance was at 232 μm with an extinction coefficient of 18,100. This result confirmed the presence of a conjugated system in the compound, and suggested that the material was a conjugated diene rather than an α,β-unsaturated aldehyde whose maximum absorbance would have been near 220 μm.

Microozonolysis (Beroza and Bierl, 1967) of ca. 1 μg of GLC purified (~96%) pheromone resulted in >99% loss of biological activity, disappearance of the original peak (OV-1 and Carbowax 20M), and the appearance of a new peak with a retention time on both columns identical to that of 1,11-undecanedial. The presence of the latter compound was confirmed by mass spectrometry. However, this compound would result from the ozonolysis of either 11,13- or 2,13-hexadecadienal.

The following experiment was conducted to unequivocally establish the correct positional isomer: Ca. 3 μg of GLC purified (~96%) pheromone was reduced in 100 μl of 1 molar ethanolic $NaBH_4$. This reaction product was acetylated by the addition of 200 μl of pyridine and 50 μl of acetic anhydride. The progress of each reaction was monitored by GLC. The final reaction product was ozonized as before.

The retention times of the major ozonolysis product were 11.6 and 10.0 min, on polar (5% Carbowax 20M; 175° C) and nonpolar (3% OV-1; 130° C) GLC substrates, respectively, and were identical to those of authentic 11-acetoxyundecanal. Also, the mass spectra of the ozonolysis product and 11-acetoxyundecanal were identical. This result fixed the position of the double bond nearest to the functional group at the 11 position, and along with the GLC and UV evidence for the presence of a conjugated system, indicated that the compound isolated from pheromone gland rinses of calling *A. transitella* was one of four possible geometrical isomers of 11,13-hexadecadienal.

Synthesis and Purification of the 11,13-Hexadecadienal Isomers. Although several methods exist that enable stereospecific construction of conjugated dienes, we employed a method that would generate mixtures thereby permitting some variation in isomer composition. The final purification of each isomer would be accomplished, in any case, by HPLC and/or preparative GLC. A de novo stereospecific approach for each geometrical isomer would only be time consuming. Also, purity could not be assured by using these previously established routes.

The method chosen is outlined in Figure 2. The key step, a Wittig condensation involving an 11-carbon aldehyde ether with an allylic ylid, afforded conjugated dienes of mixed geometrical composition. The ylids were prepared from a predominantly *cis*, or *trans* allylic phosphonium salt, and the base-solvent choices were varied to guide the isomer ratio somewhat. Mixtures were chosen to facilitate purification of individual isomers.

The tetrahydropyranyl ether (THP) of 10-undecen-1-ol was hydroborated and worked up oxidatively to produce primarily the mono-THP ether of 1,11-undecanediol which was contaminated to a degree (20%) by the diol itself. The mono-THP ether was further purified by fractional distillation (bp 150–156°, 0.02 mm). Pyridinium chlorochromate (PCC) that was buffered with sodium acetate was employed to yield the THP ether of 11-hydroxy-

undecanal, I (purified by gravity-flow liquid-solid chromatography; IR 1727 cm^{-1}).

Reduction of 2-pentyn-1-ol by either *syn* addition of hydrogen (P-2 nickel) or *anti* addition (lithium aluminum hydride in refluxing tetrahydrofuran, THF) produced (*Z*)-2-penten-1-ol and (*E*)-2-penten-1-ol, respectively. These were converted to the allylic bromides with phosphorus tribromide-pyridine and then to phosphonium salts with triphenylphosphine in benzene at room temperature. Although the reductions of propargylic alcohols and their conversions to allylic halides are highly stereoselective (Truscheit and Eiter, 1962), the conversions of the resulting allylic alcohols to bromides can be troublesome (Smith et al., 1978). In particular, *cis-trans* isomerization can occur during distillation. The bromides were therefore converted, without prior distillation, directly to phosphonium salts. The II-*Z* salt melted at 191–196°, its reported melting point is 144–145° (Truscheit and Eiter, 1962). The II-*E* salt melted at 170–173°, its reported melting point is 144° (Butenandt et al., 1962) and 178–180° (Truscheit and Eiter, 1962). The mixture melting point of our two preparations showed a distinct depression, 160–185°. The salts were converted to ylids by two methods (BuLi in THF-HMPA, NaH-DMSO) and allowed to react with the aldehyde I. The diene ethers III were converted to acetates IV for GLC analysis [Ultrabond I Carbowax, 2 m × 3 mm at 150° and SP-2340 capillary (WCOT) column, 45 m × 0.25 mm at 156°]. The acetates were further characterized by HPLC on a 20%

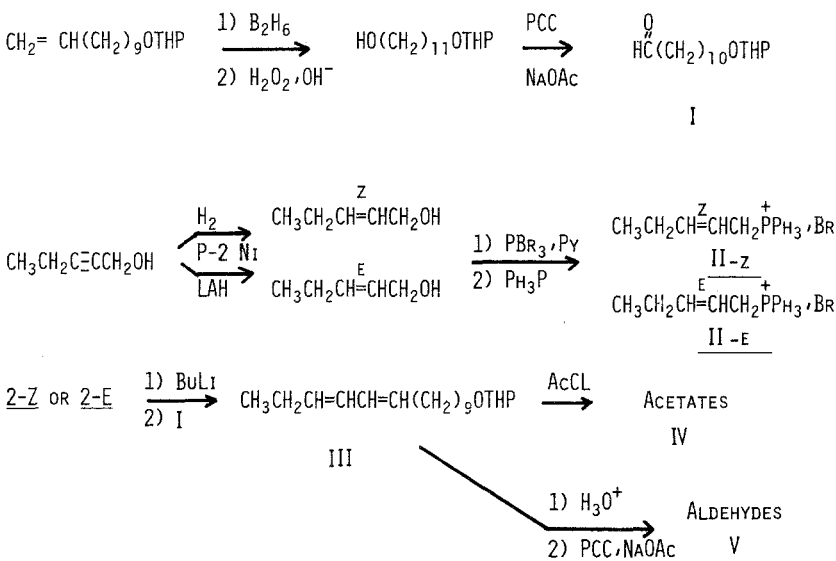

FIG. 2. Synthesis routes for geometrical isomers of 11,13-hexadecadienal.

$AgNO_3$-EF-54 column, 1.25 cm × 25 cm, 4:1 benzene-hexane at 590 psi. Diene ether preparations were chosen on the basis of the acetate analysis and were converted to aldehydes by (1) hydrolysis to the alcohols and (2) buffered PCC oxidation. The aldehydes were then collected by HPLC employing the column described. Since the 11-*Z*,13-*E* and 11-*E*,13-*Z* isomers were only partially separated by HPLC, collections were made from mixtures richer in one than the other by collecting the early or late portions of the unresolved doublet. The collected fractions were washed with aqueous sodium chloride, dried (sodium sulfate), and filtered. They were then concentrated in a slow stream of nitrogen and trapped after passage through a 4% OV-101 column, 2 m × 3.2 mm at 200° C. The aldehydes were then analyzed with the capillary column described above. This GLC analysis did not effect a complete separation of the *Z,Z* isomer from the (*E,Z*)-11,13-isomer. However, a judicious use of both HPLC and capillary GLC permitted a reliable assessment of the constitution of each collected aldehyde.

Because conjugated dienes have been previously determined as insect sex pheromones, a limited amount of chromatographic data concerning these materials has been published (Goto et al., 1975; Hall et al., 1975; Roelofs et al., 1971, 1974; Tamaki et al., 1973). These data deal with 7,9-dodecadien-1-ol acetate, 8,10-dodecadien-1-ol, and 9,11-tetradecadien-1-ol acetate. The pheromone of *A. transitella* is the second determination of a conjugated dienal, and we felt that inclusion of our chromatographic data for the synthesized diene acetates and aldehydes would be of value (Table 1). The estimated purities of the isomeric aldehydes are: (*E,E*); ⩾98%; (*E,Z*) 96%, contaminated with

TABLE 1. CHROMATOGRAPHIC PROPERTIES OF GEOMETRICAL ISOMERS OF 11,13-HEXADECADIENAL AND 11,13-HEXADECADIEN-1-OL ACETATE[a]

Compound	HPLC[b]	UB-I-CW (GLC)[c]	SP2340 (GLC)[c]
(*E,E*) aldehyde	1.00		1.05
(*E,Z*) aldehyde	1.30		1.03
(*Z,E*) aldehyde	1.24		1.00
(*Z,Z*) aldehyde	1.52		1.03
(*E,E*) acetate	1.00	1.69	2.24
(*E,Z*) acetate	1.23	1.57	2.19
(*Z,E*) acetate	1.17	1.50	2.09
(*Z,Z*) acetate	1.38	1.59	2.17

[a]Chromatographic columns as described in text.
[b]Retention volumes relative to (*E,E*) isomer.
[c]Retention times for aldehydes relative to (*Z,E*)-11,13 aldehyde isomer; for acetates relative to 1-hexadecanol acetate.

TABLE 2. ACTIVATION RESPONSE OF *A. transitella* MALES TO UNPURIFIED SEX PHEROMONE AND GEOMETRICAL ISOMERS OF 11,13-HEXADECADIENAL AT 1×10^{-4} μg

Treatment	Purity (%)[a]	% Male response (SE)
Unpurified pheromone[b]		90.0 (2.0) a[c]
Z,Z	≥99	90.0 (3.2) a
Z,E	~93	10.0 (2.0) b
E,Z	~96	10.0 (4.8) b
E,E	>98	23.8 (6.0) b
All 4 isomers[d]		92.5 (2.5) a
Blank		12.2 (3.8) b

[a]Purity determined by capillary GC; columns as described in text.
[b]Concentration determined by GLC as described in text.
[c]Means followed by same lower case letter are not significantly different at $P = 0.05$.
[d]1×10^{-4} μg of each isomer.

(*Z,E*); (*Z,E*) 93%, contaminated with (*E,Z*); and (*Z,Z*) ≥99%. Characteristic infrared bands were observed for the diene structures (Roelofs et al., 1974): (*E,E*), 986 cm^{-1}; (*Z,E*) and (*E,Z*), 945 and 979 cm^{-1}; (*Z,Z*), no absorption in this region of the spectrum.

Mass spectra of the synthesized isomers of 11,13-hexadecadienal were identical with that of the purified natural product.

Laboratory Bioassays of Synthesized Isomers of 11,13-Hexadecadienal. All bioassays (activation and orientation) were conducted as previously described by using hexane solutions of the four geometrical isomers of 11,13-hexadecadienal and unpurified pheromone gland rinse. Estimates of pheromone quantity in the latter solution were based on GLC of aliquots of the material. Unpurified pheromone was used as a standard for comparison of relative biological activity of the four isomers in all bioassays.

Initial trials with the synthetic compounds were made with 1×10^{-4} μg in the activation bioassay apparatus. The results of this series of tests (8 replications) are shown in Table 2. Male response to the (*Z,Z*) isomer was the same as that recorded for an estimated equal quantity of the unpurified gland rinse. The response of *A. transitella* males to the remaining three isomers was not significantly different from male response to untreated pheromone dispensers. A mixture of the four compounds (1 : 1 : 1 : 1) had no significant effect upon male response relative to the (*Z,Z*) isomer alone (4 replications).

Subsequently, additional bioassays (activation, 8 replications; orientation, 12–15 replications) were conducted over a range of concentrations (1×10^{-4} to 1×10^{-8} μg) with the unpurified gland rinse and (*Z,Z*)-11,13-hexadecadienal. The results of these bioassays are summarized in Figures 3

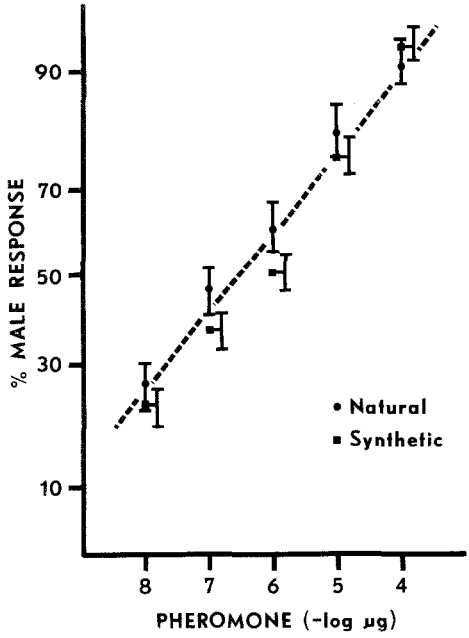

FIG. 3. Activation response of *A. transitella* males to (*Z,Z*)-11,13-hexadecadienal and unpurified pheromone. Background (control) response = 11.0 ± 1.9 (SE)%.

and 4, and indicate the qualitative and quantitative similarity of the response in both the activation and orientation bioassays. The quantitative similarity is evident from the *Y* values for each test material. The qualitative similarity is inferred from the generally similar slopes.

DISCUSSION

The chemical and biological data presented in this paper are strong evidence that (*Z,Z*)-11,13-hexadecadienal is a pheromone of *A. transitella*. Because of the very small quantities of material produced by the female, the losses during extraction and purification (ca. 50%), and the similar gas chromatographic properties of the various isomers, especially the (*Z,Z*) and (*E,Z*), the presence in the natural pheromone of small amounts of the other three stereoisomers is not ruled out. The possibility that the complete pheromone may be a mixture of compounds with different functions cannot be established from our laboratory bioassay data. Determination of an optimum blend will, in all likelihood, be established only with field tests. Such tests are underway and the preliminary data (Curtis et al., unpublished) indicate significant attractiveness of the (*Z,Z*) isomer alone. Other isomers were inactive. Additional tests designed to determine the efficacy of different

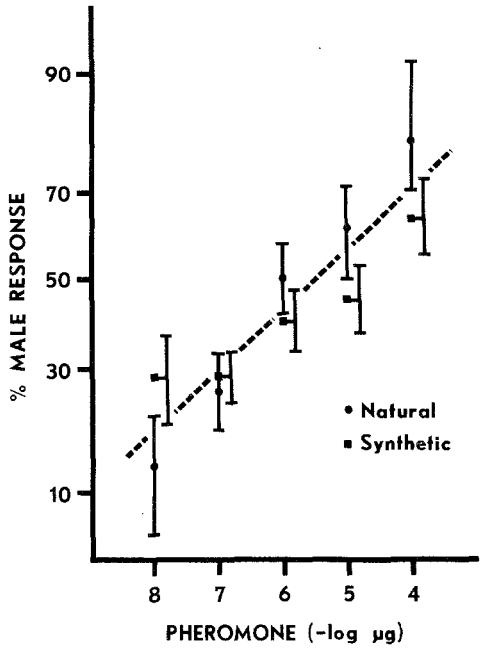

FIG. 4. Orientation response of *A. transitella* males to (*Z,Z*)-11,13-hexadecadienal and purified pheromone. Background (control) response = 9.4 ± 2.8 (SE)%.

substrates for releasing the synthetic pheromone and tests to evaluate the stability of the material are in progress.

A conjugated dienal (*E,Z*)-10,12-hexadecadienal was recently reported by Kasang et al. (1978) to be a sex pheromone for the silkmoth, *Bombyx mori* L., but the *A. transitella* pheromone is the first such compound to be reported as a pheromone based on behavioral data.

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Erratum: In the paper "Sex Pheromone Cross-Attraction Among Four Species of Pine Tip Moths, *Rhyacionia* Species," by C.W. Berisford et al. [*J. Chem. Ecol.* 5(2):205–210], the name of the journal in Reference 5 should be changed to read: *J. Econ. Entomol.*